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Neurogenic genes and vertebrate neurogenesis

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The neurogenic genes of the Delta–Notch signalling pathway mediate lateral inhibition – a mechanism that controls cell commitment in many tissues and serves in the developing nervous system to single out cells for a neural fate. Recent work has revealed the outlines of the signal transduction pathway from Notch to the nucleus, has clarified the mechanisms by which lateral inhibition causes adjacent cells to become different, and has shown that vertebrates use essentially the same lateral inhibition machinery as flies and worms to regulate neurogenesis.

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Abbreviations

AS-C	*achaete–scute* complex
bHLH	basic helix-loop-helix
E(spl)-C	*Enhancer-of-split* complex
HES	*hairy-and-Enhancer-of-split*
SMC	sensory mother cell
Su(H)	*Suppressor of Hairless*

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Introduction

A neuron in a fly or a worm is recognizably the same type of cell as a neuron in a vertebrate; but is it generated in the same way? Has evolution conserved not only the cell type, but also the machinery for its production? At first sight, the answer appears to be no: if we compare the developmental anatomy of the CNS of a vertebrate with that of *Drosophila*, for example, the structures appear radically different. The vertebrate has a dorsally located neural tube, with neurons differentiating within the neuroepithelium, whereas *Drosophila* has instead a ventrally located chain of segmental ganglia, with neurons generated from neuroblasts that are derived by delamination from the neuroectoderm. Anatomical appearances can be deceptive, however, and in the past few years it has become increasingly clear that remarkably similar sets of genes are involved in neurogenesis in the different classes of animals. Thus, *Drosophila* and *Caenorhabditis elegans* offer a key to the understanding of neurogenesis in vertebrates.

This review focuses on one particular subset of the genes that regulate neurogenesis. The members of this subset are called the *neurogenic genes* because, in *Drosophila*, mutations in them result in massive over-production of neurons. The first part of this review introduces the neurogenic genes through an outline of their role and mechanisms of action in *Drosophila* neurogenesis, where there have been some important recent advances. Against this background, the second half of the review will discuss new findings on the role of the neurogenic genes in vertebrate neurogenesis.

Neurogenic genes and lateral inhibition in *Drosophila* neurogenesis

Work over many years in flies and worms [1–3,4•,5•] has identified the products of the neurogenic genes as the components of a cell–cell signalling mechanism whereby, in the nervous system, a cell that becomes committed to a neural fate inhibits its neighbours from doing likewise. In this process of *lateral inhibition*, the receptor for the inhibitory signal is a transmembrane protein encoded by a neurogenic gene called *Notch* in *Drosophila*, and the ligand, expressed in the cell delivering inhibition, is another transmembrane protein encoded by a neurogenic gene called *Delta* in *Drosophila*.

The inhibitory signals are exchanged within groups of cells, so-called proneural clusters, that all have the potential for a neural fate; the role of lateral inhibition is to ensure that only a scattered subset of the cells actually become committed to that fate. The proneural clusters are defined by expression of proneural genes, chiefly the members of the *achaete–scute* complex (AS-C). The products of the AS-C genes are basic helix-loop-helix (bHLH) transcription factors, which have an initial upstream role as inducers of the expression of neurogenic genes throughout the proneural cluster. They also have a subsequent downstream role, in that their own expression is, in turn, regulated by the lateral inhibition machinery: as cells become committed to a neural fate, their content of AS-C proteins increases, whereas that of their neighbours in the cluster decreases [6]. The heightened expression of AS-C genes in the nascent neural cell is transient, as is the expression of *Delta* [7,8].

In the embryonic CNS of *Drosophila*, neuroblasts are generated from the neuroectoderm in successive waves [9], with the proneural and neurogenic genes controlling the proportion of cells that are singled out for this fate in each wave of neurogenesis. Each neuroblast will then undergo repeated asymmetric divisions to generate neurons and/or glial cells (see review by Doe and Skeath, this issue, pp 18–24). In the PNS, the same genes control the singling-out of cells from the epidermis to be sensory mother cells (SMCs; also called sense-organ precursors, or SOPs), each of which will undergo two or more asymmetric divisions to generate the neuronal and supporting cells of a single bristle or other sensillum [2]. In this latter

process the neurogenic genes are required a second time, providing lateral inhibition to prevent all the progeny of the precursor from becoming neurons [10]. Outside the nervous system, the same genes govern cell differentiation in tissues as varied as the gut epithelium [11•], the musculature [12], and the Malpighian tubules [13]. In each case, the same lateral inhibition mechanism appears to operate, generating a fine-grained pattern in which individual cells are singled out for commitment to a particular mode of differentiation, while the adjacent cells are inhibited from entering upon that commitment.

How to make neighbours different

In all the examples mentioned above, and in comparable processes in vertebrate neural development, neighbouring cells have contrasting fates. What makes them behave differently? Why, for example, don't all the cells in a proneural cluster remain locked in a state of mutual inhibition, each cell both delivering inhibition to its neighbours and receiving inhibition from them? Two mechanisms have been proposed, with good evidence in *Drosophila* for both. They are not mutually exclusive, and both must be borne in mind when considering how neighbouring cells come to adopt different fates during vertebrate neurogenesis.

Lateral inhibition with feedback

The first mechanism can be called *lateral inhibition with feedback*. It rests on an elaboration of the basic lateral inhibition mechanism, such that the more inhibition a cell receives, the less it is able to deliver [3,14,15]. In molecular terms, this means that increased activation of Notch in a given cell causes a decrease in the amount or activity of Delta in that cell. In a system of two or more cells, this creates a positive feedback loop (Figure 1), because the cell that delivers more inhibition to its neighbours drives down the level of inhibition that it receives back from them, and thereby still further increases its own ability to deliver inhibition. Thus, any initial asymmetry—even a small one—between two adjacent cells will be self-magnifying, leading to a state where one cell predominantly receives inhibition and does not deliver it, while the other predominantly delivers it and does not receive it.

Persuasive evidence for this mechanism has come from studies of the way in which cells are singled out to become SMCs in the developing PNS. For example, at the border of a mutant clone where cells with different numbers of copies of the *Delta* gene confront one another, it is the cells with more *Delta* gene copies that become SMCs, whereas their neighbours with less copies are inhibited [15]. Strong evidence for lateral inhibition with feedback has come also from work in *C. elegans*, where the *Notch* homologue *lin-12* and the *Delta* homologue *lag-2* act in this way to control the genesis of differences between initially equivalent cells in the gonad and elsewhere [3,16•,17].

Recent work has demonstrated at least one pathway by which lateral inhibition with feedback can come about (Figure 1). Activation of Notch induces expression of genes of the *Enhancer-of-split* complex (E(spl)-C) [18,19•]; these code for repressive bHLH proteins that act, in conjunction with the co-repressor Groucho [20,21•], to downregulate expression of genes of the AS-C [22•]; the AS-C genes, in turn, code for activator bHLH proteins that promote expression of *Delta* [23]. The net effect is that Notch activation reduces *Delta* expression by reducing AS-C expression [22•].

Asymmetric cell division

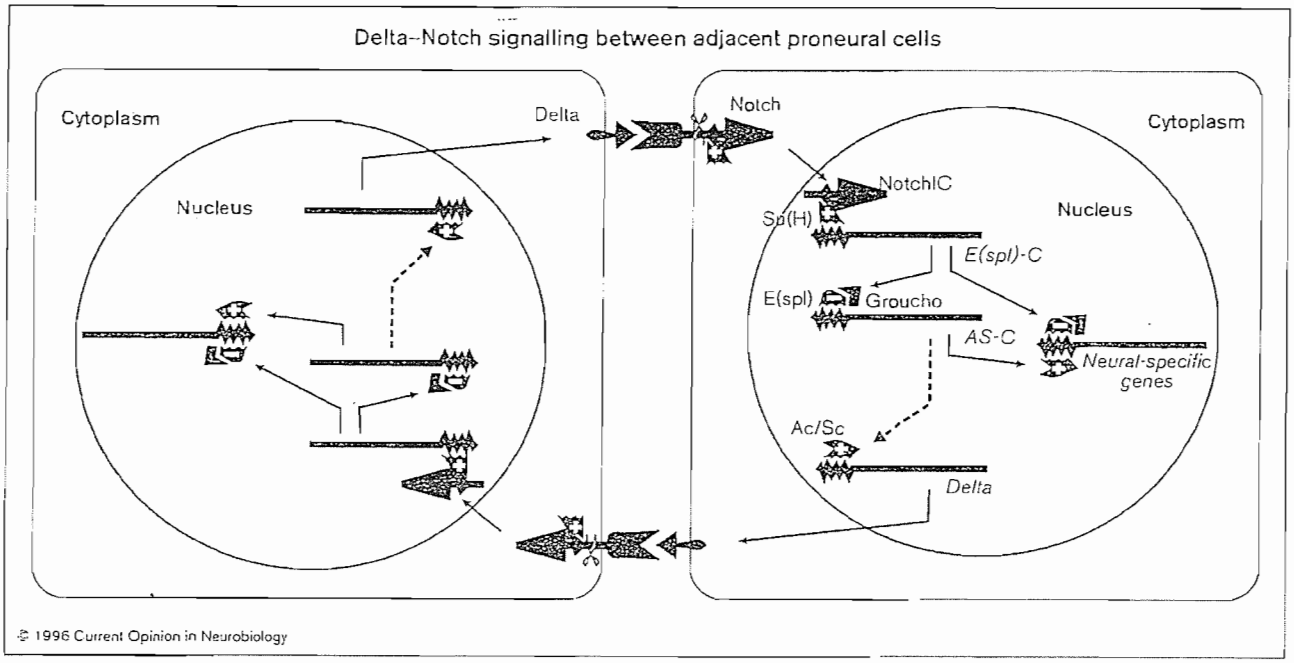
The other mechanism for generating asymmetric cell fates during *Drosophila* neurogenesis depends on asymmetric cell division: two sister cells adopt different fates because they inherit different quantities of a regulatory protein (or set of proteins) that becomes concentrated at one pole of the parent cell. As reviewed elsewhere ([24••]; see also the review by Doe and Skeath, this issue, pp 18–24), at least two proteins, Numb and Prospero, behave in this way both during the asymmetric divisions of neuroblasts in the CNS and during those of SMCs in the PNS [25–29]. These proteins are necessary and sufficient to confer a neural fate on the daughter that inherits them.

In the case of the SMC, there seems at first to be a paradox, in that Delta–Notch signalling is also required to ensure that the daughters of the SMC become different and do not both become neural [10]. It is not hard to see, however, how lateral inhibition may operate in concert with neural determinants such as Numb and Prospero. Suppose, for example, that in the daughters of the SMC, lateral inhibition operates without feedback, so that the daughters persist in delivering inhibition to one another symmetrically. If the neural determinants are absent, both daughters will remain non-neural; if one daughter inherits the neural determinants, and these make her immune to the inhibitory signal, she alone will become neural; and if Delta-Notch signalling is inoperative, neither daughter will be inhibited, and both will become neural; all this fits the facts. There is indeed evidence suggesting that lateral inhibition between the progeny of the SMC does operate without feedback: the AS-C gene products disappear from the SMC before its first differentiative division and play no part in controlling the fate of its progeny [7], so there cannot be any feedback of Notch on Delta via AS-C genes in this case.

From membrane to nucleus: the mechanism of signal transduction by Notch

Regardless of whether lateral inhibition operates with feedback or without, Delta–Notch signalling poses the perennial problem of signal transduction: how does activation of Notch at the cell membrane bring about changes in the pattern of gene expression? Several recent studies, in flies, worms, and vertebrates, together make a strong case for a novel and unusually direct mechanism

Figure 1



The Delta–Notch pathway for lateral inhibition according to the current view, depicted for a pair of adjacent proneural cells. Only the components discussed in this review are shown; although the signalling pathway is known to depend on several additional components, their specific roles are still obscure. DNA-binding proteins that function as repressors are marked with a '–', those that function as activators with a '+'. A dashed arrow symbolizes a regulatory link between AS-C genes and *Delta* that provides for feedback in the lateral inhibition of one cell by the other; Ac/Sc stands for Achaete/Scute proteins – the products of the AS-C. The regulatory links shown probably do not represent the only means by which Notch activation exerts its effects. Some evidence suggests, for example, that the main control of Delta activity during the singling-out of neural cells in *Drosophila* is post-translational rather than transcriptional [8].

(Figure 1): ligand binding causes cleavage of the Notch protein, releasing from it an intracellular fragment, called NotchIC, which then moves into the nucleus to regulate transcription [30••,31••,32]. Experiments in mammalian cells show that mutant forms of Notch lacking most of the extracellular domain but retaining the transmembrane domain undergo cleavage to release NotchIC, even in the absence of ligand binding [31••]. In transgenic fly and worm embryos, and in cultured mammalian P19 cells, both NotchIC and these other truncated forms of Notch have a dominant antineurogenic effect, inhibiting neural differentiation in the manner expected if there is constitutive activation of the Notch signalling pathway [32–37]. NotchIC has nuclear localization signals and includes a domain that mediates binding to the product of another neurogenic gene, *Suppressor of Hairless* (*Su(H)*) [38••,39•]. In *Drosophila* S2 cultured cells, activation of Notch by Delta leads to a movement of Su(H) protein into the nucleus [38••]. In the nucleus, Su(H) acts by binding to regulatory regions of the E(spl)-C and inducing transcription of E(spl)-C genes [40•], and studies of the homologous genes in mammalian cells indicate that this action of Su(H) depends on the presence of NotchIC bound to it [30••].

'Activated Notch' constructs — that is, NotchIC and other forms of Notch lacking the extracellular domain — are powerful tools for the analysis of Notch-dependent signalling mechanisms. For example, they have been used in combination with loss-of-function mutations of other neurogenic genes to discover the epistatic ordering of these genes; these tests confirm that the E(spl)-C lies downstream from *Notch*, that *Delta* is upstream from *Notch*, and that the neurogenic genes *mastermind* (*mam*), *big brain* (*bib*), *neuralized* (*neu*) and *almondex* (*amx*) also lie upstream from *Notch* [32] — that is, the presence of activated Notch causes an antineurogenic phenotype regardless of whether these genes are functional. The precise roles of *mam*, *bib*, *neu* and *amx* remain a mystery, however.

Neurogenic genes in vertebrates

In vertebrates, there have been only two reports of neurogenic genes discovered by the methods of classical genetics: one is called *white tail* and was found in the Tübingen zebrafish mutagenesis screen [41••], and the other is called *mind bomb* and was found in the Boston zebrafish screen [42•]. It is possible that they both are alleles of the same gene. By comparison, the number of vertebrate genes identified by homology with *Drosophila*

neurogenic genes is large. First to be discovered was *Xotch*, a *Xenopus Notch* homologue, now renamed *X-Notch-1* [43]. Since then, multiple *Notch* homologues have been found in rat, mouse, human, zebrafish and chick (reviewed in [5•]). More recently, homologues of *Delta* have been found [44•,45••,46•,47], as well as homologues of the *Delta*-related gene *Serrate* [48••,49•]. There is a whole family of homologues of E(spl)-C bHLH genes [50••,51], known as *HES* genes—for *hairy-and-Enhancer-of-split* homologues—because they also resemble the *Drosophila* gene *hairy*. There is also a family of vertebrate *groucho* homologues, called *TLE* genes [52,53]. *Su(H)* turns out to have as its homologue a vertebrate gene previously identified in another context as coding for a transcription factor variously called RBP-Jκ, CBF1 or KBF2 [39•,54,55]. The vertebrate *achaete–scute* homologues (*ASH* genes) are again numerous; their role in vertebrate neurogenesis is reviewed elsewhere [56•], and is a large topic that will not be covered here except in passing.

Vertebrate *Notch* and *Delta* homologues and lateral inhibition in primary neurogenesis

In vertebrates, neurons are generated in at least three distinct ways: within the central neuroepithelium that forms the neural plate and neural tube; by migration from the neural crest; and by delamination from the cranial placodes. Although placodal neurogenesis shows the most intriguingly close anatomical parallels with insect neurogenesis [57], I shall concentrate here on central neurogenesis, on which most of the recent relevant research has been done.

In the central neuroepithelium, the first neurons appear as scattered postmitotic cells in a number of distinct regions (comparable with proneural clusters in the insect neuroectoderm), where they are mingled with undifferentiated proliferative neuroepithelial cells. The pattern is most strikingly displayed in the neural plate of amphibians and fish, where production of a set of primary neurons is separated by a time-lag from production of subsequent sets of neurons [58,59]. *Notch-1*, and possibly other *Notch* homologues, are expressed by all the cells in the regions where primary neurogenesis is occurring. Meanwhile, expression of the *Delta* homologue *Delta-1* is restricted—in *Xenopus* and chick, at least—to a scattered subset of cells that have ceased dividing and appear to be the nascent neurons [45••,46•]. This suggests that Delta–Notch signalling is operating as in the fly to single out cells for a neuronal fate.

Gene functions can be tested directly in *Xenopus* by injecting RNA into the embryo at the two-cell stage. When RNA coding for full-length Delta-1 protein is injected, the production of neurons is inhibited [45••]: forced expression in all cells of a gene normally expressed only in nascent neurons inhibits all cells from becoming nascent neurons. This behaviour, at first sight paradoxical, is the hallmark of a gene whose function is to deliver lateral inhibition: normally, isolated cells express the gene and inhibit their neighbours, from whom they receive no inhibition; but when all cells are forced to express the gene, they all inhibit one another. Injection of RNA coding for a modified Delta-1 protein with a truncated intracellular domain has a contrary effect, causing excess production of neurons, as though it is blocking the normal delivery of inhibition by the endogenous Delta-1.

Injection of RNA coding for activated Notch—either NotchIC, or Notch-1 with a truncated extracellular domain—not only prevents the production of neurons, as expected if NotchIC inhibits commitment to a neural fate, but also inhibits expression of the endogenous *Delta-1* gene [45••]. This implies the existence of a feedback in the lateral inhibition system, of the type described above for *Drosophila* (see Figure 1): a cell expressing *Delta-1* more strongly than its neighbours will not only tend to inhibit them from becoming neurons, but will also inhibit them from delivering inhibition reciprocally and so will reinforce its own advantage. In this way, starting perhaps with uniform low-level expression of *Delta-1* or with uniform expression of some other *Delta* homologue, cells within an initially homogeneous population may become singled out for a neural fate.

Vertebrate *Notch* and *Delta* homologues in later neurogenesis

Is this conserved machinery of lateral inhibition with feedback a peculiarity of primary neurogenesis, or does it operate in subsequent neurogenesis too? After the first neurons in a vertebrate have been generated, the undifferentiated neuroepithelial cells of the embryonic CNS serve as stem cells for the production of further neurons. Over a period of many days, they continue to divide, with a proportion of their progeny remaining proliferative and a proportion withdrawing from the cell cycle and embarking on differentiation as neurons [60,61]. A layered structure soon develops, with the cell bodies of the proliferative cells confined to the ventricular zone (at least in hindbrain and spinal cord), and the postmitotic nascent neurons moving out of this region into the mantle zone as they begin to differentiate. Studies in the chick show that *Notch-1* meanwhile is expressed in apparently all cells in the ventricular zone, while *Delta-1* is expressed in a non-dividing subset of cells that are scattered within the ventricular zone and appear, once again, to be nascent neurons [49•]. Expression of both genes becomes switched off by the time the nascent neuron moves from the ventricular zone to the mantle zone.

All this amounts to strong circumstantial evidence for continuing control of neurogenesis by the same Delta–Notch signalling mechanism as in *Drosophila*. More direct proof comes from experiments on the developing chick retina [62••]. Activated Notch, carried into the cells by a retroviral vector, inhibits production of neurons; conversely, antisense oligonucleotides targeted against Notch-1 cause

increased production of neurons. And when the retinal cells are co-cultured with *Drosophila* cells expressing *Delta*, they are inhibited from giving rise to neurons. Similarly, in the *Xenopus* retina, activated Notch inhibits neuron production [63].

The neuroepithelium of the developing vertebrate CNS can thus be compared with the neuroectoderm of *Drosophila*, and the nascent neurons of the one with the neuroblasts of the other. In both cases, scattered cells within a neuroepithelium become singled out for a neural fate, transiently express high levels of Delta, and segregate from their close neighbours expressing Notch, which are inhibited from differentiating and remain to generate subsequent batches of neural cells. There are, however, some important differences: in the vertebrate CNS, the nascent neurons have stopped dividing, whereas in *Drosophila*, the neuroblasts continue to divide; and in the vertebrate CNS, the nascent neurons stay within the neuroepithelium, whereas in *Drosophila*, the neuroblasts escape from it. Vertebrate placodal neurogenesis is more similar to insect neurogenesis in these respects, and indeed *Notch* and *Delta* homologues are expressed in placodal epithelium at sites of neurogenesis (J Adam, D Henrique, A Myat, unpublished data).

It is not clear what part, if any, the Delta–Notch signalling mechanism plays in creating the differences among neuronal and glial cell types in the vertebrate CNS, and there is reason to suspect that asymmetric cell divisions, as well as lateral inhibition with feedback, are important. In the developing cerebral cortex of a mammal, cells in the proliferative zone are seen to divide symmetrically when generating pairs of daughters that both remain in the proliferative zone, but asymmetrically and with a differently oriented mitotic spindle when one of the daughters is destined to emigrate from the proliferative zone (presumably to become a neuron) [64••]. In the latter case, the asymmetry is marked chemically by the distribution of Notch-1 protein; puzzlingly, this becomes concentrated at the basal pole of the dividing cell and is segregated to the emigrating (neuronal) daughter (as one might expect for Numb and Prospero proteins, rather than Notch, in corresponding circumstances in an insect).

Alternative ligands for Notch

From the examples studied so far, it seems that Delta–Notch signalling has a universal role in the control of vertebrate neurogenesis. There is, however, at least one important qualification: *Drosophila* possesses another gene, *Serrate*, that also codes for a Notch ligand, and it seems that homologues of Serrate also have a role as Notch ligands during vertebrate neurogenesis in certain regions of the nervous system. Serrate is structurally related to Delta and contains a similar highly conserved Notch-binding domain (the DSL domain [65]), but it acts in different circumstances—for example, in *Drosophila*, it does not control neurogenesis, but controls the creation of the wing margin, a specialized band of tissue with a key organizing role in the growth of the wing imaginal disc [66–68]. In artificial conditions, Serrate can nevertheless serve to regulate neurogenesis [69]. In the rat, a homologue of *Serrate*, called *Jagged*, is expressed in the CNS in restricted domains of the ventricular zone, and, in an *in vitro* assay, is capable of activating Notch-1 to control gene expression [48••]. Studies of the corresponding chick gene, called *C-Serrate-1*, show that it is expressed in the ventricular zone of the neural tube in narrow stripes that extend from the anterior part of the hindbrain all the way down the spinal cord [49•]. These stripes correspond exactly to gaps in the expression domain of *C-Delta-1*, and the cellular pattern of *C-Serrate-1* expression within them suggests that C-Serrate-1 may here act instead of C-Delta-1 as a Notch ligand. The analogy with the fly, however, would suggest that C-Serrate-1 is more than just a stand-in for C-Delta-1, and that something special is happening at these sites. A still richer vein of speculation is opened up if, as one school of thought has argued, Notch proteins act as receptors not only for members of the Delta/Serrate family, but also as receptors for the signalling protein Wingless and its vertebrate relatives, the Wnts [66,70].

Vertebrate *E(spl)* homologues: lessons from transgenic mice

Notch and *Delta* are not the only neurogenic genes for which there is direct evidence of a conserved function in neurogenesis. The mouse *hairy-E(spl)* homologue *HES-1*, for example, is also expressed throughout the ventricular zone, and its role there has been tested both by overexpressing the gene using a retroviral vector and by knocking it out by homologous recombination [50••,51]. In accordance with the function of E(spl)-C genes in *Drosophila*, cells forced to express *HES-1* persistently are inhibited from adopting a neuronal or glial fate and remain in the ventricular population, eventually becoming ependymal cells or dying. Conversely, in the *HES-1* knockout animal, some classes of neurons are generated prematurely. These effects, again as expected by analogy with the fly, correlate with changes in the expression of AS-C homologues. Knockouts of *Notch-1* and *RBP-Jκ/Su(H)* have been less informative, perhaps because of genetic redundancy [71,72]: in both cases, the mutant embryos die half way through gestation, and the relatively mild abnormalities seen in the cytoarchitecture of the neural tube at this stage are difficult to decipher [55,73,74].

Conclusions

Lateral inhibition mediated by Delta–Notch signalling appears as a key mechanism in the control of neurogenesis. It seems to be a general principle that neurons are generated, and subsequently function, within a matrix of cells of other types; lateral inhibition provides the means to single them out from their neighbours for their special fate. The recent discoveries have revealed in outline how the signalling pathway works, and have shown, for several

of the main components, that it is similar in vertebrates, flies and worms. The course of evolution has indeed conserved not only the neuron as a specialized cell type, but also a major part of the developmental mechanism that gives rise to it. The task now is to pursue the many components of the Delta–Notch signalling pathway that remain mysterious (e.g. see [75•]), to find out more about how this mechanism of lateral inhibition is combined with other developmental controls, and to define precisely the cell-fate decisions that it governs, not only in the nervous system but also outside it.

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REVIEW ARTICLES

HES and HERP Families: Multiple Effectors of the Notch Signaling Pathway

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Notch signaling dictates cell fate and critically influences cell proliferation, differentiation, and apoptosis in metazoans. Multiple factors at each step—ligands, receptors, signal transducers and effectors—play critical roles in executing the pleiotropic effects of Notch signaling. Ligand-binding results in proteolytic cleavage of Notch receptors to release the signal-transducing Notch intracellular domain (NICD). NICD migrates into the nucleus and associates with the nuclear proteins of the RBP-Jκ family (also known as CSL or CBF1/Su(H)/Lag-1). RBP-Jκ, when complexed with NICD, acts as a transcriptional activator, and the RBP-Jκ-NICD complex activates expression of primary target genes of Notch signaling such as the HES and enhancer of split [E(spl)] families. HES/E(spl) is a basic helix-loop-helix (bHLH) type of transcriptional repressor, and suppresses expression of downstream target genes such as tissue-specific transcriptional activators. Thus, HES/E(spl) directly affects cell fate decisions as a primary Notch effector. HES/E(spl) had been the only known effector of Notch signaling until a recent discovery of a related but distinct bHLH protein family, termed HERP (HES-related repressor protein, also called Hey/Hesr/HRT/CHF/gridlock). In this review, we summarize the recent data supporting the idea of HERP being a new Notch effector, and provide an overview of the similarities and differences between HES and HERP in their biochemical properties as well as their tissue distribution. One key observation derived from identification of HERP is that HES and HERP form a heterodimer and cooperate for transcriptional repression. The identification of the HERP family as a Notch effector that cooperates with HES/E(spl) family has opened a new avenue to our understanding of the Notch signaling pathway. J. Cell. Physiol. 194: 237–255, 2003. © 2003 Wiley-Liss, Inc.

The evolutionarily conserved Notch signaling pathway controls cell fate in metazoans through local cell–cell interactions (Egan et al., 1998; Greenwald, 1998; Artavanis-Tsakonas et al., 1999). Notch signaling dictates cell fate and critically influences cell proliferation, differentiation, and apoptosis (Miele and Osborne, 1999). Components in the Notch pathway, such as *Notch, bigbrain, Delta, mastermind, neuralized* and *enhancer of split complex*, were isolated originally as neurogenic genes in *Drosophila*, since embryos lacking the function of these genes showed an increased number of neuroblasts at the expense of epidermal precursors (Egan et al., 1998; Greenwald, 1998; Artavanis-Tsakonas et al., 1999). However, it has subsequently been demonstrated that the Notch pathway is involved not only in neurogenesis but also in the development of many other organs derived from all three germ lines (Hartenstein et al., 1992). In vertebrates also, Notch receptors, ligands and other components are expressed in various organs from all three germ lines. Mutations for Notch receptors and ligands lead to abnormalities in many tissues, including vessels, thymus, craniofacial region, limb, rib, somite, central nervous system, heart, kidney as well as hematopoietic cells (Swiatek et al., 1994; Conlon et al., 1995; de la Pompa et al., 1997; Hrabe

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de Angelis et al., 1997; Sidow et al., 1997; Jiang et al., 1998; Kusumi et al., 1998; Hamada et al., 1999; Xue et al., 1999; Krebs et al., 2000; McCright et al., 2001; Dunwoodie et al., 2002). Thus, the Notch pathway plays crucial roles in the development of most organs.

Interaction of Notch receptors with their ligands such as the Delta and Jagged families leads to cleavage of the transmembrane Notch receptor, giving rise to the Notch intracellular domain (NICD) that migrates into the nucleus (Fig. 1) (Weinmaster, 1998; Mumm and Kopan, 2000). NICD has a transcriptional activation domain, but no DNA binding domain of its own. In the nucleus, NICD associates with a transcriptional factor, RBP-Jκ (also known as CSL for CBF1/Su(H)/Lag-1) (Egan et al., 1998; Greenwald, 1998; Weinmaster, 1998; Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000), and activates transcription from the RBP-Jκ binding DNA site. In the absence of NICD, RBP-Jκ associates with a corepressor complex and acts as a transcriptional repressor from its DNA binding site (GTGGGAA) (Ling et al., 1994; Kao et al., 1998). The NICD-RBP-Jκ complex up-regulates expression of primary target genes of Notch signaling such as HES in mammals, and E(spl) (for *Enhancer of Split*) in *Drosophila* [hereafter HES/E(spl)] (Egan et al., 1998; Greenwald, 1998; Artavanis-Tsakonas et al., 1999). The HES/E(spl) family is a basic helix-loop-helix (bHLH) type trancriptional repressor and acts as Notch effectors by negatively regulating expression of downstream target genes such as tissue-specific transcription factors (Ohsako et al., 1994; Van Doren et al., 1994; Ishibashi et al., 1995; Chen et al., 1997a). Consistent with this view, HES1 and HES5, for instance, were shown to be up-regulated by NICD and necessary to prevent neuronal differentiation of neural precursor cells from mouse embryos (Ohtsuka et al., 1999).

Although the HES family had been the only known effector of Notch in mammals, tissue distribution of Notch ligands and receptors does not always overlap with that of HES, suggesting the existence of yet undetected effectors of Notch signaling. Recently, a new bHLH family has been isolated and named as Hey/Hesr/HRT/CHF/gridlock/HERP (hereafter HERP) (Table 1). The amino acid sequence of HERP as well as its characteristic domains indicate that HERP is most closely related to the HES family among the reported bHLH proteins (Fig. 2). This finding immediately led to the speculation that HERP might be a novel Notch effector. Interestingly, HERP expression is detected in both HES-expressing and non-HES-expressing tissues. Remarkably, HES and HERP may function not only as homodimers but also as HES-HERP heterodimers in those cells co-expressing HES and HERP (Iso et al., 2001b). Although both HES and HERP act as transcriptional repressors, HERP employs different repression mechanisms than does HES (Iso et al., 2001b). HERP could thus play a critical role in mediating Notch effects in both HES-expressing and non-HES-expressing tissues either as a hetero- or homo-dimer.

CLASSIFICATION OF bHLH PROTEINS

The bHLH family of transcriptional regulators plays crucial roles in the development of various organs and cell types including the nervous system, the heart, skeletal muscles, the pancreas, endodermal endocrine organs, and hematocytes (Murre et al., 1994; Massari and Murre, 2000). Over 240 HLH proteins have been identified to date in organisms ranging from yeast to human (Massari and Murre, 2000). The bHLH proteins bind specific DNA sequences as a dimer. The basic and HLH domains have distinct functions. The basic domain is a major determinant of DNA binding specificities (Murre et al., 1994). DNA binding is mediated by a contact between each basic domain of a dimer and a specific half-site of consensus DNA sequences. The HLH domains are characterized by hydrophobic residues that allow them to form a homo- or hetero-dimer (Murre et al., 1994).

bHLH proteins can be classified into several groups according to their structural features and biochemical characteristics (Table 2A) (Murre et al., 1994; Atchley and Fitch, 1997; Fisher and Caudy, 1998; Massari and Murre, 2000). Class A proteins are transcriptional activators such as MyoD and Mash1, and bind class A sites (CANCTG) (Table 2B). Class B proteins are bHLH-lucine zipper type proteins such as Myc and Max. Both class A and B sites (CANGTG) are subtypes of the E box (CANNTG). Class C proteins are transcriptional repressors such as HES in mammals, and *hairy* and E(spl) in *Drosophila*, and are characterized by an invariant proline residue at a specific site of the basic domain. Class C proteins bind class C sites (CACGNG) as well as N-box sequences (CACNAG). Class C proteins, HES and *hairy*, are also known to bind class B sites to some degree but not class A sites (Ohsako et al., 1994; Van Doren et al., 1994; Fisher and Caudy, 1998; Jennings et al., 1999).

TABLE 1. HERP family nomenclature

Abbreviations[a]			Full name	Species	References
HERP1	HERP2	HERP3	HES-related repressor protein	Mouse, rat, human	Iso et al. (2001a, 2002)
Hesr2	Hesr1	Hesr3	Hairy/E(spl)-related	Drosophila, mouse, human	Kokubo et al. (1999), Satow et al. (2001)
Hey2	Hey1	HeyL	Hairy/E(spl)-related with YRPW	Drosophila, chicken, mouse, human	Leimeister et al. (1999, 2000a), Steidl et al. (2000)
HRT2	HRT1	HRT3	Hairy-related transcription factor	Mouse, human	Nakagawa et al. (1999)
CHF1	CHF2		Cardiovascular helix-loop-helix factor	Mouse, human	Chin et al. (2000)
Gridlock				Zebrafish, human	Zhong et al. (2000)

[a]These proteins aligned vertically are identical or homologue of other species.

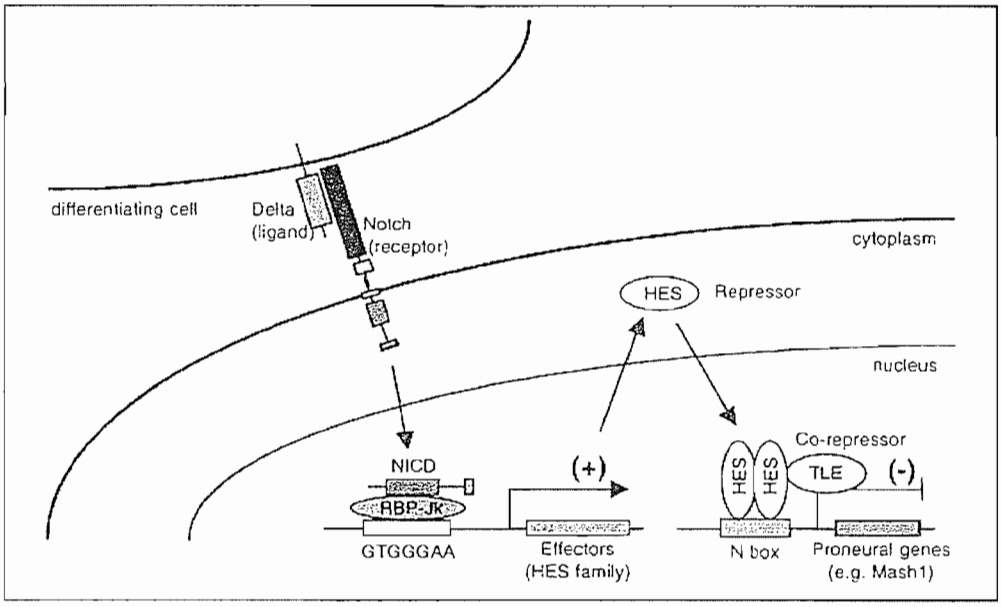

Fig. 1. Model of the Notch signaling pathway in neurogenesis in mammals. See the text for detail. NICD, notch intracellular domain, HES: *hairy* and E(spl), TLE, transducin-like enhancer of split, Mash1: mammalian *achaete-scute* homolog 1.

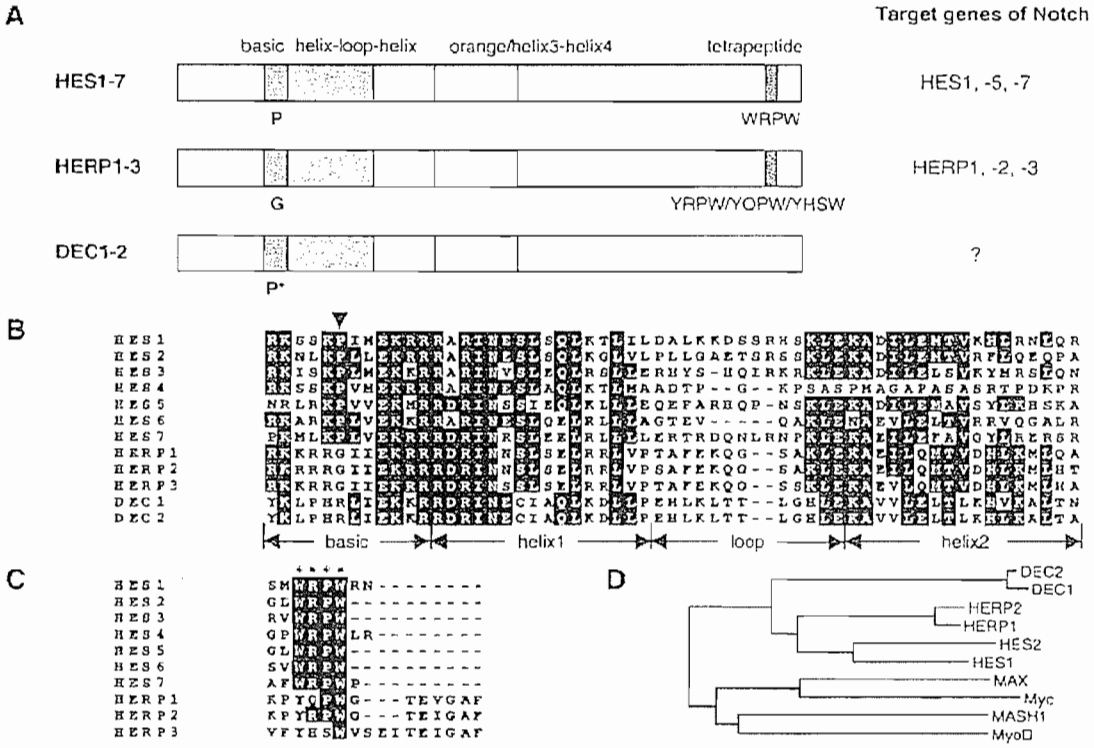

Fig. 2. Alignment of HES, HERP, and DEC amino acid sequences. A: Schematic diagram of HES, HERP, and DEC. Conserved domains are marked by distinct colors: Blue for the basic domain, green for the helix-loop-helix domain, orange for the Orange domain, and pink for the tetrapeptide motif. Potential target genes of Notch are listed on the right. DEC is shown because of its similarity to HES and HERP, but there is no data supporting DEC as a Notch target. See text for detail. B and C: Amino acid sequences of the basic helix-loop-helix domain (B) and the carboxyl termini including the tetrapeptide motif (C) were aligned by using ClustalW, and presented by BoxShade. Identical amino acids are in black and conserved residues are in gray. An arrowhead indicates the invariant amino acid residues in the basic domain of HES (proline) and HERP (glycine). Asterisks indicate the tetrapeptide motifs. D: Phylogenetic tree showing the relationship of representative members of bHLH transcription factor families. Shown is a dendrogram created by aligning the sequences of the bHLH domains of the indicated proteins by the ClustalW algorithm.

TABLE 2A. Classification of basic helix-loop-helix proteins

Fisher and Caudy (1998)	Massari and Murre (2000)	
Class A	I, II	Transcriptional activators
Class B	III, IV	bHLH-lucine zipper proteins
Class C	VI	Transcriptional repressors
	V	HLH proteins lacking basic region
	VII	bHLH-PAS proteins

STRUCTURAL SIMILARITIES AND DIFFERENCES AMONG HES AND HERP FAMILIES

To date, seven HES members (Akazawa et al., 1992; Sasai et al., 1992; Ishibashi et al., 1993; Bae et al., 2000; Hirata et al., 2000; Koyano-Nakagawa et al., 2000; Pissarra et al., 2000; Bessho et al., 2001a), and three HERP members (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Chin et al., 2000; Zhong et al., 2000; Iso et al., 2001a) have been isolated in mammals (Fig. 2). The two families share several common features (Fig. 2). They contain bHLH domain, and another domain, termed the Orange (or helix3-helix4) in the corresponding regions carboxy-terminus to bHLH region. The amino acid sequences of these domains are highly conserved within the respective family, but less so among the two different families. The most remarkable difference that distinguishes HES from HERP is a proline residue in the basic region (Fig. 2B, indicated by an arrowhead). This proline residue is invariant among HES/E(spl) family members across species from *Drosophila* to human. Because of this proline, HES members are also called proline bHLH proteins. The HERP family has a glycine at the corresponding position and this glycine also is strictly conserved from *Drosophila* to human HERPs. Thus, these prolines and glycines are hallmarks for the HES and HERP families, respectively. All HES members share the C-terminal tetrapeptide WRPW motif, whereas the HERP family has YRPW or its variants. In addition to the tetrapeptide motif, the HERP family has an additional conserved region carboxyl-terminal to the tetrapeptide motif, TE(V/I)GAF, which is absent in HES (Fig. 2C).

Both HES and HERP function as transcriptional repressors. A phylogenetic tree shows that they form a distinct subgroup in a large bHLH protein family (Fig. 2D) (Vasiliauskas and Stern, 2000; Bessho et al., 2001a; Davis and Turner, 2001; Teramoto et al., 2001). These findings indicate that HERPs are closely related to the HES family belonging to class C protein, but it forms a distinct subgroup (Fig. 2D).

REPRESSION MECHANISMS OF THE HES AND HERP FAMILIES: REPRESSION DOMAINS AND COFACTORS

Both the HES and HERP families have been reported to act as transcriptional repressors except HES6, which antagonizes the function of HES1, resulting in derepression (Bae et al., 2000; Koyano-Nakagawa et al., 2000). Although these two families have similar domains, they appear to use different repression mechanisms.

The HES/E(spl) family

Mechanisms for transcriptional repression by HES/E(spl) have been extensively studied genetically and biochemically. Three mechanisms have been proposed. The first mechanism is DNA-binding-dependent transcriptional repression, also known as active repression (Kageyama and Nakanishi, 1997; Kageyama et al., 2000). HES/E(spl) proteins form a homodimer and bind class C or N box consensus DNA sites (Sasai et al., 1992; Tietze et al., 1992; Oellers et al., 1994; Ohsako et al., 1994; Van Doren et al., 1994). They recruit the corepressor Groucho or its mammalian homologue TLE via the C-terminal WRPW motif (Paroush et al., 1994; Fisher et al., 1996; Grbavec and Stifani, 1996). The WRPW motif of HES/E(spl) is both necessary and sufficient to confer repression when expressed as a fusion protein with a heterologous DNA binding domain of Gal4. Chen et al. (1999) have shown in *Drosophila* that Groucho can recruit the histone deacetylase Rpd3, an orthologue of mammalian HDAC through poorly conseverd glycine/proline-rich domain in the central variable region of Groucho. The histone deacetylase then may repress transcription by altering local chromatin structure. Whether mammalian TLE employs the same mechanism remains to be determined.

The second mechanism is passive repression (Sasai et al., 1992; Hirata et al., 2000) involving protein sequestration. HES1, for instance, can form a nonfunctional heterodimer with other bHLH factors such as E47, a common heterodimer partner of tissue-specific bHLH factors such as MyoD and Mash1, thereby

TABLE 2B. Consensus binding site

Classification	Consensus	Examples
Class A	CANCTG	CACCTG, CAGCTG
Class B	CANGTG	CACGTG, CATGTG
Class C	CACGNG	CACGCG, CACGAG
E box	CANNTG	CACCTG, CAGCTG, CACGTG, CATGTG
N box	CACNAG	CACGAG, CACAAG

Class A and B are subtypes of E box. Class C and N box are mutually overlapping.

disrupting the formation of functional heterodimers such as MyoD-E47 and Mash1-E47.

The third mechanism is mediated by the Orange domain/helix3–helix4 (Castella et al., 2000). The Orange domain is essential to repress transcription of its own (HES1) promoter as well as the $p21^{WAF}$ promoter (Castella et al., 2000). This ability of the Orange domain is dependent on the presence of a DNA-binding bHLH domain. An important role of the Orange domain has been demonstrated in a sex determination assay in *Drosophila* (Dawson et al., 1995). Interestingly, Castella and colleagues show that the Orange domain hardly represses transcription of Gal4 dependent reporter gene constructs when fused to a Gal4 DNA binding domain (Castella et al., 2000). They proposed that the Orange domain, a putative protein interaction motif, is necessary for either the direct recruitment of an unknown corepressor and/or the stabilization or regulation of the WRPW-mediated repression function through intra- or intermolecular interaction (Castella et al., 2000).

Functional dissection of the E(spl) protein has revealed an additional important region between the Orange domain (helix3–helix4) and the WRPW motif for correct bristle development in *Drosophila*, although molecular mechanisms of how this region functions remain to be clarified (Giebel and Campos-Ortega, 1997).

The HERP family

A study using HERP1 deletion mutants fused with the Gal4-DNA binding domain unexpectedly revealed that the repression activity of HERP resides primarily in the bHLH domain rather than the C-terminal tetrapeptide (YQPW) motif (Iso et al., 2001b). This is in sharp contrast to the well established critical roles of the WRPW motif in the HES/E(spl) family (Wainwright and Ish-Horowicz, 1992; Paroush et al., 1994; Fisher et al., 1996; Giebel and Campos-Ortega, 1997). This indicates that engagement of TLE/Groucho is unlikely a critical component of repression by HERP. Indeed, the bHLH domain of HERP1 is both necessary and sufficient for recruitment of a corepressor complex including N-CoR, mSin3A, and HDAC1 (Iso et al., 2001b). Consistently, Nakagawa et al. (2000) showed that HRT2 (HERP1) represses its own gene expression and this repression required the basic domain, but not the carboxyl-terminal region containing the tetrapeptide motif. They further showed that the transcriptional repression by HRT2 (HERP1) was not affected by the addition of the histone deacetylase inhibitor TSA. The ineffectiveness of TSA is a paradox in view of the recruitment of HDAC to the complex by HERP1 (Nakagawa et al., 2000; Iso et al., 2001b). The reason for the apparent discrepancy is unknown. Perhaps HDAC is involved only in specific contexts (i.e., promoters and cell types), or HDAC functions, other than its inhibition of deacetylation, might be involved in the repression by HRT2.

Our studies show that HERP associates with N-CoR in addition to the Sin3/HDAC1 complex. However, recent data show that the purified mammalian Sin3 complex does not contain N-CoR (Zhang et al., 1997), nor is Sin3 present in the N-CoR-related SMRT complex (Guenther et al., 2000; Li et al., 2000). This suggests that the association of the two co-repressor complexes may not constitutively occur in cells. Whether HERP associates alternately with one co-repressor complex or the other, or HERP has an ability to simultaneously recruit the two co-repressor complexes remains to be clarified. Thus, despite similarities of their domains, HES and HERP appear to employ different repression mechanisms involving heterologous sets of corepressor proteins-Groucho/TLE for HES and N-CoR/mSin3A/HDAC for HERP.

It should be noted that, despite its well established repression role, the WRPW motif of the HES/E(spl) family is not always required. For instance, the WRPW domain of the HES/E(spl) family is dispensible for suppression of neurogenesis in *zebrafish*, or for suppression of *Scute* activity in the sex determination pathway in *Drosophila* (Dawson et al., 1995; Takke et al., 1999). The Orange domain of HES1, but not WRPW, is essential to repress transcription of its own gene and $p21^{WAF}$ promoters (Castella et al., 2000). Thus, the requirement for the WRPW of HES/E(spl) is not absolute, and repression functions of specific domains may be context-dependent. It is conceivable that HERP also might employ different domains for repression in a context-dependent manner, although all the available data thus far indicate that the tetrapeptide motif of HERP is not essential.

Passive repression mechanisms have also been proposed for the HERP family. CHF1 (HERP1) binds the aryl hydrocarbon receptor nuclear translocator (ARNT) and inhibits ARNT-dependent transcription of the VEGF promoter by dissociating the ARNT complex from DNA (Chin et al., 2000). In addition, Sun et al. (2001b) showed that CHF2 (HERP2) inhibits MyoD-dependent transcription of the myogenin promoter as well as muscle conversion, likely by disturbing the binding of MyoD-E47 heterodimers to E-box sites. They further demonstrated that this transcriptional repression by CHF2 requires a hydrophobic carboxyl-terminal region of CHF2 (HERP2) containing the Orange domain but neither the bHLH domain nor the YRPW tetrapeptide motif. Whether this region is involved in the inhibition of DNA binding by MyoD-E47 heterodimers is unknown.

Altogether, these findings illustrate that the HES and HERP families use distinct repression mechanisms. Although they have closely-related domains and motifs, they appear to use distinct domains for transcriptional repression.

DNA BINDING SITE SPECIFICITY AND TARGET GENES FOR HES AND HERP

HES/E(spl)

It was initially reported that *Drosophila* E(spl) proteins bind the N box (CACNAG) (Tietze et al., 1992; Oellers et al., 1994). However, the optimal site for the E(spl) proteins determined by in vitro random oligonucleotide binding site selection is a palindromic 12-bp sequence, TGGCACGTG(C/T)(C/T)A, which contains a class B core (CACGTG) and has been termed an ESE box (for E(spl) E box) (Jennings et al., 1999). Although this approach also picked up a few percent of N box (CACGAG) and class C site (CACGCG), the binding activity of E(spl) proteins for these sites are much weaker than that for class B site (Jennings et al., 1999). The in vivo significance of class B site binding by E(spl) is

confirmed by the observation that even subtle sequence changes within this class B core or flanking bases have dramatic consequences for lacZ reporter gene expression in transgenic flies (Jennings et al., 1999). Thus, the optimal sites for E(spl) proteins are likely class B sites rather than N box or class C sites in *Drosophila*.

Another HES/E(spl) family member, *Drosophila hairy* protein binds both class B and C sites in vitro (Ohsako et al., 1994; Van Doren et al., 1994). The *hairy*-binding class C site (CACGCG) was found in the promoter region of the *achaete* gene (Table 3), and the mutation of this promoter site created ectopic sensory hair organs in the fly, as in *hairy* mutants, strongly suggesting that *hairy* functions via this class C site in vivo.

Mammalian homologue HES proteins, HES1, -2, -3, and -5, have also been shown to bind an N box, but in vivo target genes have not been established except for HES1 (Table 3) (Akazawa et al., 1992; Sasai et al., 1992; Ishibashi et al., 1993; Hirata et al., 2000). Several target genes have been proposed for HES1. One such candidate is the HES1 gene itself. In reporter gene assays in cultured mammalian cells, HES1 negatively regulates its own promoter activity. When the N-box sequences in the HES1 promoter were mutated, this negative autoregulation was diminished, suggesting that HES1 regulates its own gene expression through the N box in a negative feedback loop (Takebayashi et al., 1994). Mash1 is another potential target gene of HES1. Overexpressed HES1 can repress Mash1 transcription by directly binding to the promoter region of the Mash1 via a variant class C site (CACGCA) in cultured cells (Chen et al., 1997a). Consistently, targeted disruption of the HES1 gene in mice upregulates Mash1 mRNA levels (Ishibashi et al., 1995). These data suggest that HES1 functions as a negative regulator of neurogensis by directly repressing a proneural gene, Mash1. CD4 is another candidate target gene for HES1 (Kim and Siu, 1998). HES1 binds an N-box sequence in the CD4 gene promoter in vitro. Overexpression of HES1 leads to N-box-dependent repression of the CD4 promoter as well as downregulation of endogenous CD4 expression in $CD4^-$ $CD8^-$ T_H cells (Kim and Siu, 1998). The acid α-glucosidase promoter is also repressed by HES1 in a class C site (CACGCG)-dependent manner in hepatoma-derived Hep G2 cells (Yan et al., 2001). Although a cyclin-dependent kinase inhibitor, $p21^{WAF}$, has also been proposed as a candidate target gene for HES1, the presence of a HES1 binding site in the $p21^{WAF}$ gene promoter has not been confirmed (Castella et al., 2000).

Collectively, these data indicate that class C sites and N boxes are likely critical in vivo binding sites for HES1 in mammals. Whether class B sites are in vivo targets of HES, as they are for E(spl) in *Drosophila* remains to be determined. Given the high amino acid similarity between *Drosophila* E(spl) and mammalian HES within the basic domains, however, class B sites seem to be good candidates in mammals as well.

HERP

The DNA binding site for HERP1 has also been determined by electrophoretic mobility shift assays (Iso et al., 2001b). The amino acid sequence in the basic domain of HERP1 is most similar to that of HES1 among reported bHLH proteins (Fig. 2B). Indeed, HERP1, like HES1, can bind both class B and C sites, albeit with different preferences than HES1 (Table 3). Surprisingly, HERP1 also binds class A sites (CAGGTG), raising the possibility that HERP1 may directly compete with tissue-specific bHLH activators for the E box. What are the potential target genes of HERP? It has been reported that HRT2 (HERP1) negatively regulates its own gene expression (Nakagawa et al., 2000). However, no class A, B, or C binding sites were found in the shortest reporter gene construct of the HRT2 promoter, and therefore, the apparent negative feedback regulation by HRT2 toward its own promoter may be mediated either by yet undetermined HRT2 binding sites or by indirect mechanisms.

DNA binding sequences for HERP2 seem essentially identical with those of HERP1, as expected from the very similar amino acid sequences of their DNA binding basic domains (Iso et al., 2001b). If HERP1 and HERP2 bind the same sequences, however, why are two HERPs required? One simple explanation is that HERP1 and HERP2 may be expressed in different cell types. This

TABLE 3. DNA binding activity for E(spl), hairy, HES, and HERP

	Class A	Class B	Class C	E box	N box	Target gene	References
E(spl)m8					++	E(spl)m8	Tietze et al. (1992), Oellers et al. (1994)
E(spl)		++#1	+		+		Jennings et al. (1999)
Hairy	−	++	++			Achaete	Ohsako et al. (1994), Van Doren et al. (1994)
HES1	−	+			++	HES1	Takebayashi et al. (1994)
			+#2			hASH	Chen et al. (1997a)
			+			CD4	Kim and Siu (1998)
					+	Acid α-glucosidase	Yan et al. (2001)
HES2				++	+		Ishibashi et al. (1993)
HES3				−	++		Hirata et al. (2000)
HES5				−	++		Akazawa et al. (1992)
HES6	−		−				Gao et al. (2001)
		++#1			−		Cossins et al. (2002)
HERP1	+	++	++		+		Iso et al. (2001b)
	−	++			−	HERP1/HRT2	Nakagawa et al. (2000)
HERP2	+	++	++		+		Iso et al. (2001b)
HERP3		−					Nakagawa et al. (2000)

See text and references for details. The data are based on gel mobility shift assay. ++, strong binding; +, weak binding; −, no binding. #1, proteins strongly bound E(spl) E box, or ESE box, but not classical E box. #2, the sequence of class C in hASH gene is a variant, CACGCA.

TABLE 4. HERP1 and HERP2 are expressed in distinct domains within individual tissues

	HERP1	HERP2	References
Heart	Ventricle	Atrium	This paper, Leimeister et al. (1999), Nakagawa et al. (1999)
Craniofacial region	Tissue surrounding whisker follicles	Whisker follicles	Leimeister et al. (1999)
Retina	The outer and inner regions of the inner nuclear layer	The middle region of the inner nuclear layer	Satow et al. (2001)

appears to be the case at least in several tissues and organs. For instance, HERP1 and HERP2 show a mutually complementary expression pattern in subdomains of heart and brain during embryogenesis (Table 4) (Leimeister et al., 1999; Nakagawa et al., 1999). An intriguing possibility derived from this striking mutual exclusivity is that HERP1 and HERP2 may regulate different sets of target genes and thereby contribute to establishment of distinct subdomains (i.e., cardiac atrium vs. ventricle) within a single organ. In addition, given that HERPs are transcriptional repressors, HERP members might repress each others gene expression to create a mutually exclusive expression pattern as we speculate in Figure 3. These possibilities need to be addressed in the future.

The strict conservation of proline and glycine in the basic domains of all the HES and HERP family members, respectively, suggests a potentially important role of these residues (arrowhead in Fig. 2B). However, a role for these residues in the DNA binding of HES/HERP has not been rigorously determined. In one study, a proline to asparagine mutation in a E(spl) protein largely diminishes its DNA binding activity, whereas a mutation of the same proline to threonine appears to have little effect (Tietze et al., 1992; Oellers et al., 1994). In addition, a glycine to proline mutation in CHF2 (HERP2) did not significantly affect repression of the myogenin promoter activity in cultured cells or of myogenic conversion of 10T1/2 cells by MyoD (Sun et al., 2001b). A potential role of the proline/glycine in defining the DNA binding specificity as well as other functions of HES and HERP families remains to be clarified. Hitherto, in vivo target genes for HERP have not been determined. In addition to the above mentioned auto- and cross-regulation between different HERP members, the previously established HES1 target such as Mash1 seems to be a target candidate for the HES1-HERP heterodimer (see below), if both are co-expressed in the same cells.

HERP: A NEW HETERODIMER PARTNER FOR HES

HES and HERP are co-expressed in certain cells (summarized in Table 5), and they both can bind the same DNA sequences in vitro. Why then do cells need the two effectors for Notch signaling if they both target the same genes? One possibility is that such occupancy may depend simply on the relative abundance of HES and HERP proteins in a given cell. Alternatively, DNA binding affinities of HES and HERP homodimers may be modulated in vivo, for instance, by post-translational modification and protein–protein interaction. Phosphorylation of HES1 at specific residues in the basic domain results in the loss of its DNA-binding activity (Strom et al., 1997). Since these residues are not present in HERP, such a phosphorylation event in HES1 would selectively lower its DNA binding affinity, and might allow HERP to occupy the promoter.

A more intriguing possibility is that HES and HERP positively interact with each other to enhance DNA binding. Indeed, HES and HERP associate with each other as a hetero-oligomer both in vitro and in intact cells in the absence of DNA (Leimeister et al., 2000a; Iso et al., 2001b). Gel-shift assays have demonstrated that a heterodimer of HES-HERP binds far more efficiently than the respective homodimers to class C DNA sequences (Fig. 4) (Iso et al., 2001b). Consistently, reporter gene assays using multimerized class C sites showed synergystic repression by HES-HERP heterodimers (Iso et al., 2001b). The marked increase in DNA binding activity shown by HES-HERP heterodimers, accompanied by their functional synergy, strongly suggests that HES-HERP heterodimers are likely to form and act in cells co-expressing them. Heterodimerization of the two Notch effectors provides an efficient means for signal amplification.

MUTUALLY EXCLUSIVE EXPRESSION OF HERP1 AND HERP2

Notch signaling is best known for lateral inhibition (Egan et al., 1998; Greenwald, 1998; Artavanis-Tsakonas et al., 1999). Lateral interaction occurs within a population of equivalent cells and results in the generation of different cell types. Once a small difference is created between equivalent cells, a cell that has obtained a specific fate prevents surrounding cells from assuming the same cell fate through cell–cell interaction. This process, termed lateral inhibition or lateral specification, is mediated by Notch signaling and exemplified by *C. elegans* gonadogenesis and *Drosophila* sensory organ development (Egan et al., 1998; Greenwald, 1998; Artavanis-Tsakonas et al., 1999).

In mammals, the Notch pathway dictates cell fate decisions of bipotential precursor cells to generate distinct subpopulations, a phenomenon reminiscent of lateral specification. Examples of such mammalian cell fate decision by Notch include T lymphoid versus B lymphoid cells, pancreatic exocrine versus endocrine cells, and arteries versus veins (Apelqvist et al., 1999; Pui et al., 1999; Lawson et al., 2001).

It is tempting to speculate that HERP1 and HERP2, as direct targets of Notch, might also contribute to dictating cell fate dicisions. As discussed in the earlier section, tissue distribution of HERP1 and HERP2 are often observed in a strikingly complementary fashion within single organs (Table 4) (Leimeister et al., 1999; Nakagawa et al., 1999). For instance, expression of HERP1 mRNA in the heart is limited to ventricles

TABLE 5. Co-expression of HES and HERP

	HES	HERP	References
Embryonic heart	HES1[a] (whole heart)	HERP1 (ventricles) HERP2 (atria)	This paper, Sasai et al. (1992), Leimeister et al. (1999), Nakagawa et al. (1999)
Presomitic mesoderm	HES1, -5, and -7	HERP1, -2, and -3	Kokubo et al. (1999), Nakagawa et al. (1999), Jouve et al. (2000), Leimeister et al. (2000a), Bessho et al. (2001a), Dunwoodie et al. (2002)
Olfactory epithelium	HES1, -3, -5, and -6	HERP1 and -2	Lobe (1997), Kokubo et al. (1999), Nakagawa et al. (1999), Leimeister et al. (1999), Bae et al. (2000), Cau et al. (2000), Vasiliauskas and Stern (2000), Pissarra et al. (2000)
Wisker follicle	HES3	HERP2	Lobe (1997), Leimeister et al. (1999)
Dorsal root ganglia	HES3 and -6	HERP1 and -3	Lobe (1997), Nakagawa et al. (1999), Bae et al. (2000), Leimeister et al. (2000a), Pissarra et al. (2000), Vasiliauskas and Stern, (2000)
Thymus	HES1 and -6	HERP3	Sasai et al. (1992), Nakagawa et al. (1999), Leimeister et al. (2000), Pissarra et al. (2000)

[a]Although those genes listed in the table seem to be expressed within the same organ, it is not certain whether or not they are expressed within the same cells. Based on results of many different reports, the data are combined. All data are derived from in situ hybridization except HES1 expression in an embryonic heart, which is detected only by RNA protection assay.

whereas that of HERP2 is confined to atria in mouse embryos (Fig. 5) (Leimeister et al., 1999; Nakagawa et al., 1999). In the craniofacial region, HERP2 is observed in the whisker follicles while HERP1 is expressed in the surrounding tissue (Leimeister et al., 1999). Given that HERPs are transcriptional repressors, HERPs might play a direct role in establishing distinct cell fates from equi-potential primordial cells by repressing cell-type specific genes. For instance, HERP1 expressed in heart ventricle might eliminate atria-specific gene expression in cells of a future ventricle-forming region to establish ventricular identity during embryonic heart development (Fig. 3). Unlike the HERP family, the HES family does not show such expression patterns, and hence HERP might play a more critical role than HES in establishing subdomains of an organ.

What are the upstream signals that restrict expression of one HERP member or another in cells of a specific subdomain of an organ? Given the multiple Notch ligands and receptors, specific ligands and receptors might be responsible for distinct HERP member expression. In this regard, HES1 expression is induced by co-culture of C2C12 myoblast cells with Dll1 (Delta like 1)—but not with Jagged1-expressing cells (Shawber et al., 1996; Jarriault et al., 1998; Kuroda et al., 1999), suggesting ligand-specific regulation for HES1. Are HERP family members also regulated in a ligand-specific manner? In contrast to HES1, both ligands, Dll1 and Jagged1, equally induce HERP2 mRNA expression in C2C12 myoblast (Iso et al., 2001a). However, ligand- and receptor-specific regulation for HERP members have not been extensively addressed and remains a good possibility.

ROLES OF HERP IN DEVELOPMENT

HERP in angiogenesis

Evidence is mounting that Notch signaling is involved in the development of the vascular system (Ruchoux et al., 1995; Joutel et al., 1996, 2000; Uyttendaele et al., 1996, 2000, 2001; Zimrin et al., 1996; Hrabe de Angelis et al., 1997; Wong et al., 1997; Shen et al., 1997a; Xue et al., 1999; Chin et al., 2000; Krebs et al., 2000; Shutter et al., 2000; Zhong et al., 2000, 2001; Henderson et al., 2001; Lawson et al., 2001; Lindner et al., 2001; Villa et al., 2001). Both HERP1 and HERP2 mRNA are highly expressed in the aorta (Chin et al., 2000; our unpublished observation), and another HERP member HeyL (HERP3) mRNA is also detected in aortic smooth muscle layer of the mouse embryo (Leimeister et al., 2000b). Expression of mouse CHF1 (HERP1) is induced during differentiation of neural crest-derived Moncl cells into vascular smooth muscle cells (Chin et al., 2000). Expression of mRNAs for both HERP1 and HERP2 is induced in cultured smooth muscle cells by stimulation of Notch ligands such as Dll1 and Jagged1 (Iso et al., 2002). In endothelial cells also, Hesr1 (HERP2) mRNA expression is induced during endothelial cell tube formation, a well-characterized in vitro angiogenic process, and gain- and loss-of-function studies show that Hesr1 (HERP2) is involved in proliferation, migration, and network formation of endothelial cells (Henderson et al., 2001).

In zebrafish, the gridlock mutation (grl ^{m145}), originally isolated in a large-scale chemical mutagenesis screen for developmental mutations of the zebrafish, shows an abnormal assembly of the aorta (Zhong et al., 2000). The *grl* gene (HERP1 homologue) is strongly expressed in the dorsal aorta, but not in the axial vein. The grl ^{m145} mutation changes the stop codon to Gly and extends the protein by 44 amino acids at the carboxyl-terminus, resulting in a phenotype showing selective disturbance of assembly of the aorta. Injection of wild type grl RNA in the mutant restores a normal phenotype. In addition, gridlock is required for arterial-venous differentiation during embryonic vascular development (Zhong et al., 2001). Loss of grl function ablates regions of the artery, and expands contiguous regions of the vein.

Collectively, these findings indicate that HERPs are involved in multiple aspects of vascular development including smooth muscle differentiation, angiogenic processes, arterial-venous cell fate determination, and vascular morphogenesis. Given that HERP1 and HERP2 are immediate targets of Notch and that expression of HES genes has not been observed in the vascular system, HERP family members may be the effectors of the Notch pathway in vascular development.

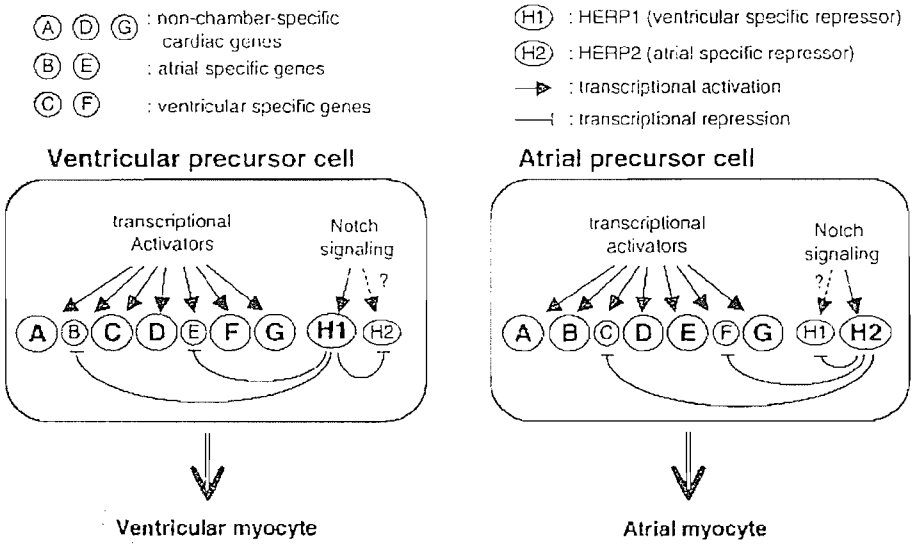

Fig. 3. Model for cardiac chamber formation by HERP1 and HERP2. Cardiac-specific transcriptional activators such as Nkx2.5, MEF2C, GATA4 are expressed in both ventricles and atria, and upregulate expression of cardiac-specific genes (i.e., genes for contractile proteins, genes A to G) in both chambers. Expression of the transcriptional repressors, HERP1 and HERP2, however, are confined to ventricles and atria, respectively. HERP1 expression in the ventricular precursor cells may inhibit expression of atrial-specific genes (B, E, and HERP2). In contrast, HERP2 expression in atrial precursor cells may block expression of ventricle-specific genes expression (C, F, and HERP1). In this manner, HERP1 and HERP2, in combination with the activators, may contribute to defining the atrial and ventricular chambers. HERP1 and HERP2 might mutually repress each others expression.

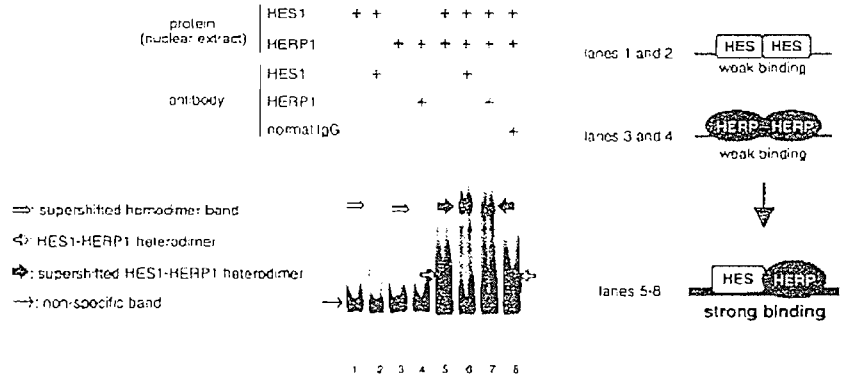

Fig. 4. HES1 and HERP1 form a beterodimer. A representative gel shift assay showing HES1-HERP1 beterodimer formation. A gel shift assay was performed using nuclear extracts from the cells transfected with the expression vectors for HERP1 and HES1. These proteins were incubated with a class C DNA probe. Note that nuclear extracts from the cells expressing both HES1 and HERP1 showed a dramatic increase of DNA binding activity (compare lane 5 with lane 1 or 3). This intense band (lane 5) contains a HES1-HERP1 heterodimer since addition of antibodies against either HES1 or HERP1 shifted this band (lanes 6 and 7). The simultaneous addition of the two antibodies generates a further shifted band (not shown). Homodimers of HES1 (lane 1) or HERP1 (lane 3) are not detectable with this short exposure time, although addition of the antibodies do show up marginally visible supershifted bands (lanes 2 and 4).

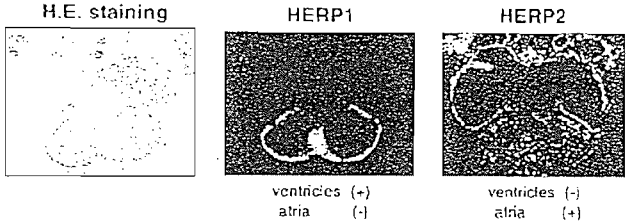

Fig. 5.

Fig. 5. In situ analysis of expression of HERP1 and HERP2 mRNA transcripts. Serial horizontal sections of mouse embryos (E12.0) at heart level were prepared. Hematoxylin eosin (HE) staining (left). Dark field micrographs for mRNA expresion of HERP1 (center) and HERP2 (right). Note that HERP1 mRNA is limited to ventricles while HERP2 mRNA is predominantly expressed in the atria.

HES and HERP in somitogenesis

Somites appear as epithelial spheres in a head-to-tail order in an oscillating fashion from a mesenchymal unsegmented tissue called the presomitic mesoderm (PSM) (Hartenstein et al., 1992; Maroto and Pourquie, 2001). Somites generate the skeletal muscles of the body as well as the axial skeleton and the dermis of the back.

Mutations for Notch1, Dll1, Dll3 and other components of Notch signaling such as presenilin, RBP-Jκ, and lunatic fringe (Lfng) exhibit defects of somite segmentation (Swiatek et al., 1994; Conlon et al., 1995; Oka et al., 1995; Hrabe de Angelis et al., 1997; Wong et al., 1997; Evrard et al., 1998; Kusumi et al., 1998; Zhang and Gridley, 1998; Donoviel et al., 1999; Huppert et al., 2000; Koizumi et al., 2001; Dunwoodie et al., 2002). Recent studies also demonstrate that Notch signaling regulates the synchronously oscillating gene expression in the PSM. Expression of mRNA for c-hairy1, c-hairy2, HES1, HES5 as well as Lfng oscillates in the PSM (Palmeirim et al., 1997; Forsberg et al., 1998; McGrew et al., 1998; Aulehla and Johnson, 1999; Jouve et al., 2000; Dunwoodie et al., 2002). Oscillations of these genes are likely to be involved in a clock linked to somite segmentation. The dynamic expression of HES1 mRNA as well as compartmentalization of somites was disrupted in the Dll1 null mutant (Hrabe de Angelis et al., 1997; Jouve et al., 2000). Mouse Dll3 null mutants also show delayed and irregular somite formation and the disrupted expression of HES1 and HES5 in PSM (Dunwoodie et al., 2002). These findings suggest that Notch signaling regulates the cyclic expression of HES1 and HES5 mRNA and somite formation. However, mutation of the HES1 gene does not recapitulate the Dll1 mutant phenotype (Ishibashi et al., 1995), suggesting that HES1 may not be required for somitogenesis or the HES1 defect can be compensated by other factors.

It has recently been reported that the expression of the newly isolated HES7 gene oscillates in the PSM and that targeted disruption of the HES7 gene in mice resulted in disorganized segmentation of somites and loss of cyclic expression of Lfng in the PSM (Bessho et al., 2001b), indicating essential functions of HES7 in somitogenesis.

All three HERP members also are expressed in the PSM. HRT1 (HERP2) and HRT3 (HERP3) also are expressed in a dynamic pattern in the PSM and somites, but expression of HRT2 (HERP1) is much weaker than HERP2 and HERP3 (Nakagawa et al., 1999). Leimeister et al. (2000a) also showed that expression of mHey2 (HERP1) in the PSM was severely affected in Dll1 and Notch1 knockout mice. They also found that expression of cHey2, a chicken homologue of HERP1 oscillates in the chicken PSM and overlaps precisely with that of c-hairy1 throughout the PSM (Leimeister et al., 2000a). In addition, Dunwoodie et al. (2002) found that normal expression of mHey1 (HERP2) in PSM is disrupted in the Dll3 null mutant.

These findings suggest that all three HERP family members as well as HES1, HES5, and HES7 are likely to play crucial roles in somitogenesis. Analysis of somite formation in HERP null mutant mice is needed to clarify the roles of HERPs in somitogenesis.

HERP in myogenesis

Given the critical roles of Notch signaling in developing somites (see the preceding section HES and HERP in Somitogenesis), one wonders whether Notch signaling might also be involved in muscle differentiation in the myotomal compartment of the somite. Consistent with this idea, mouse HRT1 (HERP2) mRNA is predominantly detected in the dermomyotome within the somite (Nakagawa et al., 1999).

Coculture of C2C12 myoblast cells with Notch ligand-expressing cells blocks muscle differentiation by inhibiting expression of muscle differentiation markers such as myogenin, myosin light chain 1, -2 and -3, α-myoglobin, and troponin T (Lindsell et al., 1995; Luo et al., 1997; Jarriault et al., 1998; Kuroda et al., 1999). Whereas Notch-ligand stimulation induces strong and continuous HERP2 mRNA expression in C2C12 cells (Iso et al., 2001a), HES1 mRNA expression was only transiently and very weakly induced in these muscle cells (Shawber et al., 1996; Jarriault et al., 1998; Kuroda et al., 1999; Iso et al., 2001a). Furthermore, forced expression of HES1 did not inhibit muscle differentiation of C2C12 cells (Nofziger et al., 1999). These findings suggest that HERP2 may play a more important role than HES1 in inhibiting muscle differentiation. To further support this idea, Sun et al. (2001b) showed that CHF2 (HERP2) mRNA is expressed at a high level in undifferentiated C2C12 myoblast cells, but it declines as muscle differentiation proceeds. They further show that exogenously overexpressed CHF2 may inhibit MyoD-induced myogenic conversion of 10T1/2 cells by associating with MyoD and inhibiting DNA binding of MyoD-E47 heterodimers (Sun et al., 2001b). These data strongly suggest a potential role of HERP2 as a negative regulator of muscle differentiation, although further loss-of-function studies for HERP2 are necessary to demonstrate that HERP2 is a true effector of the Notch pathway in inhibiting muscle differentiation.

HES and HERP in gliogenesis

Notch signaling is generally thought to inhibit the differentiation of neural precursor cells and keep them in an undifferentiated state. However, this notion has recently been challenged (Wang and Barres, 2000; Frisen and Lendahl, 2001). Several groups have reported that Notch1 and its effectors, HES1 and HES5, may play an instructive role in actively promoting gliogenesis rather than simply inhibiting neuronal differentiation (Furukawa et al., 2000; Gaiano et al., 2000; Hojo et al., 2000; Morrison et al., 2000). For example, cultured neural crest stem cells (NCSCs) generate Schwann cells with a high frequency when Notch is activated, either by an active form of Notch or by soluble ligands (Morrison et al., 2000). An instructive role of Notch in gliogenesis has also been reported during retinogenesis. Furukawa et al. (2000) reported that retinal progenitor cells overexpressing either the NICD or HES1 by retroviral infection differentiated into Muller glia cells (MGCs), while forced expression of a dominant-negative HES1 gene reduced the number of glia. In another report, HES5-deficient retina showed a 30–40% decrease in MGC numbers without affecting

cell survival, whereas forced expression of HES5 by retrovirus significantly increased the population of glial cells at the expense of neurons (Hojo et al., 2000). These findings suggest an active role of Notch in gliogenesis, which may be mediated by HES family members.

Hesr2 (HERP1) may also mediate Notch signaling in retinal gliogensis (Satow et al., 2001). Hesr2 (HERP1) is predominantly expressed in the middle region of the inner nuclear layer of the retina, which mainly contains MGCs and other cell types. In retinal explant studies, retroviral expression of exogenous Hesr2 (HERP1), but not Hesr1 (HERP2) or Hesr3 (HERP3), promoted gliogenesis while inhibiting neurogenesis (Satow et al., 2001), indicating a specific role of HERP1 in promoting gliogenesis. Consistent with an active role of HERP1 in retinal gliogenesis, a double mutation of neuronal determination genes, Mash1 and Math3, led to an increase in the number of MGCs and upregulation of Hesr2 (HERP1) expression (Satow et al., 2001). These results suggest that Hesr2 (HERP1) may be a Notch effector in promoting retinal gliogenesis.

It should be noted, however, that whether the gliogenesis by Notch is a purely instructive or default mechanism (due to inhibition of neurogenesis), is not completely established. If it turns out to be an instructive effect of Notch, a central issue in the future is to determine the molecular targets and specific effectors of Notch signaling that result in gliogenesis.

REGULATION OF HES AND HERP GENE EXPRESSION BY NOTCH SIGNALING

HES and HERP as a primary target of notch signaling

Several lines of evidence have suggested that HES-1, -5, -7, and HERP-1, -2, -3 are potential target genes of Notch (Figs. 2A and 6). The promoters of HES1, -5, and -7 as well as those of HERP1, -2, and -3 are activated by the constitutively active NICD in transiently transfected reporter gene assays (Jarriault et al., 1995; Nishimura et al., 1998; Maier and Gessler, 2000; Nakagawa et al., 2000; Bessho et al., 2001a; Iso et al., 2002). In addition, endogenous HERP-1 and -2 mRNA expression (but not HES1) were strongly upregulated after the NICD expression in several different cell types including C2C12, 10T1/2, 293T, and U2OS cells (Iso et al., 2001a). Although these results suggest that these members of the HES and HERP families may be potential targets of Notch, the question of whether they are the direct and physiological targets of the NICD-RBP-Jκ complex remains unsolved by these studies using overexpressed NICD. It is formally possible that HES and HERP expression may be upregulated secondary to expression of other genes after NICD overexpression. Further, overexpressed NICD may cause physiologically irrelevant cellular reactions. This latter concern is substantiated by the observation that endogenous NICD is hardly detected by immunostaining, whereas transfected NICD is expressed at a readily detected level (Lieber et al., 1993; Kopan et al., 1994; Nye et al., 1994; Ahmad et al., 1995; Jarriault et al., 1995; Zagouras et al., 1995; Capobianco et al., 1997).

To circumvent these potential concerns associated with overexpression studies, several groups used a co-culture approach with Notch ligand-expressing cells, which is considered to generate a more physiological level of Notch signaling (Shawber et al., 1996; Jarriault et al., 1998; Kuroda et al., 1999; Iso et al., 2001a). Use of cyclohexamide, an inhibitor of de novo protein synthesis in combination with the co-culture system eliminates secondary effects by expression of other proteins, and allows one to assess direct effect of Notch signaling on upregulation of HES and HERP mRNA (Kuroda et al., 1999; Iso et al., 2001a, 2002). These studies have now provided strong evidence that HES1, HERP1, and HERP2 are primary targets of Notch in tissue culture.

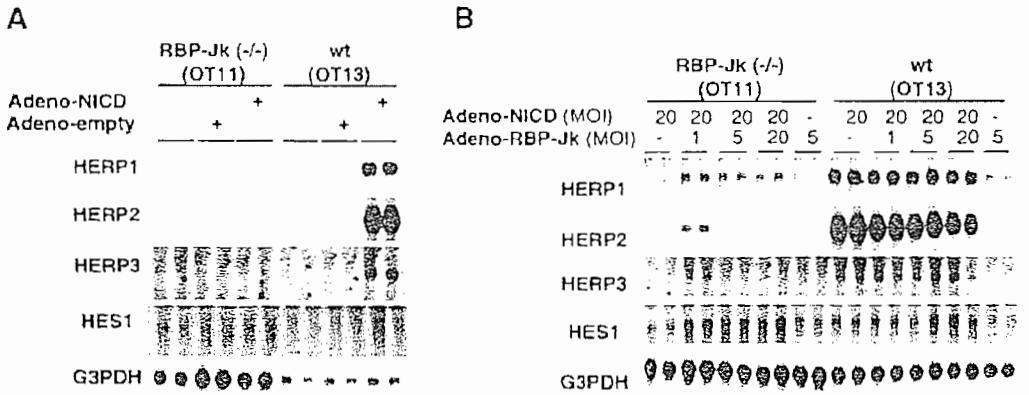

Fig. 6. A: Constitutively active Notch fails to induce HERP and HES mRNA expression in RBP-Jκ-deficient OT11 cells. RBP-Jκ-deficient OT11 (derived from mice with targeted RBP-Jκ gene disruption) or control OT13 (RBP-Jκ positive) cells were infected with recombinant adenovirus expressing NICD (Adeno-NICD) or control virus (Adeno-empty). Northern blot analysis was performed with RNA from duplicate infections. Note the robust induction of HERP1 and HERP2 mRNA, weak induction of HERP3, and marginal induction of HES1 by Adeno-NICD only in OT13 cells, but not in OT11 cells. B: Restoration of HERP and HES mRNA induction by exogenous RBP-Jκ expression in RBP-Jκ-deficient OT11 cells. OT11 and OT13 cells were infected with adenovirus expression vectors at the indicated multiplicity of infection (MOIs). Note that HERP1 and HERP2 mRNA induction is restored in OT11 cells only when Adeno-RBP-Jκ is co-infected with Adeno-NICD. Expression of HERP3 and HES1 mRNA are less conspicuous in these cells. Thus the expression of RBP-Jκ protein was sufficient to rescue HERP and HES mRNA induction by Notch in RBP-Jκ-deficent OT11 cells.

Tissue-specific expression of HERP1

Expression of HERP1 was not induced by ligand stimulation in any of the several cell types initially tested including C2C12 muscle cells, 10T1/2 fibroblasts, 293T, and P19 teratocarcinoma cells ((Iso et al., 2001a) and our unpublished results). Because of this observation, we initially thought that HERP1 may not be an immediate target of Notch or that the HERP1 gene might be under cell type-specific regulation. The observation that zebrafish gridlock (HERP1) plays a central role in the development of the aorta (Zhong et al., 2000) suggested a cell type specific role for HERP1 in vascular tissue. Consistent with this, when an aortic smooth muscle cell line, A10, was used in coculture studies, expression of endogenous HERP1 (as well as HERP2) mRNA is induced by Notch in the absence of de novo protein synthesis (Iso et al., 2002). Since HERP1 mRNA expression is detected in multiple tissues in mice embryos, its role may not be limited to vascular cells. However, the absence of HERP1 mRNA induction in several different kinds of cells described above suggests that HERP1 might have a more cell-type-restricted role (i.e., vascular tissue) than does HERP2.

Mutual repression between members of the HERP family

Notch stimulation engendered by coculturing cells with Notch ligand-expressing cells can induce expression of both HERP1 and HERP2 mRNA in A10 smooth muscle cells (Iso et al., 2002). Interestingly, the time courses of these induced expression patterns are different between the two. Upon co-culture, both HERP1 and HERP2 mRNAs simultaneously start to accumulate, and yet they accumulate in a very different manner. HERP2 mRNA rapidly accumulates, reaches a plateau at 8 h that is sustained for at least 24 h. In contrast, HERP1 mRNA expression is transient. The HERP1 mRNA level rapidly decreases after reaching a peak at 6 h, and returns to basal levels by 8 h, as if HERP2 suppresses HERP1 mRNA accumulation. The decrease of HERP1 mRNA expression in the face of increasing levels of HERP2 mRNA is somewhat reminiscent of the mutually exclusive expression of HERP1 and HERP2 reported in several subdomains of different organs observed in animals (Leimeister et al., 1999; Nakagawa et al., 1999). These results raise the possibility that HERP1 and HERP2 might mutually suppress each others expression. Consistent with this idea is the finding that overexpressed HRT1 (HERP2) inhibits HRT2 (HERP1) promoter activity in reporter gene assays in transient transfection studies (Nakagawa et al., 2000) (our unpublished data). Further studies are needed to examine whether this attractive model (see also Fig. 3) is valid in vivo.

Linkage among specific Notch ligands, receptors, and effectors

The finding that HERP can be induced in a cell-type specific manner raises a question of how different members of HES and HERP are differentially regulated by Notch. There are six Notch ligands (Dll1–4, Jagged1, and Jagged2), six receptors (Notch 1–6), and six known target genes (HES-1, -5, -7, and HERP 1–3). The existence of such multiple components at each step of Notch signaling leads one to speculate that different ligands, for instance, might be linked to distinct receptors and effectors. However, tissue distribution of these components does not immediately support their specific relationships (Lindsell et al., 1996; de la Pompa et al., 1997; Leimeister et al., 1999; Nakagawa et al., 1999).

As discussed earlier, HERP1 mRNA was induced by Notch ligand stimulation only in A10 aortic smooth muscle cells, but not in several other cells tested, whereas HERP2 mRNA was induced in all these cell lines (Iso et al., 2001a, 2002). All these cells express at least Notch-1, -2, and -3 receptors (Iso et al., 2001a, 2002), and yet only A10 smooth muscle cells express HERP1 mRNA, indicating that the selective induction of HERP1 in A10 cells is not due to selective expression of the three Notch receptors. Other cellular components of Notch signaling necessary to activate HERP1 may be present only in A10 cells but not in the other cells. Recently, additional receptors, Notch-5 and -6 have been isolated in zebrafish (GeneBank accession number; Y10353 for Notch5 and Y10354 for Notch6) (Sawada et al., 2000). It remains to be studied whether mammalian homologues of these new Notch receptors as well as Notch4 might participate in the cell-type specific expression of HERP1.

Analyses of mice deficient for a component of the Notch pathway have also provided insight regarding the relationship between specific Notch ligands, receptors, and effectors. Gene disruption of Notch ligand Dll1 causes a decrease in expression of HERP1, -2, and -3 in mice (Leimeister et al., 2000a,b). In Notch1 deficient mice, expression of HES5, HERP1, -2, and -3 were greatly diminished in various tissues while HES1 expression was not affected in any tissue (de la Pompa et al., 1997; Leimeister et al., 2000a,b). In Notch2 null mutant mice, neither HES1 nor HES5 expression was altered (Hamada et al., 1999). In mice deficient for Dll3, normal expression of HES1, HES5, and Hey1 (HERP2) was disrupted in PSM (Dunwoodie et al., 2002). Mice homozygous for RBP-Jκ gene disruption exhibits reduced expression of HES5, but not HES1 (de la Pompa et al., 1997). These findings indicate that a single Notch ligand or receptor participates in the upregulation of multiple members of the HES and HERP families. It should be kept in mind, however, that expression of HES and HERP genes was not completely abolished in these mutants, suggesting that there are alternative pathway(s) to maintain their expression in animals. The unaltered HES1 expression in most of these mutants mice, despite the well-documented function of HES1 as a Notch effector, suggests that the defects of these genes may be compensated by other receptors, ligands, or an unidentified RBP-Jκ-independent pathway for HES1 expression.

Clarifying these relationships between ligands, receptors, and target genes is one of key issues to understand tissue-specificity in Notch signaling, and continued efforts toward discovery of new Notch components and their characterization are necessary toward this end.

ROLE OF RBP-Jκ IN HES AND HERP GENE EXPRESSION

It has recently been shown that RBP-Jκ is a protein component of a large native NICD complex in the nucleus (Jeffries et al., 2002). The activated Notch receptor, NICD has a domain termed RBP-Jκ associated module (RAM), through which NICD associates with RBP-Jκ (Tamura et al., 1995). The RAM domain of NICD is required for activation of target gene promoters in reporter gene assays (Nofziger et al., 1999; Iso et al., 2002). Mutations of RBP-Jκ binding sites in the promoters of HERP1, HERP2, and HES1 genes abolish activation of their promoters by Notch (Jarriault et al., 1995; Nishimura et al., 1998; Maier and Gessler, 2000; Nakagawa et al., 2000; Iso et al., 2002). These findings suggest a critical role of RBP-Jκ in the target genes regulation by Notch.

To further address the role of RBP-Jκ protein in the expression of endogenous HERP and HES mRNAs, several groups employed a mutant RBP-Jκ (RBP-Jκ R218H) that does not bind DNA and has been considered as a dominant negative mutant (Kato et al., 1997; Nofziger et al., 1999; Nakagawa et al., 2000). In *Xenopus* oocytes, expression of the dominant negative forms of X-Su(H)1, a *Xenopus* homologue of RBP-Jκ, leads to a neurogenic phenotype with an increased number of primary neurons, whereas overexpression of wild type X-Su(H)1 did not significantly alter neuronal phenotype in vivo (Wettstein et al., 1997). In cultured mammalian cells also, transactivation of HERP1 and HES1 promoters by NICD was reduced by overexpressing the RBP-Jκ mutants, R218H and RY227GS, which lack DNA binding activity (Iso et al., 2001a). In addition, Xenopus ESR1 (a Notch target closely related to HES5) mRNA induction by Notch was reduced by the dominant negative form of X-Su(H) in oocytes (Wettstein et al., 1997). In these two studies, however, even wild type X-Su(H) or RBP-Jκ/CSL has a similar degree of inhibitory effects. This may be due to uncontrolled expression of RBP-Jκ/X-Su(H) that squelches the signaling molecules and disrupts their stoichiometry, and raises a concern regarding the specificity of the putative dominant negative RBP-Jκ and the role of wild-type RBP-Jκ in expression of HES or HERP.

To consolidate the role of RBP-Jκ protein in HERP and HES mRNA expression, RBP-Jκ deficient cells (OT11) derived from homozygous RBP-Jκ null mice were employed (Iso et al., 2001a). As shown in Figure 6A, constitutively active NICD expressed by recombinant adenovirus vector failed to induce HERPs and HES1 mRNA expression in the RBP-Jκ-deficient OT11 cells. Induction of their expression is restored by simply providing exogenous RBP-Jκ protein in these cells (Fig. 6B). Thus, the absence of HERP and HES mRNA induction in OT11 cells is not due to inadvertent phenotypic changes in these cells; rather it is a direct consequence of the lack of RBP-Jκ. These findings established that RBP-Jκ is essential for HES and HERP expression in response to Notch stimulation.

ARE HES AND HERP THE ONLY PRIMARY TARGETS OF NOTCH?

A group of recently isolated genes, Stra13/SHARP/DEC genes (hereafter DEC family) have amino acid sequences most similar to those of HES among the known bHLH proteins (Fig. 2A) (Boudjelal et al., 1997; Rossner et al., 1997; Shen et al., 1997b; Fujimoto et al., 2001). DEC has a proline residue in the basic domain (not in the same position as HES) and the Orange domain. Davis and Turner named HES, HERP, and DEC families as bHLH-O proteins, as they represent a unique family of bHLH proteins bearing the Orange domain (Davis and Turner, 2001) (Fig. 2D). DEC functions as a transcriptional repressor through class B DNA sequences (Zawel et al., 2002) and is involved in the control of proliferation and/or differentiation of chondrocytes and neurons as well as hematopoiesis (Boudjelal et al., 1997; Rossner et al., 1997; Shen et al., 1997b; Sun et al., 2001a; Seimiya et al., 2002). These features of DEC make one wonder whether DEC might be a primary target of Notch. DEC expression is induced by several stimuli including hypoxia, cAMP, and TGF-β (Shen et al., 1997b, 2001; Ivanova et al., 2001; Yoon et al., 2001; Yun et al., 2002; Zawel et al., 2002). However, there is no evidence that DEC is upregulated by Notch. Nevertheless, given the similarities between DEC and HES, the possibility remains that DEC might be a Notch target in other contexts than have been tested.

Recently, it has been reported that promoter regions of a number of genes other than HES and HERP families contain RBP-Jκ binding sites, including MHC class I, CD23, interleukin6, β-globin, erbB-2, NF-kB2, and cyclin D1 genes (Israel et al., 1989; Ling et al., 1994; Shirakata et al., 1996; Chen et al., 1997b; Kannabiran et al., 1997; Plaisance et al., 1997; Lam and Bresnick, 1998; Oswald et al., 1998; Ronchini and Capobianco, 2001). The cyclin D1 promoter which has a poorly conserved consensus sequence, GCTGAGAT, is bound by RBP-Jκ in electrophoretic mobility shift assay, and cyclin D1 mRNA expression was upregulated by overexpressed NICD (Ronchini and Capobianco, 2001). Thus, the cyclin D1 gene is likely an immediate target of the NICD-RBP-Jκ complex. In the other genes mentioned above, however, the implication of RBP-Jκ in their transcription has been suggested only by electrophoretic mobility shift assays and reporter gene assays, without demonstrating induction of their mRNA expression by Notch. Conversely, induction of endogenous mRNA following overexpression of NICD has been shown in some genes, such as CD21 and NRARP (Notch related ankyrin repeat protein), although promoter analyses of these genes have not yet been reported (Strobl et al., 2000; Krebs et al., 2001; Lamar et al., 2001). Therefore, further studies, including those with ligand stimulation in addition to NICD overexpression, are awaited to determine whether these genes are directly regulated by the NICD-RBP-Jκ complex in vivo, and whether protein products of some of these putative Notch target genes might act as an effector for Notch signaling. Nevertheless, these findings are concordant with the idea that HES and HERP may not be the only primary target or effector for the Notch signaling.

ARE HERPs PHYSIOLOGICAL EFFECTORS OF NOTCH?

As we have seen, HES1, -5, -7 and HERP1, -2, -3 are likely target genes of Notch. Among these HES and HERP members, HES1 and HES5 are true effectors of Notch in animals. Kageyama's group has shown that inhibitory effect of Notch signaling on neuronal differentiation was abolished in neural precursor cells prepared from HES1–HES5 double deficient mice ($HES1^{-/-}/HES5^{-/-}$), but not in cells from either HES1 ($HES1^{-/-}$) or HES5 ($HES5^{-/-}$) single mutants, indicating that HES1 and HES5 have redundant functions in mediating the Notch effects (Ohtsuka et al., 1999). These findings, together with induction of HES1 and HES5 mRNA by Notch, provide strong evidence that HES1 and HES5 are effectors of Notch.

That HERP may be a physiological Notch effector is supported by the following observations: 1) HERP mRNA expression is directly up-regulated by Notch ligand stimulation in the absence of de novo protein synthesis in cultured cells, 2) HERP has an intrinsic transcriptional repressor activity and forms a heterodimer with HES1, the established Notch effector, and 3) the HES-HERP heterodimer binds the same group of target DNA sequences as HES homodimer but with much higher efficiency. However, physiological relevance of these observations needs to be confirmed in target gene promoters.

CONCLUSIONS AND FUTURE DIRECTIONS

In this review, we summarized similarities and differences between HES and HERP in molecular structure, repression mechanisms and tissue distribution. Several members of both families are primary target genes of Notch (Figs. 2, 6, and 7). They are directly induced by Notch ligand stimulation in the absence of de novo protein synthesis, and some of them in a cell type-specific manner. Although both HES and HERP families possess very similar domains, belong to class C bHLH protein families and function as transcriptional repressors, they utilize distinct domains and corepressors to repress transcription (Figs. 2 and 7).

The differential expression of HES and HERP in different tissues suggests that HES and HERP may work separately as the respective homodimers. In other tissues where both of them are co-expressed, Notch signaling may rely on heterodimer formation between HES and HERP with distinctive repression mechanisms (Fig. 7). Because both HES and HERP are conserved from *Drosophila* to human, the heterodimerization of HES-HERP is likely to be conserved throughout evolution. In vivo target genes for HES and HERP have not been firmly established except for a few cases (Ishibashi et al., 1995; Chen et al., 1997a), and identification of target genes for a HES-HERP heterodimer as well as HERP homodimers is among the most important issues to be addressed in the future. We have discussed the possibility that the two HERP isoforms, HERP1 and HERP2, might regulate subdomain-specific gene expression within a single organ, such as cardiac ventricle versus atrium (Fig. 3). Identification of such HERP isoform-specific target genes may greatly facilitate our understanding how distinct subdomains of a single tissue or organ (i.e., cardiac chamber formation) is established.

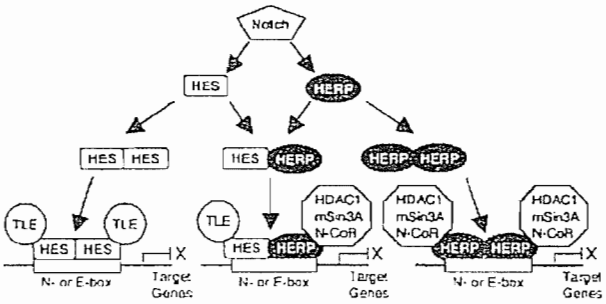

Fig. 7. Model for HES and HERP cooperation in Notch signaling. Upon notch stimulation, HES and HERP expression may both be induced. In tissues where only HES or HERP is expressed, the respective homodimer binds promoters of target genes. The HES homodimers recruit TLE via their C-terminal WRPW motif, whereas the HERP homodimers recruit an N-CoR/mSin3A/HDAC complex via their bHLH domain. In tissues where both HES and HERP are coexpressed, the HES-HERP heterodimers become the predominant complex that avidly binds a specific DNA site, which may be newly defined by the two heterologous basic domains of HES and HERP. Because of the higher DNA binding activity of the heterodimers, a lower concentration of HES and HERP may be sufficient to achieve repression. Repression by HES-HERP heterodimers may be reinforced by their ability to recruit a more diverse set of corepressors including both TLE and N-CoR/mSin3A/HDAC. The model is based on experimental data derived largely from HES1 and HERP1. Because of the significant similarity among members of each family, however, such a model may be apt for other members including those in human, mouse, and *Drosophila*. The association of HERP with N-CoR is based on co-transfection and GST-pull down studies (Iso et al., 2001b). N-CoR has been recently shown to associate with HDAC3 (Wen et al., 2000; Guenther et al., 2001), but not with the Sin3/HDAC1 complex (Guenther et al., 2000; Li et al., 2000). Further studies are under way to clarify the significance of the N-CoR interaction with HERP. See text for further discussion.

Ever increasing numbers of isoforms of Notch components—ligands, receptors, and effectors—are certainly adding complexities to Notch signaling. Each ligand isoform, for instance, may be linked to only specific isoforms of receptors, which in turn may be linked to particular effector isoforms. Such a link among specific isoforms of ligand, receptor, and effector might create cell-type specific sub-pathway of Notch signaling, and contribute to generation of distinct cell fates, provided that different effector isoforms regulate distinct sets of target genes.

This hypothesis is supported partly by the tissue-specific distribution of different isoforms of Notch components. For instance, HERP1 appears to be important particularly in the development of vascular tissue, and HERP1 might be regulated by vascular-specific isoforms of ligands and receptors such as Dll4 and Notch4/int-3 that are predominantly expressed in vascular endothelial cells (Uyttendaele et al., 1996; Shirayoshi et al., 1997; Shutter et al., 2000). Distinct functions of each isoform in animals are clearly demonstrated at least for Notch receptors and ligands, by the gene disruption studies for three receptors (Notch1, Notch2, and Notch4) and four ligands (Dll1, Dll3, Jagged1 and Jagged2). Mice with a mutation of one of these genes show different phenotypic changes, indicating distinct roles of

the isoforms (Swiatek et al., 1994; Conlon et al., 1995; Hrabe de Angelis et al., 1997; Sidow et al., 1997; de la Pompa et al., 1997; Jiang et al., 1998; Kusumi et al., 1998; Hamada et al., 1999; Xue et al., 1999; Krebs et al., 2000; McCright et al., 2001; Dunwoodie et al., 2002).

The formation of HES-HERP heterodimers might also allow cells to regulate different target genes than the respective homodimers, by redirecting target DNA binding specificity, although such a change was not obvious in gel-shift assays (Iso et al., 2001b). Alternatively, quantitative differences in degrees of affinity of target genes by homodimers versus heterodimers might also lead to different phenotypes.

HES and HERP have been viewed as primary targets/effectors of Notch. However, we have detected a low level of HES1 and HERPs mRNAs even in the absence of RBP-Jκ in cultured cells. Consistently, RBP-Jκ null mice show no change in spatial distribution of HES1 transcripts (de la Pompa et al., 1997). These results make one wonder whether Notch signaling can express these effectors by means other than RBP-Jκ, or whether there are Notch-independent mechanisms for HES and HERP expression. In this regard, there are several examples of cross-talk between Notch and other signaling pathways (Axelrod et al., 1996; Guan et al., 1996; Price et al., 1997; Ordentlich et al., 1998; Oswald et al., 1998; Bash et al., 1999; Carmena et al., 2002; Chu et al., 2002), and such signals other than Notch might be involved in the regulation of HES1 and HERPs expression.

Finally, mutations of Notch components are implicated in human diseases such as acute T cell lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991), cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Joutel et al., 1996), and Alagille syndrome (arteriohepatic dysplasia manifest by a paucity of biliary ducts in the liver and a variety of cardiovascular abnormalities including the great vessels) (Oda et al., 1997). These diseases are due to mutations of Notch ligands and receptors. Unregulated expression of components of Notch is also related to many malignant tumors (Zagouras et al., 1995; Daniel et al., 1997; Chen et al., 1997a; Gray et al., 1999; Jang et al., 2000; Ito et al., 2001; Shou et al., 2001). Given that certain HES and HERP members are Notch target/effectors, one would wonder whether misexpression of HES and HERP genes might also be involved in the etiology of human diseases. HERP genes have been mapped by fluorescence in situ hybridization both in mouse and human, and HERP mutations have already been screened in several candidate diseases whose genetic loci map in the vicinity of the chromosomal location of HERP genes. However, initial screening did not reveal any diagnostic alterations in the coding region (Steidl et al., 2000).

Continued efforts to dissect the regulatory mechanisms of HES and HERP as well as discovery of new factors in the Notch pathway should lead us to a deeper understanding of Notch signaling. Such information would hopefully lead, eventually, to the development of diagnostic and therapeutic modalities for Notch-related diseases. This quest will be greatly facilitated as the Human Genome Project approaches completion over the next several years.

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The many faces of REST oversee epigenetic programming of neuronal genes

Nurit Ballas and Gail Mandel

Nervous system development relies on a complex signaling network to engineer the orderly transitions that lead to the acquisition of a neural cell fate. Progression from the non-neuronal pluripotent stem cell to a restricted neural lineage is characterized by distinct patterns of gene expression, particularly the restriction of neuronal gene expression to neurons. Concurrently, cells outside the nervous system acquire and maintain a non-neuronal fate that permanently excludes expression of neuronal genes. Studies of the transcriptional repressor REST, which regulates a large network of neuronal genes, provide a paradigm for elucidating the link between epigenetic mechanisms and neurogenesis. REST orchestrates a set of epigenetic modifications that are distinct between non-neuronal cells that give rise to neurons and those that are destined to remain as nervous system outsiders.

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Introduction

Epigenetic regulation is a compelling mechanism for controlling developmental events [1,2]. In this form of regulation, distinct patterns of gene expression are inherited by chromatin modifications, such as DNA and histone methylation, that do not involve changes in DNA sequence. Neurogenesis, a process central to vertebrate development, requires the acquisition of neural cell fates within the developing nervous system and, in parallel, maintenance of non-neural cell fates outside the nervous system [3]. These two complementary events must be coordinated precisely for correct formation of the nervous system. Furthermore, neurogenesis requires that, within the developing nervous system, only post-mitotic neurons will express neuronal genes, because neural stem cells or progenitors have not yet committed to a neural lineage [4]. These requirements raise the fundamental question of how neuronal gene chromatin is epigenetically programmed in different cellular contexts. How, for example, does neuronal gene chromatin in non-neural cells, where neuronal genes are never expressed, compare to that in neurons where these genes are expressed? In multipotent neural stem or progenitor cells, neuronal genes are repressed, but the cells have the capacity for subsequent expression in response to a developmental signal. Does neuronal gene chromatin in the progenitors reflect a state that is intermediate between suppression and activation, or is there a switch between a silenced and active state upon differentiation? Finally, what is the status of neuronal gene chromatin in pluripotent embryonic stem (ES) cells that have the unique capacity to differentiate into all cell lineages of the developing embryo?

For the establishment of epigenetic modifications representing distinct stages of differentiation, chromatin modifiers, such as DNA methyltransferases, histone methyltransferases and histone acetyltransferases, are recruited to specific genomic loci by DNA binding proteins, either repressors or activators [5]. A compelling candidate for orchestrating epigenetic events is the DNA binding protein, REST (RE1 silencing transcription factor; also called NRSF). REST was discovered in 1995 as a repressor of neuronal genes containing a 23 bp conserved motif, known as RE1 (repressor element 1 or NRSE) [6,7]. Several lines of evidence now point to REST as a key protein for regulating the large network of genes essential for neuronal function [8]. Here, we discuss the most recent studies on epigenetic mechanisms, orchestrated by REST, that characterize specific stages of mammalian neurogenesis.

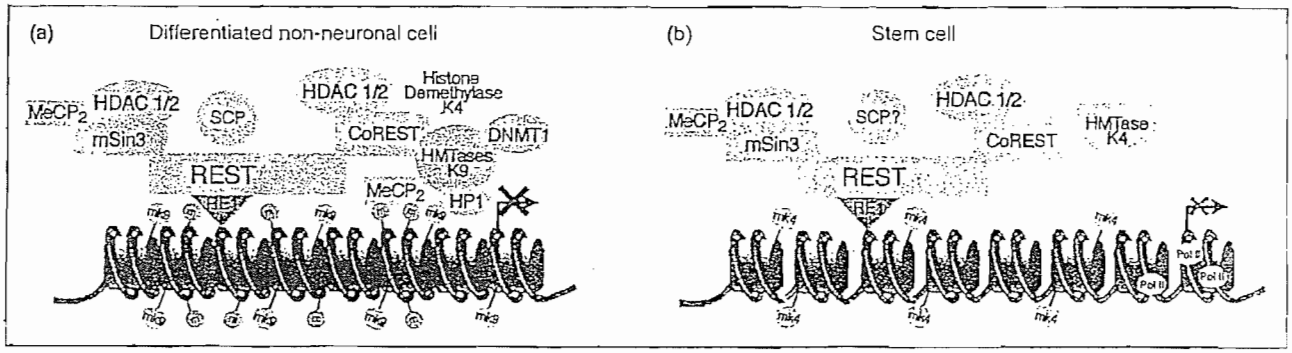
Wiring a genetic network for permanent silencing of neuronal genes outside the nervous system

REST is obligatory for the correct development of vertebrates, because perturbation of REST expression or function in the developing embryo results in ectopic expression of neuronal genes in non-neuronal tissues and early embryonic lethality [9]. In terminally differentiated non-neuronal tissue, neuronal genes are presumably in a long-term silencing state. How does REST direct this mode of silencing? The answer lies in part with its signature functional domains. REST harbors three functional domains: a DNA binding domain containing eight zinc-finger motifs that binds to the RE1 motif, and two independent repressor domains one

located at the amino- and one at the carboxy- terminus of the protein [10]. The amino terminal repressor domain interacts with mSin3, a corepressor found in all eukaryotes that recruits histone deacetylases (HDACs) [11–14]. The mSin3–HDAC complex, however, is associated primarily with a dynamic mode of repression that can alternate between repression and activation and, therefore, by itself, would probably be inadequate for long-term silencing of neuronal genes. This conundrum was solved by the discovery of the corepressor CoREST, which interacts directly with the carboxy terminal repressor domain of REST [15,16] and, similar to mSin3, exists stably in complexes with HDACs [16–18]. Interestingly, unlike mSin3, CoREST is present only in organisms with a nervous system [19], pointing to CoREST as a more specialized corepressor. Several recent studies indicate that the REST–CoREST complex recruits chromatin modifiers for long-term silencing of neuronal genes [20–22] (Figure 1a). Specifically, CoREST can form immuno-complexes not only with HDACs but also with the histone H3 lysine 9 (H3–K9) methyltransferase G9a [23] and with the newly discovered histone H3 lysine 4 (H3–K4) demethylase LSD1 [24••] (that is also known as KIAA0601 or BHC110) [25], both of which mediate modifications associated with gene silencing. Importantly, these histone-modifying enzymes are required for REST–CoREST silencing in non-neuronal cells [22,24••]. Furthermore, CoREST recruits to the REST–RE1 site other silencing machinery, including the methyl DNA-binding protein MeCP2 and the histone H3–K9 methyltransferase SUV39H1 [21]. Heterochromatin protein 1 (HP1), which causes compaction of chromatin and is associated with histone H3–K9 methyltransferases, is also present on the neuronal gene chromatin [21], specifically on the RE1 region [22]. The effects of these modifications are manifested in histone deacetylation, an absence of H3–K4 methylation, and presence of H3–K9 methylation, which creates binding sites for HP1 and condensation of the targeted chromatin (Figure 1a). Additionally, the recruitment of silencing machinery by REST–CoREST might result in the propagation of silencing across a large chromosomal interval containing several neuronal genes that do not have their own REST binding sites [21], suggesting a relationship between higher order chromatin structure and patterns of gene expression.

The methylation of cytosine residues in CpG dinucleotides in the genome represents an additional epigenetic modification of biological importance [26]. DNA methylation can interfere with transcription by repulsing or attracting DNA binding proteins. The REST binding site (RE1) contains a CpG dinucleotide and recent studies reveal that the RE1 and surrounding region of neuronal genes is methylated in differentiated non-neuronal cells [27••]. Furthermore, the DNA methyltransferase DNMT1, which interacts with histone H3–K9 methyltransferases [28], is associated with the RE1 region of neuronal gene chromatin (J Chenoweth and G Mandel, unpublished). Binding of REST to the RE1 site, how-

Figure 1



REST–CoREST orchestrates differential epigenetic mechanisms to inactivate neuronal genes in non-neuronal cells. (a) REST–CoREST recruits a silencing complex to neuronal genes in terminally differentiated non-neuronal cells. Neuronal gene chromatin is a substrate for chromatin modifying enzymes including histone deacetylases (HDAC 1,2), histone H3 lysine 4 demethylase (histone demethylase K4), and histone H3–K9 methyltransferases (HMTases K9). Methylated lysine 9 residues (mK9) are binding sites for heterochromatin protein 1 (HP1), which causes chromatin condensation. The REST binding site (RE1) and adjacent region is methylated at CpGs (m) and associated with the methyl DNA binding protein MeCP2. MeCp2 is also associated with Sin3–HDAC complexes. DNA methyltransferase 1 (DNMT1) is recruited to the methylated RE1 site. The small carboxyl terminal domain (CTD) phosphatase (SCP) might block RNA polymerase II activity. (b) REST–CoREST–mSin3 recruits a repressor complex to neuronal genes in embryonic stem and progenitor cells. HDAC 1 and 2 are predominant modifiers mediating repression of neuronal genes. RNA polymerase II (Pol II) is associated with neuronal gene chromatin probably because of a relatively low state of chromatin compaction (compare a with b). The RE1 sequence and adjacent region is not methylated and histones are marked by methylation of K4 (mK4). The presence of methylated K4 suggests that histone H3–K4 methyltransferase (HMTase K4) is probably present on the RE1 site. The presence of SCP is not confirmed, but functional studies suggest it is probably present on the RE1. SCP could function to keep Pol II minimally active.

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REST Acts through Multiple Deacetylase Complexes

The RE1 binding silencer protein REST represses neuronal-specific gene expression in nonneuronal cell types. In this issue of *Neuron*, Ballas et al. show that REST inhibits gene expression via the recruitment of multiple histone deacetylase complexes.

As our understanding of cell fate determination in the developing nervous system grows, it has become clear that negative factors as well as positive regulators play important roles in cell fate specification. One such negative regulator is the RE1 binding silencer protein REST (also called NRSF), which acts to repress transcription of a class of neuronal genes in both neural progenitors and nonneuronal cell types. The REST protein contains nine noncanonical zinc finger domains and is related to the Gli-Krüppel family of transcriptional repressors. REST was initially identified as a factor from nonneuronal cell types which binds to a negative *cis*-acting element (repressor element-1 [RE1]) within the promoters of both the type II sodium (*NaII*) channel and *SCG10* genes (Chong et al., 1995; Schoenherr and Anderson, 1995). The RE1 was subsequently identified in a host of neuron-specific gene promoters, suggesting a broad role for REST in repressing neuronal gene expression outside the nervous system. In support of this idea, REST is required for mouse viability, with REST-deficient animals showing evidence of precocious neuronal differentiation (Chen et al., 1998). Expression of a dominant inhibitory form of REST in nonneuronal tissue results in the ectopic expression of several REST target genes. Furthermore, overexpression of REST in developing chick spinal cord neurons causes repression of neuronal-specific gene expression and significantly increases the frequency of axon guidance errors, suggesting that effective REST downregulation is required for proper neuronal differentiation (Paquette et al., 2000).

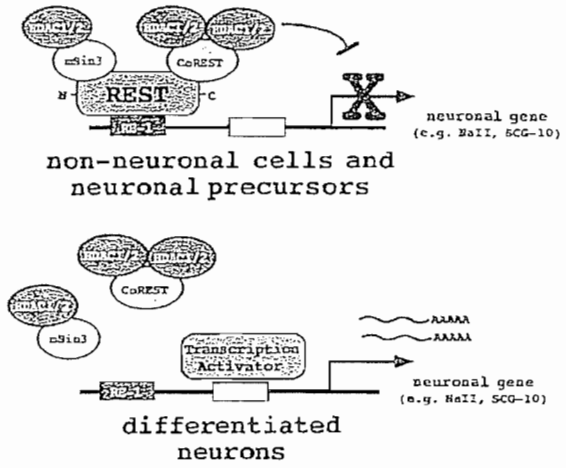
In this issue of *Neuron*, Gail Mandel and colleagues describe an elegant study that significantly advances the understanding of REST-dependent gene silencing (Ballas et al., 2001). The authors generated a pheochromocytoma cell line (PC12) in which REST is conditionally expressed and examined the effect of enforced expression of REST on nerve growth factor (NGF)-induced neuronal differentiation. Induction of REST was found to inhibit NGF-induced differentiation, blocking both sodium channel currents and neurite outgrowth. In addition, similar effects were observed in cultured cortical neurons infected with a REST-expressing virus. Together with the data from REST-deficient mice, the current findings strongly suggest that downregulation of REST is required for both the induction and maintenance of a neuronal phenotype.

Mandel and coworkers also examine the molecular mechanism by which REST acts to block gene transcription. Previous work revealed the presence of two distinct repressor domains that lie near the N and C termini of the REST protein. Several groups have demonstrated that the N-terminal domain acts to repress transcription by recruiting the corepressor mSin3 that in turn associates with a histone deacetylase (HDAC) (Huang et al., 1999; Naruse et al., 1999; Grimes et al., 2000). The acetylation state of highly conserved lysine residues present in the core histones has been shown to modulate both chromatin structure and gene expression. HDACs catalyze the removal of acetyl-lysine groups from the histone N termini and have been found to be a pervasive mechanism of transcriptional repression in eukaryotes.

The Mandel group has also identified a novel corepressor, termed CoREST, which interacts with the C-terminal repressor domain of REST (Andres et al., 1999). Although CoREST was shown to function as a transcriptional repressor when recruited to artificial promoters as a Gal4-REST fusion protein, the exact mechanism of this repression was unknown. In the current study, the Mandel group demonstrates that, like mSin3, CoREST associates with an HDAC complex. Experiments utilizing either the HDAC inhibitor tricostatin A or the microinjection of neutralizing antibodies reveal that CoREST-mediated repression requires deacetylase activity. The authors go on to show by chromatin immunoprecipitation that in the presence of REST the CoREST/HDAC complex occupies the promoter of the native *Nav1.2* voltage-dependent sodium channel in nonneuronal cells. In contrast, in neuronal cells that express sodium channels and lack REST, the corepressor complex is not present on the *Nav1.2* promoter. Thus, REST restricts the expression of neuronal genes by associating with two distinct corepressors, mSin3 and CoREST, which in turn recruit HDACs to the promoters of REST-regulated genes (Figure).

This study by Mandel and colleagues raises a number of important issues that remain to be addressed. First, does CoREST function as a corepressor for silencing factors in addition to REST? Of note, immunopurification of CoREST-associated polypeptides from HeLa extracts did not isolate REST, perhaps due to the extraction conditions used, but did purify ZNF217, an eight zinc finger Krüppel-like protein which has been implicated as a candidate oncogene (You et al., 2001). The importance of this factor in transcriptional silencing awaits further characterization. In addition, the Mandel study sets the stage for the comprehensive identification of REST target genes. Utilization of the PC12 cell line conditionally expressing REST should permit microarray experiments to identify genes affected by REST induction. These results when combined with the existing knowledge of the RE1 consensus sequence should provide an understanding of the true scope of REST-regulated gene expression.

An intriguing question raised by the current work is why two distinct HDAC complexes are utilized by REST to shut off transcription. Chromatin immunoprecipitation experiments show that both mSin3 and CoREST complexes are present on the *Nav1.2* promoter. How-



Model of REST-Mediated Transcriptional Repression

ever, it remains unclear whether both can associate with a single REST molecule at the same time. The possibility that different types of REST silencing complexes may exist in cells suggests that there may be several distinct mechanisms by which REST acts to repress transcription. Interestingly, the authors demonstrate that the two repressive domains of REST function neither synergistically nor additively when assayed using transfected or microinjected DNA constructs. The reason REST has evolved the ability to recruit two HDAC complexes remains unclear, but the answer may involve differences between Sin3 and CoREST complexes. Recruitment of HDAC activity by mSin3 has been observed for multiple signal-responsive transcription factors, and this interaction appears to be subject to dynamic regulation. CoREST has recently been identified as a component of a novel HDAC complex that lacks many of the components of the mSin3 complex (You et al., 2001; Humphrey et al., 2001). Whether this CoREST complex possesses distinct activity or is subject to differential regulation remains to be explored.

The expression patterns of the various repressor molecules change during development, also suggesting that the composition of the REST complex may be dynamically regulated. Early in development, CoREST exhibits a restricted expression pattern, in which CoREST is concentrated in the head mesenchyme but absent in the somites and presomitic mesoderm (Grimes et al., 2000). In contrast, mSin3 and REST are widely expressed throughout the embryo. As development proceeds, CoREST expression broadens, and, by E11.5, CoREST is ubiquitously expressed in the developing mouse. The observed patterns of expression raise the possibility that REST recruitment of the mSin3 complex may play a role in the dynamic changes in gene expression that occur in the developing animal. Subsequent association of the CoREST deacetylase complex may be required to produce a stable, long-lasting repressive state, locking the cell into a nonneuronal fate.

Differences in the function and mechanism of action of the two corepressors may also be important for the regulation of REST target genes in the adult nervous system. Although REST is downregulated in postmitotic neurons, detectable levels are still found in the adult central nervous system. A neuronal-specific splice variant of REST, termed REST4, has been identified, which lacks the C-terminal repressor domain and thus would be unable to bind CoREST (Palm et al., 1998). Provocatively, REST4 is induced in hippocampal and cortical neurons following kainate-induced seizures, suggesting that REST may regulate gene expression in the adult nervous system in response to external stimuli. However, the true role of REST and its splice variants in the adult nervous system awaits the generation of animals harboring a conditional allele of REST to circumvent the lethality associated with REST null mice.

A remaining mystery is what regulates REST. REST mRNA and protein levels are known to be downregulated as neural progenitors differentiate and migrate out of the ventricular zone (Chong et al., 1995; Schoenherr and Anderson, 1995), and this downregulation of REST is critical for the proper development of the nervous system. We can expect that in the future characterization of the various *cis*- and *trans*-acting factors that control REST expression will provide important insight into the mechanisms of cell fate determination in the developing nervous system.

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Selected Reading

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Sequential expression and role of Hu RNA-binding proteins during neurogenesis

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SUMMARY

We have identified three avian (chicken) Hu/elav family RNA-binding protein genes. cHuD and cHuC are expressed specifically in neurons of both the central and peripheral nervous systems. Although cHuA is expressed in a wide variety of tissues, including neurogenic precursor cells, it is transiently down-regulated, and is then re-expressed in maturing neurons. Misexpression of cHuD in cultured neural crest cells results in a dramatic increase in the proportion of cells exhibiting neuronal morphology, molecular markers for neurons, and neurotrophin dependence. These data confirm that cHuD protein is involved in regulating neuronal differentiation.

Key words: Hu, *elav*, RNA-binding protein, neurogenesis, neural crest, apoptosis, chicken

INTRODUCTION

Segregation of different cell types from multipotent precursor cells must involve differential gene activity. The regulation of gene expression by transcription factors and *cis*-elements has been well documented. However, the role of posttranscriptional regulation, such as alternative splicing of pre-mRNA, regulation of mRNA stability, and intracellular localization of mRNA, as a mechanism of regulating cell differentiation has only recently been addressed (reviews: Javier-Lopéz, 1995; Curtis et al., 1995; St Johnston, 1995).

Hu proteins were first identified as neuron-specific antigens recognized by autoimmune antiserum from patients with small cell lung cancer (Graus et al., 1985; Furneaux and Posner, 1990; Dalmau et al., 1990). Subsequently, several *Hu* genes, including HuD (Szabo et al., 1991), ple21/HuC (Sakai, et al., 1994; Liu et al., 1995), Hel-N1 (Levine et al., 1993) and HuR (Ma et al., 1996) have been cloned from humans, and also from other vertebrates (Good, 1995; Perron et al., 1995). Proteins encoded by these genes share a high degree of homology to the *Drosophila* RNA-binding proteins ELAV (Campos et al., 1985, 1987; Robinow et al., 1988; Yao et al., 1993) and RBP9 (Kim and Baker, 1993). Genetic analysis of *elav* mutant alleles has shown that *elav* gene function is essential for the development and maintenance of the *Drosophila* nervous system (Campos et al., 1985, 1987). Therefore, *Hu* gene products have been suspected also to be involved in the development and maintenance of the nervous system in vertebrates.

Members of the Hu/ELAV family of proteins all have three RNA recognition motifs (RRMs) and bind to AU-rich RNA sequences. Although the in vivo target RNA(s) have yet to be identified, in vitro analyses have shown Hu proteins are able to bind to AU-rich 3′ untranslated regions (UTR) of mRNAs encoding several transcription factors and cytokines (Levine et al., 1993; King et al., 1994; Liu et al., 1995; Ma et al., 1996). Since AU-rich sequences of 3′ UTRs are known to be important for the control of mRNA stability (review: Chen and Shyu, 1995), the potential function of Hu proteins during the development and subsequent maintenance of the nervous system may result from modulating the ultimate use of subsets of mRNA species. Moreover, association of Hel-N1/2 proteins in polysomes reported recently (Gao and Keene, 1996; see also Jain et al., 1997) suggested possible involvement in the control of translation and mRNA metabolism.

The expression of different *Hu* family genes during early neural development has been only partially described. In *Xenopus*, expression of *Xel-1*/elrB, the homologue of Hel-N1 (Good, 1995; Perron et al., 1995), is first detected at early tadpole stages, and is expressed only in the post-mitotic neurons in both the central nervous system (CNS) and peripheral nervous system (PNS; Perron et al., 1995). Neuron-specific expression of Hu protein(s) has also been described in the avian tissues using autoimmune serum (Marusich and Weston, 1992) and the monoclonal antibody 16A11, which potentially recognizes more than one species of Hu proteins (Marusich et al., 1994; Barami et al., 1995). Double-labeling of chicken embryos with 16A11 and BrdU showed 16A11-positive cells in the spinal cord were generally post-mitotic (Marusich et al., 1994). The early expression of Hu proteins during nervous system development suggests the importance of Hu gene(s) for the early neuronal differentiation.

To examine the role(s) of Hu proteins during neuronal development, especially in neural crest-derived peripheral neurons, we cloned Hu sequences from a chicken cDNA library. We identified three gene products, chicken HuA (cHuA), chicken HuC (cHuC) and chicken HuD (cHuD), equivalent to

HuR/elrA, HuC/elrC and HuD/elrD, respectively. Here, we describe the sequential expression of these genes during avian neurogenesis. In addition, we show multiple effects of misexpression of cHuD in neural crest cells. We suggest an involvement of *Hu* genes in nervous system development, and in particular, the regulatory function of cHuD in neuronal differentiation by neurogenic neural crest cells.

MATERIALS AND METHODS

Animals

White Leghorn X New Hampshire F_1 chicken (*Gallus gallus*) and Japanese quail (*Coturnix coturnix japonica*) embryos (Oregon State University, Poultry Program, Animal Sciences Department, Corvallis, Oregon) were used for histological analyses and for neural crest culture experiments, respectively. Embryos were staged according to Hamburger and Hamilton (1951).

cDNA cloning

A cDNA library, obtained from whole brain of day-13 chicken embryos (Funahashi et al., 1993) was screened with an 1136 base pair *Eco*RI-*Kpn*I fragment, including the zebrafish elrD coding region of *zfeld-1* (Good, 1995). Eight positive clones were subcloned into pBluescriptII (Stratagene) or pGEM11 (Promega) and sequenced by standard didcoxynucleotide sequencing with Sequenase version 2.0 (United States Biochemical). From their sequence homology, six of the isolated clones (chh1, 2, 3, 4, 5 and 11) are derived from same gene, which we designate cHuD. All cHuD clones except chh3 had a long, single open reading frame and a short 3′ untranslated region (UTR), probably due to priming with oligo(dT) at A rich sequences. Chh3 had a carboxy-terminal portion of the coding region and a long 3′UTR. A hairpin loop sequence in the 3′UTR which is conserved in HuD/elrD of other species (Good, 1995) was also found in cHuD (data not shown). Three sequence variants were found at the 5′ end of cHuD cDNAs. Two non-cHuD clones, chh7 (1314 base pairs) and chh13 (1282 base pairs), represented distinct sequences, and were named cHuC and cHuA, respectively. Both chh7 and chh13 had a single open reading frame and short 3' UTR like many of the cHuD clones.

In situ hybridization

Riboprobes were prepared by in vitro transcription with T7, T3 or SP6 RNA polymerase and with digoxigenin-11-UTP (Boehringer-Mannheim) according to the manufacturer's instructions. An 881 base pair *Eco*RV fragment of the 3′ UTR of chh3 was subcloned into pBluescriptII, and was used as a template for the cHuD riboprobe. cDNA clones chh7 and chh13 in pGEM11 were used as templates for cHuC and cHuA riboprobe, respectively.

For in situ hybridization on sections, 10 μm frozen sections were prepared as described previously (Wakamatsu et al., 1993). Pretreatment of sections and hybridization were done as described previously (Wakamatsu and Kondoh, 1991), except digoxigenin-labeled probe was used instead of ^{35}S-labeled probe. After washing in 2× SSC containing 50% formamide at 65°C for 30 minutes, sections were treated with 20 μg/ml RNase A in 10 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA for 30 minutes, then washed in 2× SSC for 20 minutes and in 0.2× SSC for 20 minutes. Then, sections were incubated overnight with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim). Color reaction with NBT/BCIP was performed according to Barth et al. (1994) for 1 day (cHuD) or 2 days (cHuC and cHuA). Sections were either mounted with glycerol or processed for immunohistological staining as described below.

Whole-mount in situ hybridization was performed as described previously (Yamaguchi et al., 1992), except the color reaction was performed in the presence of 10% polyvinyl alcohol (Barth et al., 1994) for 3-5 hours (cHuD) or overnight (cHuA and cHuC).

Immunological stainings

Immunological staining on histological section and in cell cultures were done as described previously (Wakamatsu et al., 1994). 16A11 (mouse IgG2b; Marusich et al., 1994), 5C5 (mouse IgG1; Marusich, Furneaux and Weston, unpublished), anti-neurofilament 160 kDa (mouse IgG2a), HNK-1 (mouse IgM) were prepared from hybridomas, and were provided by the monoclonal antibody facility, Institute of Neuroscience, University of Oregon. 5C5 monoclonal antibody was raised against human HuDmex, a minor splice variant of HuD, but also recognizes HuR on western blot and histocytochemistry (Marusich and Furneaux, personal communication). Anti-MAP-2 (mouse IgG1) was purchased from Sigma. Anti-FLAG D8 (rabbit IgG) was purchased from Santa Cruz Biotechnology. Biotin-conjugated donkey anti-mouse IgG antibody (Jackson), TRITC-conjugated goat anti-mouse IgM antibody (Southern Biotechnologies), FITC-conjugated donkey anti-rabbit IgG antibody (Jackson), Avidin-FITC (Vector) and Avidin-Texas Red (Vector) were used for detection of primary antibodies. After staining with antibodies, nuclei were labeled with DAPI. Stained sections and cultures were mounted either in glycerol or Vectashield (Vector).

For whole-mount antibody staining, embryos were fixed with 3.5% paraformaldehyde in PBS for 3 hours. After washing in TBST (0.1 M Tris-HCl, 0.15 M sodium chloride, 0.5% Triton X-100, pH 7.5), embryos were heated at 63°C for 30 minutes. Embryos were then incubated with blocking solution (TBST containing 10% heat-inactivated normal goat serum) for 2 hours, followed by incubation overnight at 4°C with 16A11 antibody diluted with blocking solution. After washing in TBST for 5 hours, embryos were incubated overnight at 4°C with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Southern Biotechnology). Embryos were then washed with TBST for 5 hours. The color reaction with NBT/BCIP and mounting procedure were the same as that of whole mount in situ hybridization.

Construction of expression vectors

The N-terminal end of cHuD, cHuA and mutant jellyfish green fluorescent protein (pREST-S65T, Heim et al., 1995) cDNAs were tagged with FLAG epitope by subcloning into pyDF30. For FLAG-cHuD, clone chh5 was chosen, because it had only 31 base pairs of the 3′untranslated region. For FLAG-cHuA and FLAG-cHuC, chh13 and chh7 were used, respectively. All FLAG-tagged cDNAs were subcloned into pmiwSV expression vector (Suemori et al., 1990; Wakamatsu et al., 1997).

Cell culture and transfection

50 μl of Lipofectin (Gibco) and 100 μl of Opti-MEM (Gibco) were mixed gently, and were incubated at room temperature for 30 minutes. 15 μg of expression vector plasmid in 50 μl of water was mixed with 100 μl of Opti-MEM. Then, the Lipofectin suspension and DNA solution were mixed gently, and incubated for 30 minutes at room temperature. This lipofection solution was diluted with 700 μl of Opti-MEM, prior to use for transfection.

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For transfection, the culture was washed with Opti-MEM, then incubated in lipofection solution for 2 hours. After transfection, the culture was maintained for 24 hours, fixed and labeled with antibodies as described above.

Stage 12-14 quail neural tubes were prepared as described previously (Loring et al., 1981; Glimelius and Weston, 1981). For a culture of neurogenic neural crest cells, neural tubes were placed onto tissue culture dish in neurogenesis permissive medium (Henion et al., 1995; Ham's F12 supplemented with 15% fetal bovine serum and 4% chicken embryo extract), and were cultured for 12-13 hours. After washing with Opti-MEM, dispersed neural crest cells and neural tubes were incubated in lipofection solution at 38°C for 1 hour. Neural tubes were removed with a micro-spatula made from a sewing needle. After incubation with culture medium for 1 hour, transfected neural crest

cells were dissociated with 0.25% trypsin, 1 mM EDTA in Hanks' balanced salt solution. Dissociated crest cells were re-plated at 4×10^3 cells/10 mm diameter Sylgard well (Dow Corning; Marusich and Weston, 1992) in the neurogenesis permissive medium. Preparation of non-neurogenic neural crest cells was performed as described before (Vogel and Weston, 1988). In the case of cell death or neuronal marker expression assay, the medium was changed to a reduced medium (containing 7.5% fetal bovine serum and 2% chicken embryo extract) with or without 20 ng/ml neurotrophin-3 (Peprotech, or Promega).

Staurosporine treatment of cultured neural crest cells

Two-day old culture of the crest cells were treated with 5 μM of staurosporine (Calbiochem, Weil et al., 1996) for 6 hours with or without 20 ng/ml neurotrophin-3, then the medium was changed to neurogenesis permissive medium (see above). Two days after the treatment, cultures were processed for antibody staining as described above.

Detection of cell death in culture

Cultured neural crest cells were fixed with 4% paraformaldehyde in PBS for 10 minutes. After washing in PBS, cultures were pre-incubated in TdT buffer (0.2 M sodium cacodylate, 1.5 mM cobalt chloride, 50 μg/ml BSA, 25 mM Tris-HCl, 0.2% Tween 20, pH 6.6) for 10 minutes, then incubated with TdT buffer containing 120 units/ml of terminal deoxynucleotidyl transferase (Boehringer-Mannheim) and 8 μM of biotin-14-dATP (Gibco) at 37°C for 30 minutes. Labeled cells were detected with avidin-Texas red. Detection of transfectants with anti-FLAG and nuclear-staining with DAPI were performed as described above.

BrdU labeling

BrdU labeling of cultured crest cells was performed as described previously (Marusich et al., 1994). In brief, transfected crest cultures were pulse labeled with 10 μg/ml of BrdU (Boehringer-Mannheim) for 1 hour. Then, anti-FLAG staining was performed as described above. After postfixation in 4% paraformaldehyde in PBS, cultures were treated with 2N HCl for 30 minutes. Anti-BrdU (Boehringer-Mannheim) staining was performed as with other immunostainings described above.

RESULTS

Identification of chicken homologs of *Hu* genes

A chicken cDNA library made from embryonic brain (Funahashi et al., 1993) was probed with the zebrafish HuD homolog elrD (Good, 1995). Six cDNA clones were derived from the same gene product, which were homologous to human HuD (96.5%; Szabo et al., 1991), and therefore designated chicken HuD (cHuD; Fig. 1). All the cHuD clones contained the sequence that encodes the peptide used as the antigen for producing monoclonal antibody 16A11 (Marusich et al., 1994; Fig. 1).

One clone showed the highest homology to human HuC (82.5%; Liu et al., 1995), so, this gene was designated chicken HuC (cHuC; Fig. 1). Another clone showed high sequence similarity to human HuR (98.2%; Ma et al., 1996;

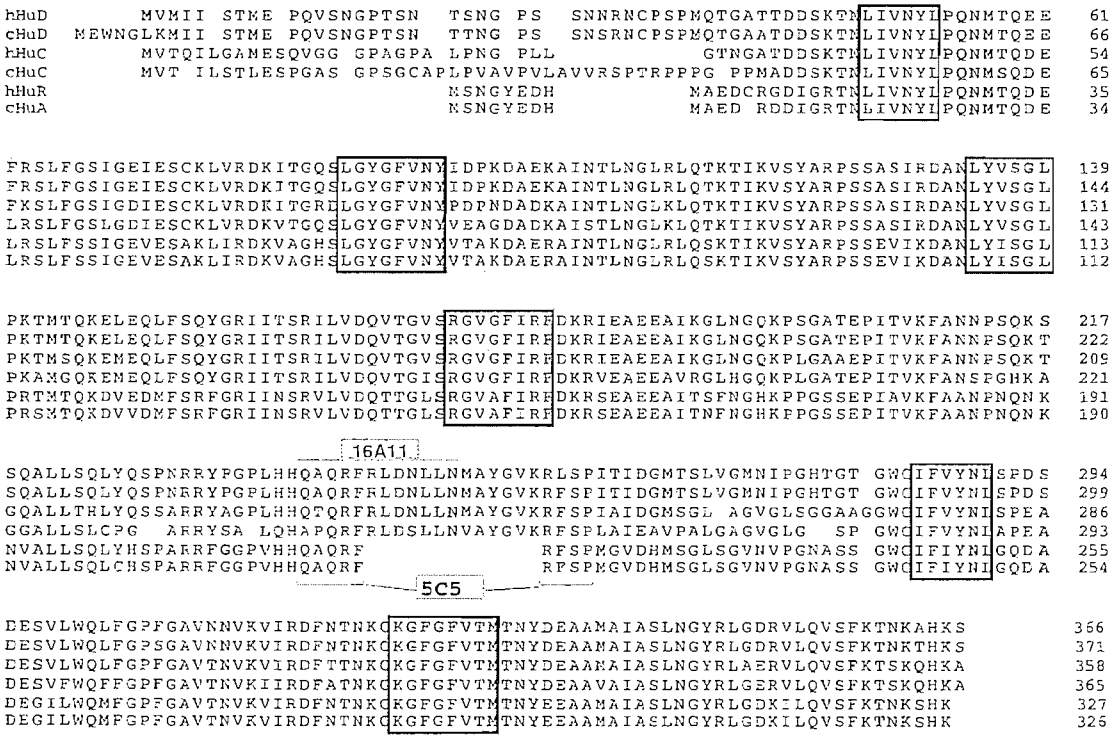

Fig. 1. Amino acid sequence comparison of human and chicken Hu proteins. Amino acid residues conserved in more than three proteins are shaded. RNP sequences of RNA recognition motifs are outlined. The peptide sequence used as an immunogen to produce monoclonal antibody 16A11 is also indicated. This sequence is completely conserved in human and chicken HuD, and is partially conserved in cHuC and cHuA. cHuA possesses only a half of the 16A11 epitope, and 5C5 epitope is present.

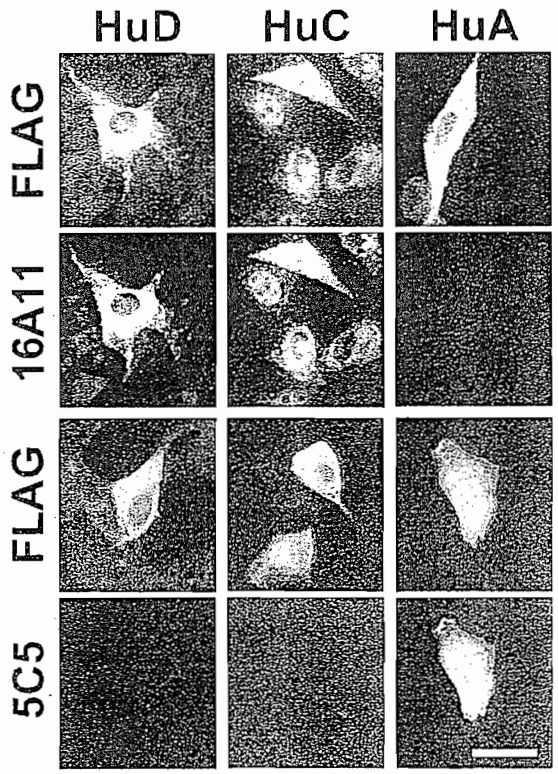

Fig. 2. Immunoreactivities of NIH3T3 cells transfected with FLAG-tagged *Hu* gene constructs. FLAG-cHuD and FLAG-cHuC transfected cells show both anti-FLAG and 16A11 immunoreactivities, but not 5C5 immunoreactivity. FLAG-cHuA transfected cells are immunoreactive to anti-FLAG and 5C5, but not to 16A11. Note anti-Hu immunoreactivity in both cytoplasm and nuclei. Scale bar, 10 μm.

Fig. 1) and *Xenopus* elrA (95.7%; Good, 1995), so we refer to it as chicken HuA (cHuA; Fig. 1).

In ribonucleoprotein consensus sequences (RNPs), the core domains of RRMs, are identical between human HuD, cHuD, human HuC and cHuC, but gene specific amino acid substitutions were found throughout HuR and cHuA sequences, particularly in three RNPs (Fig. 1).

cHuC and cHuA do not contain the complete coding sequence of the 16A11 epitope (Fig. 1). To test the immunoreactivity of cHuD, cHuC and cHuA to the 16A11 antibody, therefore, FLAG-epitope tagged expression vectors of cHuD, cHuC and cHuA were constructed in the pmiw expression vector (Suemori et al., 1991; Wakamatsu et al., 1997; see also Materials and Methods). pmiwFLAG-cHuD, pmiwFLAG-cHuC and pmiwFLAG-cHuA expression vectors (referred to as FLAG-cHuD, FLAG-cHuC and FLAG-cHuA) were transfected into an NIH3T3 mouse fibroblast cell line. The non-transfected line was not immunoreactive to either anti-FLAG antibody or 16A11. Cells that received expression vector were easily detected with anti-FLAG antibody. FLAG-cHuD and FLAG-cHuC transfectants were also immunoreactive with 16A11 (Fig. 2). In contrast, FLAG-cHuA transfectants were not recognized by 16A11 (Fig. 2).

Immunoreactivity of a monoclonal antibody 5C5, which recognizes the HuR sequence (Furneaux and Marusich, personal communication, see also Materials and Methods), were also tested on the transfected cells. In contrast to the results with 16A11, FLAG-cHuA transfected cells were immunorective to 5C5, but FLAG-cHuD and FLAG-cHuC transfected cells were not (Fig. 2). These results were consistent with the sequence data, since the peptide sequence used to raise 5C5 is conserved in cHuA and HuR, but shares only part of the sequence used to raise 16A11 with cHuD and cHuC (Fig. 1). Moreover, these results indicated that the expression vector-derived Hu proteins were intact, at least as far as the epitopes. In most cases, FLAG-Hu immunoreactivity was detected in the cytoplasm (Fig. 2). However, many of transfectants also displayed nuclear staining (Fig. 2).

Expression of chicken *Hu* genes

Whole-mount analysis

The expression of cHuA, cHuC and cHuD mRNA during early development was examined by whole-mount in situ hybridization. In stage 16 (Hamburger and Hamilton, 1951) embryos, cHuD mRNA was detected in the anterior spinal cord, ventral midbrain and nascent trigeminal ganglia in the head (data not shown). At stage 17, in addition to the expression in the CNS and trigeminal ganglia, cHuD expression was detected in vestibulo-occular, glosso-pharyngeal and nodose ganglia (Fig. 3A). At stage 17-18, expression of cHuD in trunk crest-derived cells was observed for the first time dorsally between the somite and neural tube (Fig. 3B). Subsequently cHuD$^+$ cells were present in nascent dorsal root ganglia. Although HNK-1$^+$ cells were present ventrally (data not shown), no cHuD$^+$ cells were observed between the dorsal root ganglia and the location of sympathetic chain. We conclude, therefore, these cHuD$^+$ cells are likely to be precursors of dorsal root ganglia neurons. Expression of cHuD in sympathetic ganglia was first detected at stage 18 in the brachial level (data not shown). At this stage, cHuD expression was also detected in the olfactory placode region, and at later stages, some migrating cells from the olfactory placode were also seen (Fig. 3E, and data not shown). At stage 21-23, the number of cHuD$^+$ cells increased both in the CNS and the PNS (Fig. 3E). Tissue distribution of cHuD mRNA was similar to that of 16A11 immunoreactivity (Fig. 3E).

The tissue distribution of cHuC is almost identical to that of cHuD (Fig. 3F), but cHuC expression was delayed by at least one stage. For example, expression of cHuC was first detected in the dorsal root ganglia at stage 19 (data not shown), and unlike cHuD, no cHuC expression could detected in migrating crest cells at stage 18 (Fig. 3C, also see Fig. 3B).

In contrast to the nervous system-specific expression of cHuD and cHuC, expression of cHuA was detected in a wide variety of tissues but not ubiquitously (Fig. 3D). For example, at stage 23, cHuA expression was not detected in heart (Fig. 3D), whereas abundant expression was observed in the mesonephros and liver primordium (data not shown).

Analysis of tissue sections

The expression of different *Hu* genes was also compared by in situ hybridization of transverse histological sections. At stage 17, cHuD expression was detected in differentiating neurons in the periphery of spinal cord (Fig. 4A). Double-labeling with HNK-1 antibody revealed cHuD$^+$ neural crest-derived cells migrating

on the ventromedial pathway (Fig. 4A, and data not shown). 16A11 immunoreactivity in adjacent sections revealed similar signal distribution to the cHuD mRNA in HNK-1$^+$ migrating crest cells (Fig. 4B, C; see also Fig. 3B).

cHuC and cHuA mRNA expression was examined on neighboring sections. cHuC mRNA was not detected in stage 17 embryos except for very faint staining in the developing ventral horn (data not shown, but see faint staining in the neural tube in Fig. 3C). In contrast to the neuron-specific expressions of cHuD and cHuC, cHuA mRNA was detected throughout the neural tube, in migrating neural crest cells and in mesodermal tissues (data not shown).

At stage 22, cHuD mRNA expression was observed in neurons of the spinal cord, dorsal root ganglia (Fig. 4D,E), and sympathetic ganglia (data not shown). cHuD mRNA distribution precisely overlapped 16A11 immunoreactivity (Fig. 4E,F). Counter-labeling with HNK-1 antibody revealed that cHuD expression was present in the center, but absent in the periphery of dorsal root ganglia (data not shown; also see Marusich et al., 1992, 1994).

cHuC mRNA was expressed in the spinal cord and dorsal root ganglia (Fig. 4G). Counter-immunostaining with 16A11 showed that a subpopulation of 16A11-immunoreactive cells also expressed cHuC mRNA at this stage (data not shown). Unlike cHuD, which was highly expressed in newly differentiating neuronal precursors (Fig. 4D, arrowheads), cHuC was weakly expressed in the neurons in the periphery of the spinal cord (Fig. 4G).

cHuA mRNA expression in the nervous system was different from both cHuD and cHuC. cHuA expression was high in the ventricular zone and abundant along the boundary of the ventricular zone and the neuronal layer (Fig. 4H, I). Counter-labeling with 16A11 antibody which recognizes cHuD and cHuC but not cHuA (see above), revealed 16A11-immunoreactive cHuA$^+$ cells in the boundary of ventricular zone and neuronal layer, and a subpopulation of 16A11-immunoreactive cHuA$^+$ cells in the periphery of dorsal root ganglia (Fig. 4I,J), suggesting that these cells co-express cHuA and one or both of cHuD and cHuC. Although the cHuA mRNA expression

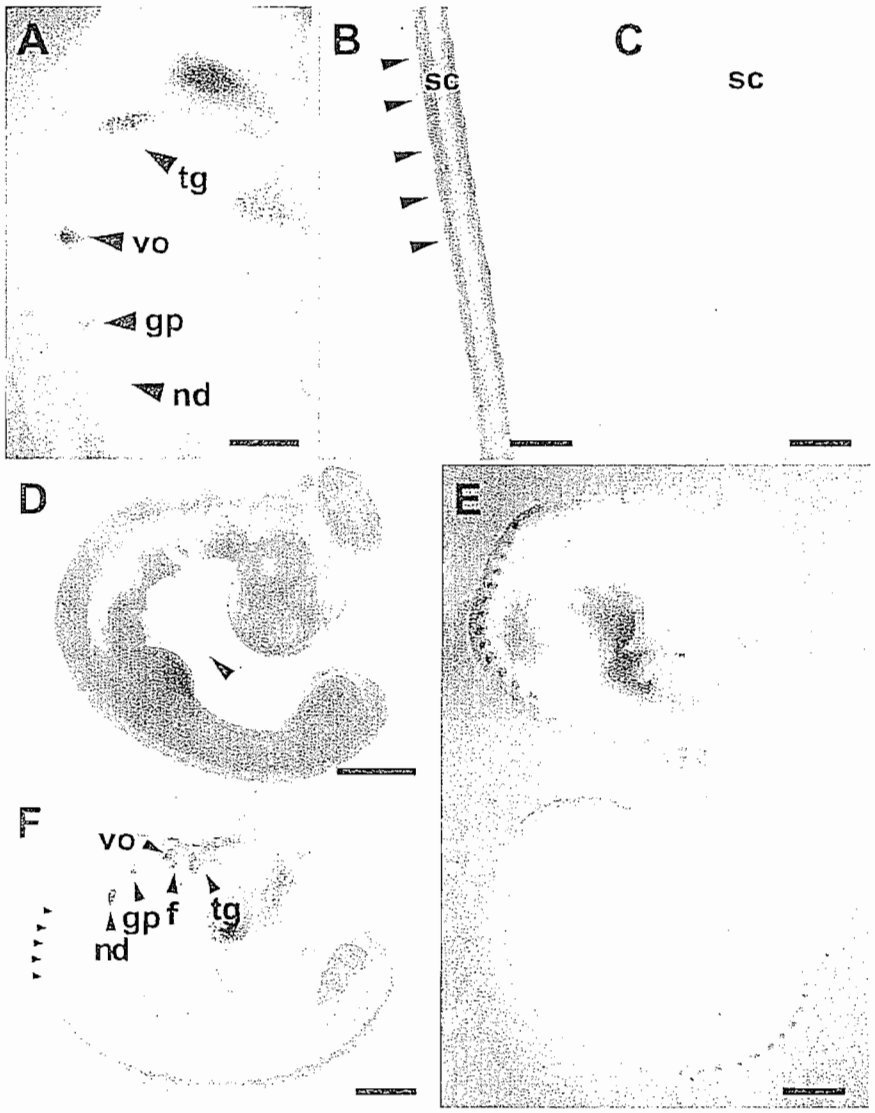

Fig. 3. Whole-mount analysis of *Hu* gene expressions. (A) Lateral view of cHuD mRNA expression in cranial ganglia stage 17 chicken embryo. High expression in the ventral part of mesencephalon and diencephalon is also visible adjacent to the trigeminal ganglion (tg). (B) cHuD expression in neural crest-derived sensory neuron precursor cells (arrowheads) of stage 18 mid trunk (dorsal view). (C) cHuC mRNA expression of stage 18 mid trunk (dorsal view), showing faint staining in the spinal cord (sc), and no staining can be detected in the PNS. (D) cHuA mRNA expression is observed in wide variety of tissues, but not in the heart (arrowhead). (E) Whole-mount staining of stage 22 embryos with 16A11 antibody (above) and cHuD antisense riboprobe (below). Staining in cranial ganglia, dorsal root ganglia, brain, spinal cord and olfactory placode are similar in these preparations. Staining in the ventral part of the embryo is an artifact, since the same staining was also detected without primary antibody (data not shown). Red blood cells in the trunk and black pigment retina are also visible in the 16A11-labeled embryo, due to the lack of bleaching step (See Methods). (F) Staining of stage 22 embryo with cHuC antisense riboprobe. Tissue distribution of cHuC mRNA is similar to that of cHuD mRNA. f, facial ganglion; gp, glossopharyngeal ganglion; nd, nodose ganglion; sc, spinal cord; tg, trigeminal ganglion; vo, vestibulo-occular ganglion. Scale bars, (A-C) 300 μm; (D-F) 1 mm.

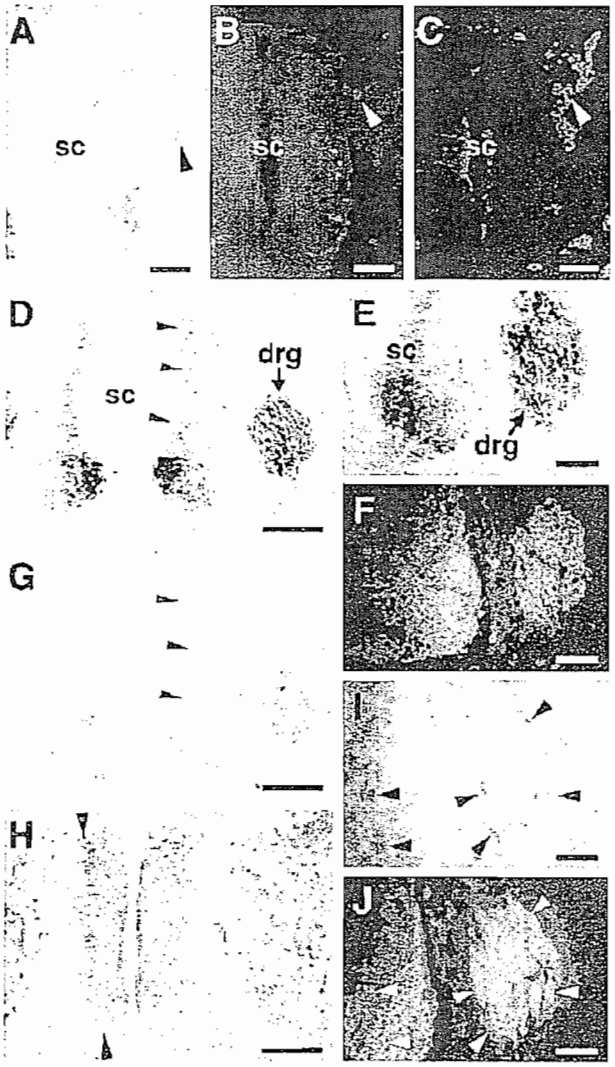

Fig. 4. Distributions of Hu RNAs and 16A11 immunoreactivity on transverse sections. (A-C) Stage 17 embryo. In addition to the differentiating neurons in the periphery of the spinal cord, migrating crest cells are labeled with cHuD antisense probe (A, arrowhead) and 16A11 (B, arrowhead). 16A11$^+$ cell is also labeled with neural crest marker HNK-1 (C, arrowhead). At this stage, many crest cells still remain in the migration staging area (see Discussion). (D-F) Distribution of cHuD mRNA and comparison with that of 16A11 staining on sections of stage 23 embryo. cHuD is expressed in differentiating neurons, particularly high at the boundary between the ventricular zone and the neuronal layer (D, arrowheads). E and F show the cHuD mRNA$^+$ domain in the spinal cord and dorsal root ganglia (E) and the overlapping 16A11 staining pattern (F) in the same section. (G) cHuC mRNA distribution in a neighboring section to D. cHuC expression is absent in the boundary region (arrowhead). Compared to cHuD expression, fewer cHuC$^+$ cells are visible in the dorsal root ganglion. (H) cHuA mRNA distribution in a neighboring section to D and G. cHuA mRNA is high in ventricular zone and abundant in the boundary region (arrowhead). Cells in the spinal cord and dorsal root ganglion which express high levels of cHuA mRNA (I, arrowhead) are also stained with 16A11 (J, arrowhead). Scale bars, (A-C,E,F,I,J) 50 μm; (D,G,H) 100 μm.

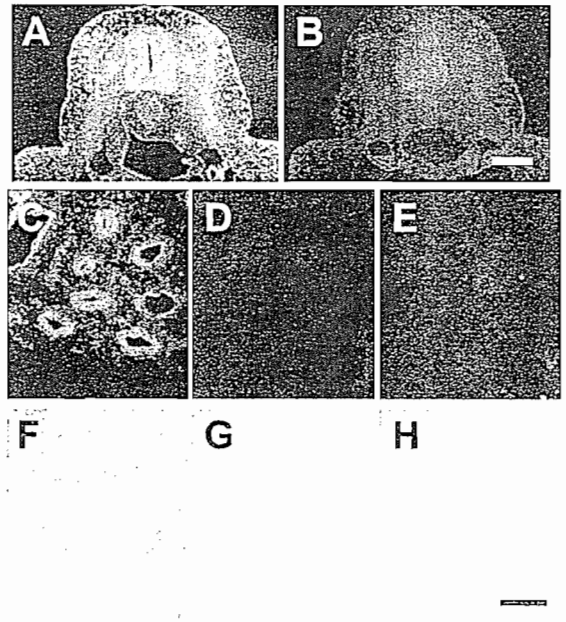

Fig. 5. Distribution of 5C5 immunoreactivity on transverse sections. Widespread staining is visible in the transverse section of stage 22 embryo with 5C5 staining (A), compared to the low background staining without primary antibody (B). Brighter staining is also visible in the neuronal layer of the spinal cord and dorsal root ganglion, possibly due to the expression of cHuDmex. 5C5 immunoreactivity is also present in lung epithelium of stage 36 embryo (C). No staining was observed in neighboring sections without primary antibody (D) or with 16A11 (E). cHuA mRNA expression was detected in lung epithelium of stage 38 embryo (F), but not cHuD or cHuC mRNA (G and H, respectively). Scale bars, (A,B) 200 μm, (C-H) 50 μm.

was broadly observed (Fig. 4H), higher level of expression was detected in developing mesonephros and liver (data not shown).

To examine cHuA protein expression in vivo, stage 22 embryo sections were stained with 5C5 antibody. Widespread 5C5 immunoreactivity was observed (Fig. 5A) where neither cHuD nor cHuC were expressed. Slightly brighter 5C5 immunoreactivity was also detected in the differentiating neurons in both CNS and PNS, probably due to the expression of cHuDmex (see Materials and Methods, see also Marusich et al., 1994). At stage 38, cHuA mRNA expression was more restricted, compared to the widespread expression at earlier stages. In addition to the expression in mesonephric tubule and liver hepatocytes (data not shown), abundant expression was also observed in lung epithelium (Fig. 5F). 5C5 immunoreactivity confirms the presence of cHuA protein in this tissue (Fig. 5C). Neither 16A11 immunoreactivity nor cHuD and cHuC mRNAs were detected in this tissue (Fig. 5E,G and H, respectively).

Expression of these genes were also tested on neighboring transverse sections of stage 38 embryonic trunk (Fig. 6A-C). At this stage, all three genes are expressed in maturing neurons in the spinal cord, dorsal root ganglia and sympathetic ganglia. Satellite cells in the ganglia and Schwann cells all lacked evidence of Hu mRNA expression.

In summary (see Fig. 7), cHuA is first detected in apparently undifferentiated cell populations. At an early stage of neuronal differentiation, cHuA increases and cHuD begins to be expressed in the same cells. Then, as cHuA expression declines, cHuC is expressed for the first time and cHuD expression is maintained. Later, cHuA expression increases in maturing neurons. Except for the early expression of cHuA in initial precursor populations, none of the three genes is expressed in glial cells.

Because it is difficult to compare the level of expression objectively in tissues at different stages of development, we attempted to confirm our inferences in a single tissue known to contain cells at various stages of neuronal differentiation. Accordingly, we examined expressions of the three *Hu* genes in longitudinal sections of stage 40 embryonic cerebellum (Fig. 6D-E). In the cerebellum at this stage, the vast majority of neurons are granule cells whose stage of differentiation can be easily identified by the location of cell bodies. In this tissue, cHuA expression was observed in the external granular layer (EGL) (Fig. 6D), and especially in the outer-most part of EGL, where committed granule cell precursors actively proliferate. Although cHuA expression diminished in the inner part of EGL, the Purkinje cell layer (PCL), and the outer part of internal granular layer (IGL), where immature granule cells migrate, cHuA mRNA again became abundant in the inner part of IGL (Fig. 6D), where mature granule cells are located. cHuD expression was observed from the middle of EGL, where it overlaps cHuA expression, and in all of the IGL (Fig. 6E). cHuC expression was detected from the inner part of EGL to the inner-most part of IGL (Fig. 6F). These observations support the idea of sequential expression of Hu genes during neuronal differentiation as described above and summarized in Fig. 7.

Misexpression of cHuD and cHuA in cultured neural crest cells

Neural crest cells were prepared from quail neural tube explants as described in Materials and Methods. After overnight culture, cells were transfected by lipofection, and the crest cell outgrowth was replated. In cultures of crest cells transfected with FLAG-tagged green fluorescent protein (GFP) (Fig. 8A), differentiated neurons, expressing neuronal markers such as neurofilament (NF) and MAP-2, melanocytes, and other derivatives including putative glial cells were observed among the transfectants (data not shown). Since the proportion of differentiated cell types of FLAG-GFP transfectants was similar to that of non-transfected cells, we concluded that transfection itself did not affect differentiation, and thereafter, used the FLAG-GFP construct as a negative control to compare with the result of FLAG-cHuD and FLAG-cHuA transfection. Transfection efficiency was approximately 4-5% with any expression constructs described above, 24 hours after the transfection. This low transfection efficiency

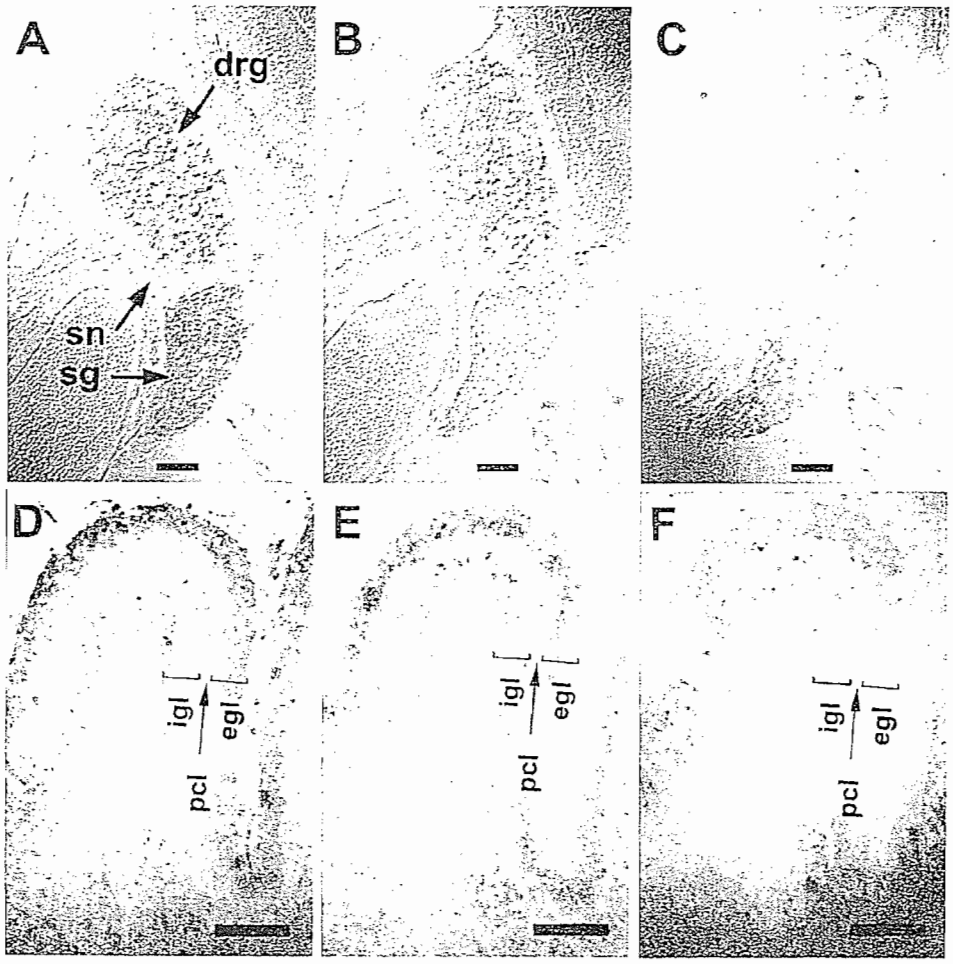

Fig. 6. Expression of *Hu* genes in the PNS and cerebellum of stage 38 embryo. A-C shows expression of cHuA, cHuD and cHuC mRNA in the peripheral nervous system on transverse sections, respectively. All three genes are expressed in neurons of dorsal root ganglia (high magnification photographs are shown at bottom right) and sympathetic ganglia, but not in Schwann cells along the spinal nerve, or in satellite glia in ganglia. D, E and F shows expression of cHuA, cHuD and cHuC mRNA in longitudinal serial sections of developing cerebellum (Only a single lobe is presented). Abbreviations: drg, dorsal root ganglion; egl, external granular layer; igl, internal granular layer; pcl, Purkinje cell layer; sg, sympathetic ganglion; sn, spinal nerve. Scale bars, 100 μm.

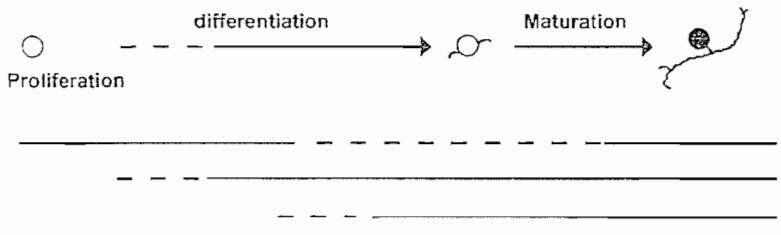

Fig. 7. Summary of sequential expression of *Hu* genes during neuronal differentiation.

ensures that the results described below are due to the cell-autonomous effect of misexpression.

To test the possible role of *Hu* genes in neurogenesis by neural crest-derived populations, crest cultures were transfected with FLAG-cHuD or FLAG-cHuA expression vector. FLAG-cHuA transfectants showed no significant morphological changes compared to FLAG-GFP controls (data not shown). In contrast, FLAG-cHuD transfected cells exhibited dramatic changes in morphology (Fig. 8). Some of the transfectants exhibited flattened cell bodies with processes (Fig. 8C), and eventually showed more advanced neuronal morphology, such as round cell bodies and long axon-like processes (Fig. 8D). However, many of the transfectants also appeared to have round cell bodies without obvious processes (Fig. 9A-c) or with short processes (Fig. 9A-a). Unlike FLAG-GFP transfected cells, FLAG-cHuD transfected cells often had abnormal nuclei, including faint staining with DAPI and evidence of nuclear fragmentation (Fig. 9A-b). These observations suggested that such FLAG-cHuD transfected cells were also undergoing apoptosis.

Effect of cHuD misexpression on apoptosis

To confirm the induction of apoptosis by FLAG-cHuD misexpression, FLAG-cHuD, FLAG-cHuA or FLAG-GFP transfected cultures were subjected to the TUNEL reaction (see Methods) to detect DNA fragmentation (Fig. 9A-c, d). As reported in Fig. 9B, FLAG-cHuD transfected cultures revealed elevated levels of TUNEL-labeling (26%) compared to FLAG-cHuA transfected cultures (17%), and FLAG-GFP transfected control (15%).

The elevated levels of apoptosis in cHuD transfected cells suggested that they had acquired an increased dependence on some environmental cues present in limiting amounts in the cultures. Accordingly, we tested whether apoptotic FLAG-cHuD transfected cells could be rescued by addition of a neurotrophic factor NT-3 to the culture media. NT-3 had no significant effect on the proportion of FLAG-GFP-, and a small effect in the proportion of FLAG-cHuA-transfected cells that underwent apoptosis. However, in the presence of NT-3, the percentage of apoptotic cells in FLAG-cHuD-transfected cultures was dramatically reduced to near basal levels (17%).

To confirm that the action of NT-3 for the survival of cHuD transfected cells was due to neuronal differentiation, we used staurosporine as an inducer of cell type-independent apoptosis, and examined the effect of NT-3. Under our culture condition, in which approximately half of the cultured crest cells underwent apoptosis with staurosporine treatment, application of NT-3 did not rescue apoptosis in the population of cells. We also examined the expression of 16A11 and neurofilament (NF) as neuronal markers, and application of NT-3 did not change the proportion of cells expressing these neuronal markers in the staurosporine-treated cultures (data not shown). We conclude, therefore, that the rescue of cHuD-induced apoptosis with NT-3 is likely to be associated with neuronal differentiation.

Effect of cHuD misexpression on neuronal marker expression

Neuron-like morphology and NT-3 dependence of cHuD transfected crest cells suggested induction of neural differentiation

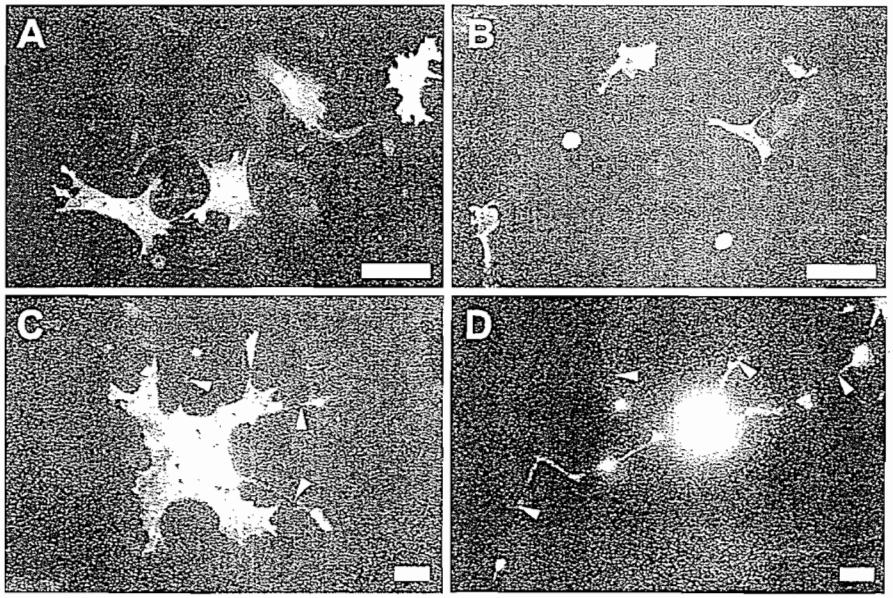

Fig. 8. Morphology of transfected crest cells. Cultured crest cells were labeled with anti-FLAG antibody 3 days after transfection. Cells transfected with FLAG-GFP (A) and FLAG-cHuD (B-D), respectively. Some of the cHuD-transfected cells appear as round cell bodies without cell processes (B), as flattened cell bodies with short processes (C, arrowheads), or long processes (D, arrowheads). Scale bars, (A,B) 50 μm, (C,D) 10 μm.

(Fig. 8C,D). To confirm these inferences, expression of neuronal markers such as neurofilament (NF) and MAP-2 were tested (Fig. 10). Three days after transfection with FLAG-cHuD, FLAG-cHuA or FLAG-GFP, cultures were stained with anti-NF or anti-MAP-2 antibody. Approximately 40% of FLAG-GFP$^+$ cells also expressed either NF or MAP-2 representing basal levels of neurogenesis in control crest cultures. FLAG$^+$ cells in the FLAG-cHuA transfected cultures also included similar number of NF$^+$ cells (33%). When the FLAG-cHuD expression vector was transfected, however, an average of 69% of FLAG$^+$ cells were NF$^+$ (Fig. 10B). Similar results were obtained by testing MAP-2 expression, where approximately 74% of FLAG-cHuD$^+$ cells were MAP-2$^+$.

Taken together, misexpression of FLAG-cHuD in cultured crest cells appears to promote increased neuronal differentiation, as revealed by morphological change, neuronal marker expression and neurotrophin dependence. In contrast, FLAG-cHuA-transfection resulted only in a small difference in neurotrophin responsiveness.

Effect of misexpression of cHuD in non-neurogenic neural crest cultures

To test the neural differentiation-promoting capability of cHuD, FLAG-cHuD was transfected into cultured neural crest cell populations that lack cells with neurogenic ability (Vogel and Weston, 1988). Transfection efficiency was 4-5%. Two days after transfection, cultures were double-stained with anti-FLAG and anti-NF antibodies (Fig. 11). No NF$^+$ transfectants were found in the culture. Regardless of whether the population contained FLAG-GFP, FLAG-cHuA or FLAG-cHuD transfected cells, approximately 70% of transfected cells were pigmented melanocytes and 30% were non-neuronal, non-pigmented cells thought to be glia (Blyss, Henion and Weston, unpublished data).

Effect of misexpression of cHuD and cHuA on cell proliferation

The higher level of cHuA mRNA expression detected in the ventricular zone of spinal cord (See Fig. 4H) suggested the possible involvement of cHuA in maintaining proliferation of neuronal precursors. To examine the effect of misexpression of cHuA and cHuD on the proliferation of neural crest cells, BrdU pulse-labeling was performed on the transfected cultures. No significant change of the proportion of BrdU$^+$ cells (7-10%) was observed as a result of transfection with FLAG-GFP, FLAG-cHuA and FLAG-cHuD.

DISCUSSION

Chicken Hu RNA-binding protein genes

In this study, three *Hu/elav* family genes were identified from a chicken cDNA library. Sequence analysis showed these

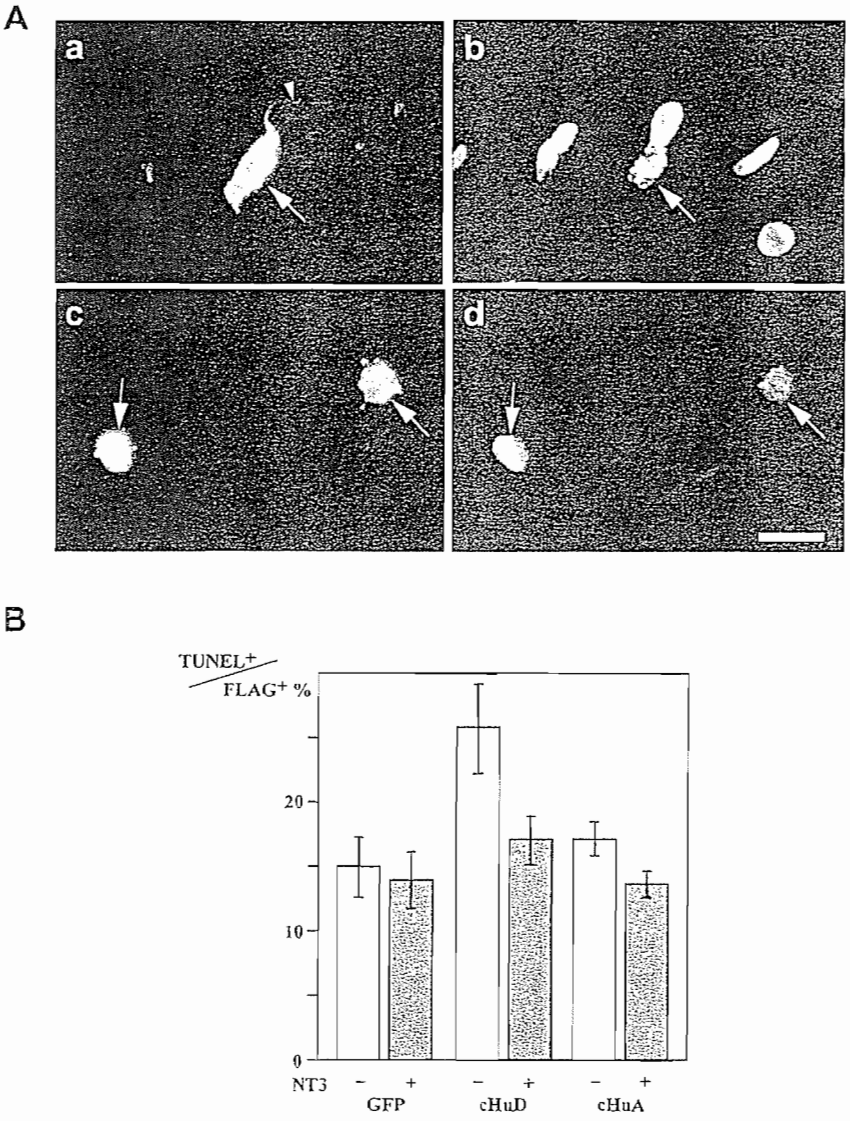

Fig. 9. Induction of apoptosis by cHuD, and rescue by NT3. (A) Examples of FLAG-cHuD transfected cells showing anti-FLAG stained cell (a, arrow), with fragmented nuclei revealed by DAPI staining (b, arrow). This cell also had a residual process (a, arrowhead). Anti-FLAG stained round cell bodies (arrows) (c), are also labeled with the TUNEL reaction (d). Scale bar, 20 μm. (B) Two days after transfection of FLAG-GFP, FLAG-cHuD or FLAG-cHuA to cultured crest cells, DNA fragmentation was assayed by the TUNEL method. These experiments were repeated 3 times, and the average percentage of TUNEL$^+$, FLAG$^+$ cells/FLAG$^+$ cells are shown. Approximately 100-200 FLAG$^+$ cells were examined in every experiment.

chicken genes were homologs of HuD/elrD, HuC/elrC and HuR/elrA (Szabo et al., 1991; Sakai et al., 1994; Liu et al., 1995; Good, 1995; Ma et al., 1996). The vertebrate Hu proteins and *Drosophila* ELAV and RBP9 have similar primary structure, such as three RNA recognition motifs (RRMs), and a stringer domain between second and third RRMs (Robinow et al., 1988; Kim and Baker, 1993). However, it is noteworthy that cHuA/HuR has gene-specific amino acid substitutions in RNP sequences (See Fig. 1A). This difference may alter the binding preference of target RNA sequences in vivo, even though an in vitro study showed that the sequence specificity of RNA binding of HuR appears to be similar to other Hu proteins (Ma et al., 1996).

Sequential expression of *Hu* genes during the neurogenesis

Neuron-specific anti-Hu immunoreactivity has been described (Marusich and Weston, 1992; Marusich et al., 1994) in the chicken embryo. However, it had not been clear which gene product(s) were recognized by the monoclonal antibody 16A11 in avian development. In this study, we have shown that expression patterns of cHuD and cHuC mRNAs overlapped with 16A11 immunoreactivity, and that both cHuD and cHuC proteins expressed in transfected fibroblast cells were recognized by 16A11. In contrast, cHuA mRNA expression did not correspond to the 16A11 immunoreactivity, and cHuA protein expressed in the fibroblast cells was not recognized by 16A11. Therefore, it seems likely that 16A11 immunoreactivity is due to the expression of cHuD and cHuC. In *Xenopus*, however, Xel-1 (the Hel-N1 homolog) mRNA is expressed in a neuron-specific manner both in the CNS and in sensory neurons of the PNS (Perron et al., 1995). Therefore, it is possible that an unidentified family member, such as a Hel-N1/elrB homolog may also contribute to 16A11 immunoreactivity in the avian embryo.

Expression of cHuD mRNA and 16A11 immunoreactivity were detected in newly differentiating neural precursors in the central nervous system and in crest-derived neuron precursor cells leaving the migration staging area (MSA; Weston, 1991), between the dorsal neural tube and the dorsal part of somite onto the medial pathway. cHuC mRNA expression was detected in differentiating neurons in both CNS and PNS one stage later than cHuD. In contrast to the neuron specific expression of cHuD and cHuC, cHuA was expressed in wide variety of tissues. As summarized in Fig. 7, cHuA expression in neural tissues is high in undifferentiated cells such as neuroepithelial cells in the ventricular zone of the neural tube and in migrating neural crest cells, and decreased during neuronal differentiation. However, cHuA expression is subsequently upregulated in maturing neurons. Therefore, the combination of expression of *Hu* genes seems to reveal the progress of neuronal differentiation and maturation. Moreover, the distinct expression pattern of cHuA from the neuron-specific expression of cHuD and cHuC suggests that these genes are functionally different.

Role of *Hu* genes in neurogenesis

Mutation in the *elav* locus results in defects in the *Drosophila* nervous system, suggesting the involvement of the *elav* gene in nervous system development and maintenance (Campos et al., 1985, 1987). Based on the pattern of expression of cHuD and cHuC RNAs and protein at early stages of neurogenesis, it seems plausible that these genes are also involved in the development and maintenance of vertebrate neurons.

In vitro studies have shown that all the Hu proteins have similar RNA-binding specificity. All Hu proteins can bind the AU-rich element of 3′UTR of transcription factors and cytokines, such as c-fos, c-myc, N-myc, Id, GM-CSF etc. (Levine et al., 1993; King et al., 1994; Liu et al., 1995; Ma et al., 1996; Chung et al., 1996). Recently, Gao and Keene showed the presence of Hel-N1/2 proteins bound to poly(A)$^+$ mRNA in a polysome fraction (Gao and Keene, 1996).

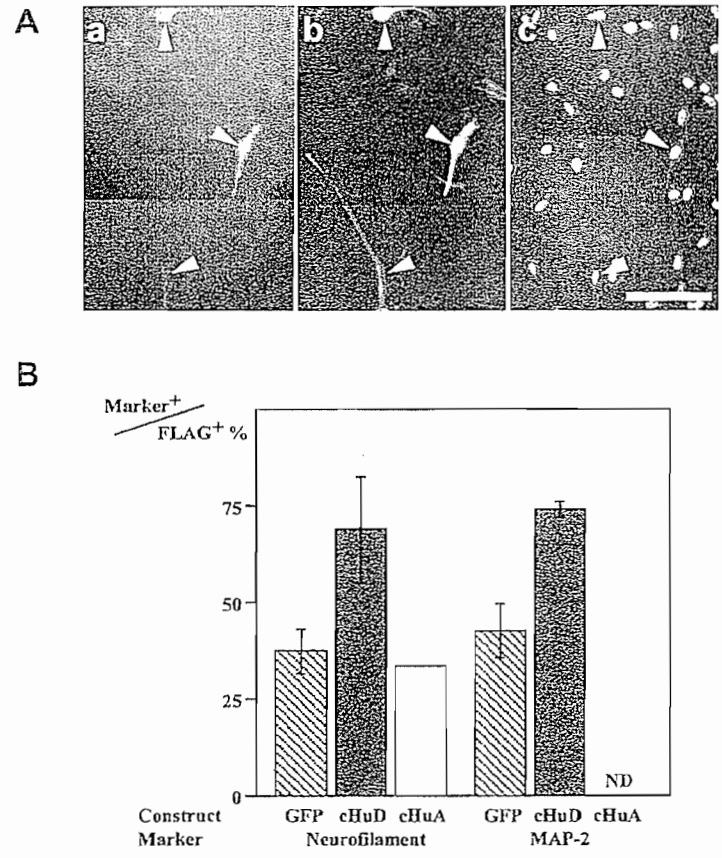

Fig. 10. Induction of neuronal marker expression by cHuD misexpression. (A) FLAG-cHuD transfected cells (a) expressing neurofilament (b) (arrowheads). (c) DAPI nuclear staining of same field as a and b. Scale bar, 100 μm. (B) Three days after transfection of FLAG-GFP, FLAG-cHuD or FLAG-cHuA, expressions of either MAP-2 or neurofilament was examined. Experiments were repeated 3 times except FLAG-cHuA transfection was done once, and the average percent of FLAG$^+$ cells that also expressed a neuronal marker are shown. Approximately 100-200 FLAG$^+$ cells were examined in every experiment. ND; not tested.

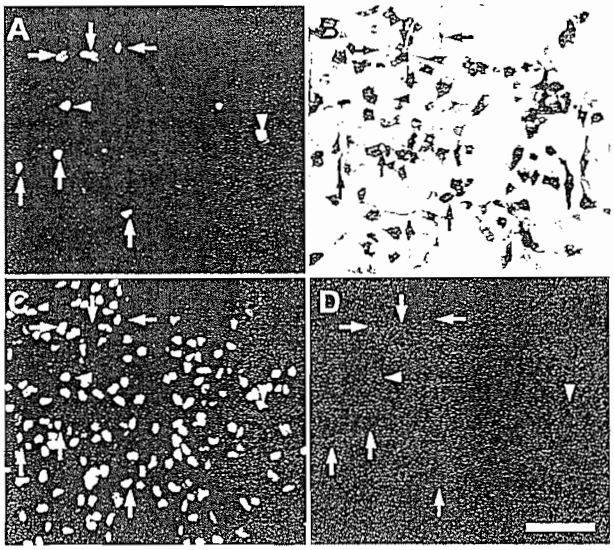

Fig. 11. Misexpression of cHuD does not affect the differentiation of non-neurogenic neural crest-derivatives. FLAG-cHuD was transfected to non-neurogenic crest cells, and stained with anti-FLAG and anti-NF antibodies. (A) Anti-FLAG, dark field; (B) bright field. (C) DAPI and (D) anti-NF. Note absence of NF^+ cells. Arrows indicate pigmented melanocytes. Arrowheads indicate non-pigmented putative glial cells. Scale bar, 50 μm.

Therefore, Hu proteins may bind to mRNA products and post-transcriptionally modulate the expression of genes that regulate proliferation, differentiation and survival. However, as described above, no significant effect was observed on the proliferation by misexpression of cHuD and cHuA, at least in cultured neural crest cells.

cHuD transfection increases the number of cells expressing neuronal markers in neurogenic populations of neural crest cells. However, cHuD could not restore the neurogenic ability in crest cell populations that lacked neurogenic ability. Therefore, it is likely that cHuD does not specify crest cells to adopt a neuronal fate, but promotes neuronal differentiation in cells that co-express neuronal precursor specific mRNAs.

Ectopic expression of cHuD in cultured neural crest cells resulted in elevated apoptosis of transfected cells. This cHuD-induced apoptosis was suppressed by NT-3. Therefore, it is possible that the apoptosis is due to increased neurotrophin dependence. Alternatively, forced expression of cHuD might alter the normal sequence of neuronal differentiation and stimulate an inherent program of cell death. However, the former idea seems more likely because the application of NT-3 failed to rescue neuronal differentiation-independent apoptosis (see above). It is known that a subpopulation of early neurogenic crest cells expresses *trkC*, the receptor of NT-3, and depends on NT-3 for survival (Henion et al., 1995). Therefore, cHuD might up-regulate the expression and/or translation of *trkC* mRNA. Alternatively, cHuD might regulate a 'live or die' signal mediated by neurotrophic factor. For example, it has been reported that $P75^{NTR}$ induces either apoptosis or survival by binding to NGF (Rabizadeh et al., 1993; Barrett and Bartlett, 1994). The activity of the *trk* receptor tyrosine kinase family is modulated by $p75^{NTR}$ (Hantzopoulos et al., 1994; Kahle et al., 1994). Thus, cHuD may be able to cause crest cells to become dependent on neurotrophic factor by stimulating the death signaling pathway mediated by $p75^{NTR}$. NT-3 might, in turn, support the survival of cHuD-expressing cells by activating *trkC*-mediated signaling, and subsequently, by suppressing the death pathway. Although these possibilities remain to be tested, it is clear, at least, that misexpression of cHuD alters the expression of various neuronal traits, including neurotrophic factor dependence, in crest cells.

The functional differences, if any, between the various *Hu* genes remain to be determined. In *Drosophila*, the *rbp9* transgene does not rescue the *elav* mutant phenotype, even though they are closely related genes, suggesting that *elav* and *rbp9* have different functions (Yao et al., 1993). Therefore, it is tempting to speculate that, although cHuD, cHuC and *Xel-1* show similar neuron-specific expression, they may have non-complementing functions. Likewise, since the expression patterns of cHuA differ from those of other family members, and since misexpression of cHuA appears to have only a minor effect on neurogenesis of neural crest cells, the role(s) of cHuA in development might differ from other Hu proteins.

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Dispatches

Cell Biology: Notch Recycling Is Numbed

During asymmetric cell division, the Notch regulator Numb segregates unequally to establish different cell fates in the two daughter cells. Numb is thought to act as an endocytic protein. Two new studies show that Numb antagonizes Notch signaling by also regulating recycling of Sanpodo–Notch complexes via AP-1.

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and Jürgen A. Knoblich*

Drosophila sensory organ precursors (SOPs) are one of the best-understood model systems for asymmetric cell division [1]. SOPs generate two daughter cells that establish different fates and are called pIIa and pIIb. It is thought that this difference is established through the asymmetric segregation of Numb, a cell-fate determinant that inhibits Notch signaling to specify the pIIb cell fate. In numb mutants, SOP cells divide symmetrically into two pIIa cells, whereas overexpression of Numb results in reciprocal cell-fate transformation [2]. Numb binds to the Notch receptor but also to a four-pass transmembrane protein called Sanpodo (Spdo) that is required for Notch signaling in the SOP lineage [3]. Notch is activated by its ligand Delta [4]. Upon activation, Notch is cleaved and its extracellular domain is trans-endocytosed together with Delta into the signal-sending pIIb cell, whereas the intracellular domain of Notch (NICD) translocates into the nucleus of the pIIa cell and acts as a transcriptional co-activator [5,6].

Several mechanisms have been proposed to establish differences in Notch signaling between pIIa and pIIb. First, the E3 ubiquitin ligase Neuralized segregates into the pIIb cell and ubiquitinates Delta [7]. This facilitates Delta endocytosis and increases its ability to activate Notch on the neighboring pIIa cell. Second, an endocytic compartment called the Sara endosome segregates asymmetrically into the pIIa cell together with Notch and Delta so that NICD gets released from the endosome and preferentially initiates signaling in pIIa [8]. Finally, Rab11-positive recycling endosomes are asymmetric, so that Delta is preferentially recycled to the plasma membrane in pIIb to enhance its signaling capacity [9]. Despite this apparent redundancy, however, the strong and highly penetrant cell-fate transformations observed in numb mutants suggest that Numb is a major contributor to asymmetric cell-fate establishment. It has therefore been unsatisfying that the mechanism by which Numb antagonizes Notch has not been fully clarified. A series of findings have suggested an 'internalization model', in which Numb acts in the pIIb cell to promote endocytosis of Notch. First, Numb localizes to endocytic organelles [10] and it co-localizes and physically interacts with the ear domain of a-adaptin, a subunit of the adaptor-protein complex 2 (AP-2) involved in endocytic trafficking [10,11]. Second, the Notch interactor Spdo localizes in vesicles in a Numb-dependent manner. In the absence of Numb, Spdo is found at the plasma membrane and activates Notch signaling [12]. Finally, antibody internalization experiments have suggested that Notch is preferentially internalized from the basal plasma membrane during cytokinesis in the Numb-inheriting pIIb cell and this does not occur in numb mutants [13].

Two studies published in this issue of Current Biology by Cotton et al. [14] and Couturier et al. [15] now add significantly to this topic by proposing that Numb primarily acts on post-internalization sorting events and inhibits recycling of Spdo–Notch (Spdo–N) complexes back to the plasma membrane. For this, the two papers combine classical Drosophila genetics and biochemical analysis with innovative imaging technologies and antibody internalization assays. Couturier et al. [15] generate fully functional GFP-tagged Numb (NumbGFP), Spdo (SpdoiGFP) and Notch (NiGFP) [13], and show that all three proteins accumulate and co-localize at sub-apical endosomes during SOP cytokinesis. These endosomes are identified as sorting endosomes, since they co-localize with Rab5 and Rab7. In numb mutants, localization of SpdoiGFP and NiGFP at sub-apical endosomes is reduced, suggesting that Numb regulates endosomal accumulation of Spdo–N complexes.

Interestingly, using antibody-internalization assays the two papers demonstrate that Spdo and Notch are both internalized in numb mutants, indicating that Numb is dispensable for the internalization of Spdo–N complexes. Previous reports revealed that endosomal localization of Spdo is dependent on AP-2 [12]. Consistent with this, Cotton et al. [14] show that fully functional mCherry-tagged Spdo is not internalized in a-adaptin mutants. From this, the authors conclude that internalization of Spdo requires AP-2 but not Numb and suggest that Numb instead might regulate post-internalization sorting events of Spdo–N complexes. Given that Numb physically interacts with AP-2, this raises the question about the functional relevance of this interaction. Couturier et al. [15] show that in mutants expressing a Numb-binding-defective form of a-adaptin, Notch is internalized normally in pIIb cells, but both Notch and Numb fail to accumulate at sub-apical endosomes. From this the authors conclude that the Numb–AP-2 interaction is specifically required for the rapid re-localization of Numb and Notch to sub-apical endosomes, but not for Notch internalization per se.

In addition to AP-2, the adaptor protein complex 1 (AP-1) is also required for correctly specifying the SOP lineage and has also been implicated in trafficking of both Notch and Spdo [16]. Cotton et al. [14] now demonstrate that the minor Notch gain-of-function phenotype in ap-1 mutants is increased in a numb heterozygous background. Conversely, Numb overexpression in

an ap-1^{RNAi} background causes a Notch loss-of-function phenotype. As Numb also binds to the AP1 subunit g-adaptin, it is plausible that Numb function is partly mediated through the AP-1 complex as well. In contrast to AP-2, which is thought to primarily regulate receptor internalization at the plasma membrane, the AP-1 complex is implicated in receptor sorting and recycling. Indeed, using an antibody uptake-recycling assay, Cotton et al. [14] can demonstrate that Spdo is internalized in both pIIa and pIIb, but recycles back to the plasma membrane in the pIIa cell only. As Spdo recycling occurs in both daughter cells in numb mutants, the authors propose that Numb inhibits AP-1-mediated recycling of Spdo–N complexes. While this hypothesis nicely explains the new data, it is still unclear how Numb might negatively regulate the recycling of Spdo–N. Numb could act as a dominant negative and compete with another protein that is crucial for AP-1-dependent sorting events. In mammals two functionally distinct AP-1 complexes exist: AP-1B is involved in basolateral targeting of transmembrane proteins, whereas AP-1A mediates transport between endosomes and lysosomes. The authors propose that Numb might inhibit AP-1 recycling function by converting an AP-1B into an AP-1A-like complex.

In conclusion, the new findings suggest a model in which Numb antagonizes Notch signaling primarily by inhibiting the AP-1-dependent recycling of Spdo–N complexes to the plasma membrane (Figure 1). How can these data be reconciled with the previous model in which Numb and AP-2 were thought to stimulate the endocytosis of Notch signaling components? Unlike AP-1, the essential role of AP-2 in specifying the SOP lineage is supported by a strong and fully penetrant numb-like cell-fate transformation phenotype observed in certain a-adaptin alleles [13]. These separation-of-function alleles affect Numb binding while leaving the cell-essential a-adaptin functions intact and therefore suggest that Numb needs to bind to a-adaptin for regulating asymmetric cell division. Besides modifying cell fate, these alleles were also shown to affect the asymmetric segregation of a-adaptin that is observed in the SOP lineage. It is conceivable that Numb is not strictly required for a-adaptin to regulate endocytosis but that the higher concentration of a-adaptin in pIIb enhances this cell's capacity to internalize signaling components. Better quantification of internalization rates would be necessary to test this hypothesis. In a second scenario, Numb could act with AP–2 on Notch signaling components other than Spdo or the Notch receptor itself, while Numb acts with AP-1 to regulate Notch recycling. Finally, it is quite possible that a-adaptin has a role in post-endocytic processes and it is this function that is specifically regulated by Numb. For example, the Numb–a-adaptin interaction could be required to speed up the rapid relocalization of Numb from the basal plasma membrane to apical sorting endosomes. In support of this, mammalian cell culture work has demonstrated a role for a-adaptin in post-endocytic trafficking through a clathrin-independent pathway [17].

Taken together, the new data suggest an alternative explanation for the Notch-inhibiting role of Numb. As well as their function in SOP cells, Numb and a-adaptin also have a role in Drosophila neural stem cells as tumor suppressors [18]. Whether or not AP-1 contributes to the stem-cell function of Numb is not known, but this can now be tested. Finally, of course, it will be exciting to explore how the new findings extend to vertebrates where the endocytic function of Numb was first described. Unlike in Drosophila, Numb does not bind to mammalian g-adaptin [10],

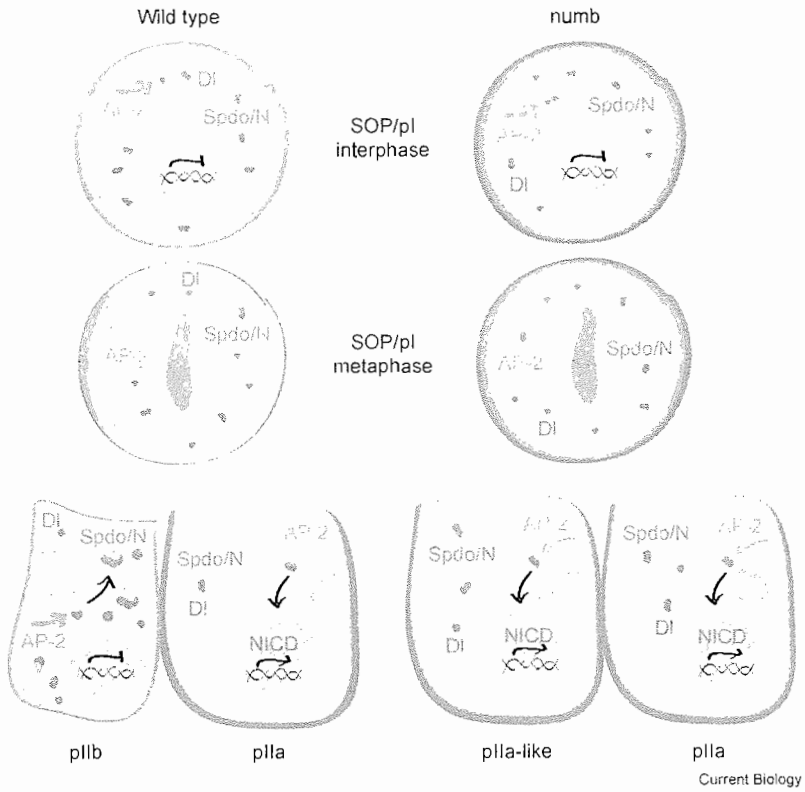

Figure 1. Numb inhibits recycling of Spdo–N complexes.

In interphase SOP cells, Spdo–N complexes (red) and the Notch ligand Delta (Dl, purple) co-localize in cellular vesicles. In numb mutants, Spdo localizes at the cell cortex, whereas Dl patterning remains unchanged. During mitosis, Numb (green) and a-adaptin (AP-2, blue) localize asymmetrically at the anterior cell cortex and segregate into the pIIb cell. In this cell, AP-2 promotes internalization of Spdo–N complexes, whereas Numb prevents their recycling back to the plasma membrane, presumably by inhibiting AP-1 (orange) function. In pIIa cells, Spdo–N complexes are recycled back to the plasma membrane after internalization. In numb mutants, Spdo–N is recycled in both daughter cells, resulting in activation of the Notch pathway, nuclear accumulation of the Notch intracellular domain (NICD), and ultimately the establishment of a pIIa-like fate in both daughter cells.

although the b-subunit of AP-1 was recently found in a proteomic-based search for Numb interaction partners [19]. In any case, the new findings reported in this issue extend the multiple roles that have been assigned to the Numb protein.

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Animal Communication: Sniffing Is About More Than Just Smell

A recent study shows that subordinate rats reduce their rate of sniffing while dominants explore their faces thus delaying dominants' subsequent aggression. Sniffing not only facilitates acquisition of olfactory information, but unexpectedly, also serves as a medium for communication.

Bennett G. Galef

When two Norway rats meet for the first time, they engage in lengthy bouts of mutual olfactory exploration, sniffing one another's faces, flanks and anogenital areas. Such intense olfactory activity promotes acquisition of information regarding the identity, sex, reproductive condition and dominance status of interacting individuals [1] and permits exchange of information as to the foods two interacting individuals have recently eaten [2]. Unexpectedly, such mutual olfactory exploratory behavior, studied for decades in one of mankind's most closely observed experimental animals (Rattus norvegicus), still contains secrets awaiting discovery. In this issue of Current Biology, Wesson [3] reports evidence that the duration and frequency of face sniffing between rats interacting for the first time (Figure 1) plays an important role in mediating the aggressive behavior of pair members.

To explore the details of sniffing in freely moving, socially interacting animals, Wesson [3] devised head-mounted, wireless, radio transmitters linked to thermocouples implanted in the nasal cavity of subjects. These contrivances allowed him to simultaneously record on video both the behavior of interacting rats and the frequency and amplitude of the sniffing of each member of pairs of rats meeting for the first time.

Wesson [3] found that when one rat investigated the face of another, some recipients of facial investigation significantly decreased the frequency with which they sniffed their partners' faces. In particular, when large male rats were paired with potentially subordinate individuals — either smaller males or females ovariectomized to increase the probability that they would behave submissively [4] — the subordinate member of many pairs significantly decreased its sniffing rate while the dominant individual examined its face. In contrast, the larger, presumably dominant member of such a pair showed either no change or an increase in sniffing while subordinates investigated their faces.

Subordinates' reduction in sniffing frequency while dominant individuals explore their faces plays a significant role in mediating agonistic interactions between pair members. The latency with which a dominant rat exhibited aggressive behavior following a bout of sniffing at the face of a subordinate was significantly correlated with the magnitude of the subordinate's decrease in sniffing frequency. The greater a smaller animal's decrease in sniffing rate (relative to baseline), the longer the latency to its larger partner's next expression of dominance asserting behaviors (boxing, kicking, standing over, and so on). Thus, rate of sniffing by submissive rats in social situations acts as a submissive or

REVIEW:

REVIEW

New Directions in Neuronal Migration

Mary E. Hatten

the past decade, genetic analyses have yielded a more molecular v
migration and its role in central nervous system development.
t many of the molecular mechanisms that guide migrations in inv
tulated in the vertebrate nervous system. These mechanisms gu
d anterior-posterior migrations and merge with radial migratory
prominent in the development of the mammalian cortex. Th
s the choreography of these different migratory mechanisms wi
of genetic approaches that have defined their molecular mechanisms.

The migration of immature neurons from germinal zones to specific positions where axon-target interactions occur is a critical step in the development of the synaptic circuitry of the brain. During development of the worm *Caenorhabditis elegans*, very few cells move from the positions where they are generated. Only 12 cell populations migrate, including three classes of neurons (HSN, CAN, and Q neuroblasts), somatic gonad precursors, and sex myoblasts (*1–3*). The more complex body plan of the fruitfly *Drosophila* is reflected in more widespread cell migration (*3*). In vertebrates, many cells undergo remarkable cell migrations, including the cells of the gonads, kidney, and the immune and nervous systems. Neuronal migration culminates in the formation of layered cortical structures in mammals where a novel form of migration, across the radial plane of the neural tube, develops.

Studies on neuronal migration in *C. elegans* have identified numerous genes that encode chemoattractants or receptors important for neuroblast migration along the body axis, either along the dorsoventral (DV) axis or anterior-posterior (AP) axis (Fig. 1) (*1–3*). The most studied of these is *unc-6* (also called *unc-6/Netrin1*), which is required for DV but not AP migrations in *C. elegans*. *unc-6* encodes a protein secreted by ventral midline cells, which guides the migration of cells in the dorsal direction via the receptor UNC5 and ventrally via the receptor UNC40 (*4*). As discussed later, UNC-6/Netrin1 and its receptors are critical for early cell migrations along the DV axis of vertebrates as well. With regard to the AP axis of *C. elegans*, MIG13 is a transmembrane protein that acts nonautonomously in anterior migrations of Q neurons (*5*) The expression of MIG13 is regulated by Hox gene activity, such that increasing doses of MIG13 causes cells to migrate further anterior. In *C. elegans*, *vab-8* functions in posterior migrations (*6*). The *vab-8* locus encodes two isoforms of an intracellular protein, one of which contains a kinesin-like motor domain. The general schema seen in *C. elegans*, of migrations along the central axes of the embryo via global positioning system genes, is now appreciated in vertebrate embryos.

Dorsoventral Migrations in Vertebrates

The *unc-6/Netrin1* mechanism is involved in the developing cerebellar system. In the cerebellum, beginning on about embryonic day 12 in the mouse, proliferating progenitor cells located along the edges of the rhombic lip at the mes/metencephalon border begin to migrate over the surface of the ventricle and around the circumference of the neural tube. Precursors of cerebellar granule neurons move in a dorsorostral pathway to cover the surface of the emerging anlage between embryonic days 12 and 16 (Fig. 2). In the developing chick embryo, real-time imaging has revealed a temporal relation between the time of emergence of cells from the rhombic lip and their migration routes (*7, 8*). Although a small population of cells from the rostral rhombic lip migrate ventrally, following the chemoattractant signal of UNC6/Netrin1, EGL cells of the cerebellum are repelled by netrin1 and move dorsorostrally across the surface of the anlagen. Indeed, progenitor cells overshoot the cerebellar territory in the spontaneous neurological mutant mouse *Unc5h3*, a disruption in a gene similar to *unc-5*. Thus, the cerebellar cortex forms, in part, as neurons evade the repulsive signal of the UNC6/Netrin1 guidance system.

Along the caudal portion of the rhombic lip, precursor cells of the precerebellar nuclei of the brainstem migrate from the rhombic lip toward the ventral midline, around the circumference of the neural tube, in a dorsal to ventral trajectory. These cell populations find netrin1 an attractive guidance cue and fail to migrate in mutant mice lacking DCC/UNC40 (*9*). Within the brainstem, the cells coalesce to form the inferior olive, a precerebellar nucleus that projects the climbing fiber afferents to the Purkinje cells. The basilar pons, another precerebellar nucleus in the brainstem, which projects mossy fiber afferents to the granule cells of the cerebellar cortex, also forms by DV migrations under the control of Netrin1 (*10, 11*). These progenitors undergo a DV migration and use UNC6/Netrin1 as a chemoattractant in their migrations. Thus, the axonal guidance system Netrin1/UNC6 plays a critical role in the formation of the cerebellar system.

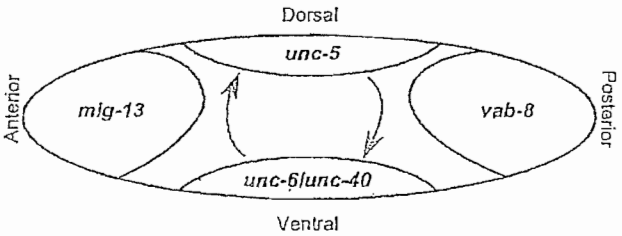

Fig. 1. Migratory pathways of neurons in *C. elegans* (*1*). UNC6/Netrin1 provides a guidance cue at the ventral midline (purple). Repulsion by UNC5 directs cells in a dorsal pathway (blue), whereas attraction by UNC6 or UNC40 directs cells ventrally. The membrane receptor MIG13 provides an anterior guidance system (yellow), whereas *vab-8* encodes two proteins for posterior cell migrations (green), one of which contains a kinesin domain.

Tangential Migrations in the Telencephalon

Another DV migration occurs in the forebrain, where proliferating precursor cells in the progenitor zone of the basal ganglia, the lateral and medial ganglionic eminences (LGE and MGE, respectively), migrate in a ventral to dorsal direction. This DV migration has been defined more precisely in genetic experiments using tissue from mice lacking the transcription factors *Dlx1,2* and *Nkx2.1* (*12*) With this tissue, it is possible to discern two populations of GABAergic inter-

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neurons migrating into the cortex. An early population of cells migrates dorsally from the MGE into the cortex, and a later population migrates along this trajectory from the LGE. The importance of this ventral to dorsal mode of migration is evidenced by the rather amazing fact that the GABAergic neurons comprise about 20% of the total neuronal population in the cortex. In addition to sending cells dorsally into the overlying cortex, the lateral ganglionic eminence also provides cells for the subventricular zone of the cortex. The migration of GABAergic neuroblasts from the MGE into the cortex involves the axon chemorepellant proteins semiaphorin 3A and semaphorin 3F. Migrating neurons express neuropilins (receptors for semaphorins), and semaphorin 3A and 3F are expressed in the striatum, the region the neuroblasts must circumnavigate to reach the cortex (*13*).

During development, a secondary proliferative zone is formed along the third ventricle of the forebrain. This progenitor zone, known as the subventricular zone (SVZ), persists in the adult, where it continues to generate neurons. The subventricular zone, once thought to provide glia to the overlying cortex late in development, is now realized to provide a steady supply of new GABAergic neurons destined for the olfactory bulb; these neurons travel from the SVZ to the olfactory bulb in the rostral migratory stream. These cells continue to proliferate as they migrate in a posterior to anterior direction to populate the olfactory bulb (*14*, *15*). Cells within this stream undergo an unusual mode of cell migration, namely, as a chain of neurons ensheathed by a protective layer of glial cells (*16*). Two classes of molecules guide the migration of SVZ cells to the olfactory bulb. Polysialated neural cell adhesion molecule (N-CAM) provides a positive guidance cue on the cell surface of migrating neurons as loss of polysialated N-CAM by targeted gene disruption or by enzymatic digestion disrupts SVZ neuroblast migration (*13*, *17*). Two proteins involved in axon guidance serve as repellants for SVZ neuroblasts. The receptor tyrosine kinases EphB1-3 and EphA4 and their transmembrane ligands ephrins-B2/3 are expressed by SVZ neuroblasts. Infusion of the ectodomain of EphB2 or of ephrinB2 into the lateral ventricle disrupts migration, suggesting a role for Eph/ephrin signaling in migration (*18*). Diffusible signals also appear to guide SVZ neuroblast migration as a gradient of the axon guidance protein Slit repels the stream of migrating cells from the overlying striatum (*19*). Great interest has arisen concerning this cell population, because it continues to generate new neurons after birth, well into adulthood, providing a supply of new GABAergic neurons for the olfactory bulb and a population of partially committed stem cells that can be differentiated into interneurons when transplanted into the septum, thalamus, hypothalamus, and midbrain of embryonic brain (*20*).

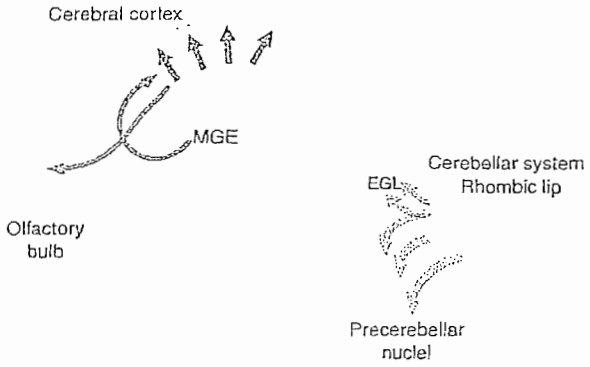

Fig. 2. DV and AP migratory pathways in vertebrate CNS migrations. Progenitors of the cerebellum arise along the rhombic lip at the midbrain/hindbrain junction and migrate dorsally, by repulsion to netrin1 and attraction to slit2 (green arrows). Progenitors of the precerebellar nuclei in the brainstem arise from more caudal portions of the rhombic lip and migrate ventrally along a netrin1 pathway (purple arrows). In the forebrain, cells from the MGE migrate dorsally into the cortex (blue arrow), while those of the rostral migratory stream follow a posterior to anterior trajectory (red arrow). A new migratory pathway emerges in higher vertebrates, namely, a radial pathway (black arrows).

Radial Migrations in Vertebrates

Before the cells migrating from the ventral forebrain arrive, the cerebral cortex begins to develop a radial migration pattern unique to higher vertebrates. The radial pathway of migration uses the processes of radial glial cells as a guide, and, importantly, provides a pathway for postmitotic neuronal migration. DV migrations, by contrast, generally involve mitotic neural precursor cells and do not use a glial scaffold. The initial hypothesis of glial guidance formed the basis for the radial unit hypothesis, which proposed that clones of cells generated in the VZ would be projected along radial glia into a columnar arrangement (*21*). This view was modified by studies on clonal dispersion in cortex, which showed widespread dispersion of some clones, and by imaging studies that showed tangential movements of some precursors (*22*, *23*). These two views have been clarified by recent findings, discussed above, that a large population of cortical interneurons arrive by a ventral to dorsal pathway of migration, from the MGE into the layers of cortex. Those cells that are widely dispersed cells from their subventricular origin are now thought to be interneurons (*24*, *25*). Cells that follow a simple radial pathway form the large output neurons of the cerebral cortex, the pyramidal cells. In contrast, GABAergic interneurons use a DV migration from the basal forebrain followed by a tangential movement into the forming layers of the cortex.

The initial movement of cells from the cortical germinal zone, on about embryonic day 11 or 12 in the mouse, generates a layer of postmitotic cells called the preplate, which is composed of Cajal-Retzius and subplate cells. As more cells exit the cell cycle and migrate through this region, they split this early zone into the outer layer of Cajal-Retzius cells and the underlying subplate forming the cortical plate. Sequential populations of cells migrate past the subplate to a position just underneath the Cajal-Retzius cells. The Cajal-Retzius cells form layer 1 and cells of the cortical plate generate layers 2 to 6. The cells within layer 6 are the earliest born neurons and those of layers 5 to 2 are sequentially later born cells, thus generating the "inside-out" arrangement described by Sidman (Fig. 3). A key insight into radial migration came from studies of the neurological mutant mouse *reeler*, which reels and stumbles from awkward coordination systems and has a cerebral and cerebellar cortex where the general pattern of neuronal layers is scrambled. Cloning of the *reelin* gene by insertional mutagenesis into the locus responsible for lamination defects in the *reeler* mutant mouse (*26*) revealed that the gene is responsible for the formation of the cortical plate, the backbone on which the neural layers are built. Reelin is a large extracellular matrix molecule, apparently synthesized by the earliest generated neurons, which binds to one of several receptor classes including VLDLR and ApoER2 (*26*, *27*). The binding of Reelin to receptors on adjacent neurons leads to a tyrosine kinase cascade that inludes phorphorylation of Dabl, an intracellular adapter protein the loss of which causes cortical malformations that resemble those in the *reeler* mouse. In *reeler*, those earliest generated cells fail to invade the preplate, thus failing to form a cortical plate with its systematic layering of cells. Expression studies indicate that the Cajal-Retzius cells, the cells of layer 1, express high amounts of Reelin and thus are the likely site of neuronal repulsion to form the underlying lamina. Thus, the Reelin pathway governs the overall patterning of cortical neurons into layers.

Mode of Movement of Immature Neurons Along Glial Fibers

The mode of movement of neurons along glial fibers, first proposed from static Golgi images, and then provided in detail from enhanced video microscopy, provides a framework for a series of genetic studies on the mechanism of cortical malformations (*28*). Here, the migration of the cerebellar granule cell has served as a paradigm for radial migration, because these cells can be purified in large numbers and used in cell-based assays of migration. Video microscopy of granule neurons first demonstrated the movement of neurons along glial fibers (*29*). Movement occurs in a salutatory cadence by formation of an adhesion junction along the length of the soma and the extension of a leading process, which projects short filopodia along the glial fiber (Fig. 4). Dynamic studies, labeling the membranes of migrating cells, showed rapid extension and retraction of this leading process over the length of the glial fiber. A critical aspect of migration concerns the cytoskeletal organization of the cells. Microtubules generate a "cage" around the nucleus (*30*) and apparently hold it in a caudal position as the cell moves. In addition, a centrosome or basal body projects a system of microtubules into the leading process. NudEL, a homolog of an *Aspergillus nidulans* gene involved in nuclear translocation, appears to localize to this structure (*31*). By video microscopy, vesicles move along this microtubule system; a cessation of vesicle movement accompanies a halt of migration. In contrast to growth cones, the microtubules seen in the leading process extend to the tip of the process. The only form of actin detected is cortical actin, a ring of which is seen in the cell soma. "Stress fibers," typical of extending growth cones, are not present in the leading process of migrating neurons. The importance of the cytoskeletal organization of migrating cells to movement is evident, because disruption of the system with pharmacologic agents halts migration. Thus, the mode of movement of neurons migrating on glial fibers is distinct from the mode of growth cone motility seen in axon extension. Indeed, the leading process of a neuron migrating along a glial process resembles a dendritic process more than it does an axon, a fact first noted by Ramon y Cajal (*32*).

In addition to revealing the cytoskeletal organization of migrating neurons, cell-based assays also proved useful in identifying *astrotactin* (*Astn1*), a gene that encodes a neuron-glial adhesion molecule. *Astn1* is expressed by neurons migrating along glial fibers in both cerebellum and cerebral cortex. A loss of *Astn1*, by targeted gene disruption, leads to a slowing of migration and to defects in the dendritic arborization of the target cell of the granule neuron, the Purkinje neuron (*33*, *34*). The disruption of Purkinje cell dendrite formation seen in mice lacking *Astn1* suggests that the slowed movement of the granule neurons, the presynaptic target cell of the Purkinje neuron, into position may provide an important timing device for dendritic development. Several other genes have been shown to function in glia-guided migration. These include neuregulin, which binds to ErbB4 on the glial surface and provides signals that maintain glial process formation (*35*, *36*). Although ASTN1 and neuregulin are expressed in postmitotic granule cell progenitors just before their migration along glial fibers, it is possible that even earlier genes act to set up the process of migration. In *C. elegans*, a number of genes act to disrupt axon formation. Homologs of one of these, *unc-51*, are expressed just after the cells leave the cell cycle, yet before they express markers of axon extension (TAG1) or neuron-glial binding (ASTN1). Unc51.1/Unc51.2 are serine-threonine kinases that provide a signaling casade for process formation in granule neurons (*37*). In *C. elegans*, *unc-51* mutants show a failure of axon outgrowth and of axon guidance, suggesting a role for this class of gene in steps required for cells to form migratory processes.

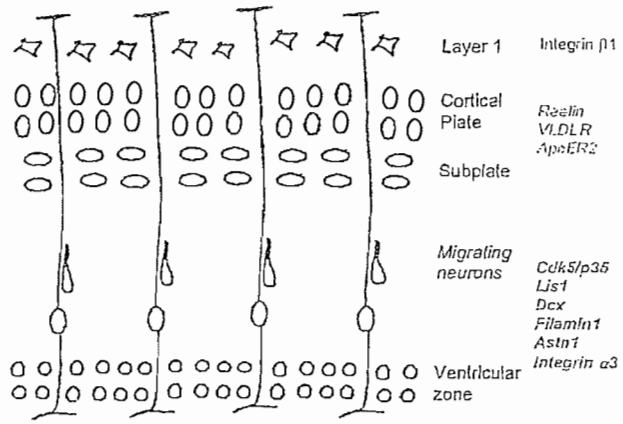

Fig. 3. Formation of the neuronal layers of cerebral cortex. The first-generated cells form layer 1 (Cajal-Retzius cells, blue), which later secrete Reelin. Thereafter, postmitotic cells migrate along the radial glial system to form the cortical plate (orange) and subplate (yellow) via the *reelin* pathway. As more cells arrive, the cortical plate generates a systematic set of layers (5 to 2). These later cells migrate along the radial glia (green) using genes that provide components of the cytoskeleton (*Lis1*, *Dcx*, *Filamin1*, and *Cdk5/p35*) or neuron-glia binding (*Astn1*, and *Integrin α3*). Continued cell division in the ventricular zone (blue) generates the most superficial layers.

Human Cortical Malformations

Studies on human cortical malformations (*28*, *38*) have described a series of neuronal migration syndromes, likely caused by disruptions in the cytoskeleton. Lissencephaly, a condition caused by less than normal sulcation of the cortex and thickening of the gray matter, is a large family of diseases. The range of malformations overlaps with two other syndromes, double cortex (DCX) and subcortical band heterotopias (SBH). The gene that is mutated in a severe form of Lissencephaly called Miller-Dicker syndrome is *Lis1*, which normally encodes a protein that binds to microtubules (*31*, *39*, *40*). Homozygous gene disruptions of *Lis1* result in lethality during embryogenesis. Heterozygotes survive but show defects in cell migration. Interestingly, the *Lis1*(+/–) cortex is not inverted, as seen in the *reeler* mouse phenotype; instead, migration appears to be slowed. Biochemical studies on the LIS1 protein show that it binds microtubules, suggesting that it may stabilize the microtubule network during cell migration. Attention has focused on the hypothesis that LIS1 also binds several NudEL homologs to regulate dynein function in the migrating neuron (*31*, *41*–*43*). This is consistent with the discovery of a *Drosophila Dlis1* gene mutation that results in defects in nuclear migration and in dendrite formation (*44*, *45*). Cdk5 phosphorylates NUDEL, suggesting that dynein motors are important in migration. Imaging studies from in vitro preparations, slice preparations, and electron microscopy studies all emphasize the posterior localization of the nucleus in neurons migrating along glia (*29*, *30*, *46*–*49*). Along with Miller-Dicker syndrome, XLIS is a common syndrome of classical lissencephaly in hemizygous males and DC/SBH is seen in heterozygous females. The Doublecortin gene *Dcx* encodes Doublecortin, another tubulin binding protein (*38*, *43*). DCX is ubiquitously expressed in developing neurons and exists both bound to the tubulin structures and cytoplasmic forms. In the search for proteins related to Doublecortin, a Doublecortin kinase was discovered. A mutant form of a related gene, *zyg-8*, has been reported in *C. elegans*, making it likely that *zyg-8* is the ancestral gene for Doublecortin (*50*). None of the genes that function in radial migration of postmitotic neurons appears to function in axon guidance. This contrasts with the migration of neuroblasts along the DV and AP (tangential) axes where the axon guidance systems Netrin1/UNC6,

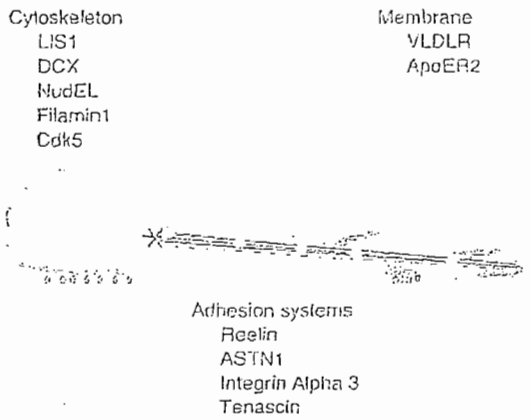

Fig. 4. Locomotion of the neuron along the glial guide. Neurons move along the glial guide in a salutatory motion, forming migrating junctions beneath the cell soma and at the tips of filopodia. Tubulin (purple) is organized into a cage surrounding the nucleus and a basal body (purple star) projects microtubules into the leading process. The latter extends short filopodia that enwrap the glial fiber as the cell moves. A ring of cortical actin (green) forms in the soma, with cortical actin seen along the distance of the leading process and extending into filopodia. As the cell moves, the nucleus remains in the posterior aspect and vesicles (orange) flow along the microtubule system.

Eph/ephrins, Semaphorin/neuropilin, and Slit play prominent roles in guiding migration.

Periventricular heterotopias highlight the function of actin binding proteins in migration. Individuals affected with periventricular heterotopias have pockets of ectopic cells lining the ventricles of the cortex. The gene mutated in this disorder, *filamin1*, encodes a protein that normally binds to actin and may be involved in the extension of filopodia as the cell moves along the glial fiber (*43*). Integrin α3, which could also act via actin filaments, appears to function in this step of migration as well (*51*). In mice, the Cdk5 kinase and its activator p35 induce actin reorganization when overexpressed in fibroblasts (*52*). Cdk5 modulates PAK kinases, effectors of the small guanosine triphosphatase (GTPase) Rac. Because PAK and Rho GTPases are implicated in actin reorganization, it may be critical for the dynamic aspects of the leading process of neurons migrating along radial glia. In mice lacking Cdk5 and p35, disruptions in cortical layering occur (*52, 53*). Unlike the Reelin mechanism, which inverts layers, a loss of the Cdk5 pathway appears to affect later migrating neurons as the cortical layers emerge from the cortical plate (*52*). New evidence indicates that *cdk5* is regulated, at least in part, by the class III POU transcription factors *Brn-1* and *Brn-2*. *Brn-1*, which is expressed in the cortex, apparently functions via regulation of p35, an activator of Cdk5. A loss of *Brn-1* results in a delayed migration to layers 2 and 3 in the mouse. This result resembles the phenotype of mice lacking Cdk5 and p35 and suggests a role for these genes in the locomotion of cells along the glial fiber system (*54*). In *C. elegans*, several mutations have been identified in Ras-like GTPases, which would be expected to remodel the actin cytoskeleton as in the formation of the leading process and its filopodia. These include *unc-73* and *mig-2*, protein products of which control the speed of migration in worm neurons. New families of genes involved in migration are therefore likely to come from a clearer understanding of the basic cell biology of migration, especially of signaling cascades critical for the specialized cytoskeletal assemblies seen in migrating neurons.

Conclusions

Vertebrates show far more widespread neural migrations than previously realized. In general, these migrations can be seen as DV or AP migrations, pathways thought to be prominent in lower organisms but not in vertebrates. Indeed, genes discovered in *C. elegans* and *Drosophila* provide molecular mechanisms for the DV and AP migrations in higher vertebrates. In the systems studied so far, these genes encode proteins that direct axon guidance in spinal cord and visual systems, and neuroblast migrations in the cerebellar system, the MGE/cortical system, and the rostral migratory stream. Genes for the radial pathway of migration have been cloned by the analysis of neurological mutant mice (Reelin pathway) and of humans with cortical malformations (*Lis1*, *Dcx*, and *Filamin1*). None of these genes has been shown to play a role in axon guidance. Model systems, with either cell-based assays (granule cells) or tissue slice preparations (cerebral cortex) identify many of the genes for radial migration as cytoskeletal or neuron-glia adhesion ligands (*Astn1*). Future experiments on the genetics of neuronal migration in invertebrates and vertebrates will reveal the scaffolds for CNS neuronal migrations. As the molecular basis of migratory pathways becomes more clear, we should begin to discern the underlying benefit of vast movements of cells to the development of the circuitry of the brain.

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Modulation of *nurr1* gene expression in mesencephalic dopaminergic neurones

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Abstract

The transcription factor/nuclear receptor Nurr1 is essential for the differentiation of midbrain dopaminergic neurones. Here we demonstrate that, during the ontogeny of rat ventral mesencephalon, *nurr1* gene expression is developmentally regulated and its levels show a sharp peak between embryonic day E13 and E15, when most dopaminergic neurones differentiate. In addition, in primary cultures from embryonic rat mesencephalon, *nurr1* gene follows a temporal pattern of expression comparable to that observed *in vivo*. We also report that exposure of embryonic mesencephalic cultures to depolarizing stimuli leads to a robust increase in *nurr1* mRNA and protein. The depolarizing effect is also detected in mesencephalic cultures enriched in dopaminergic neurones by using a combination of bFGF and Sonic hedgehog. The latter further increases the number of dopaminergic neurones in these 'expanded' cultures, an effect abolished in the presence of anti-Sonic hedgehog antibodies. Our data show that *nurr1* gene is highly expressed in midbrain dopaminergic neurones in a sharp temporal window and that its expression is plastic, both *in vivo* and *in vitro*. In addition we show that Sonic hedgehog can direct dopaminergic differentiation in proliferating dopaminergic neuroblasts *in vitro*.

Keywords: bFGF, dopamine, primary cultures, midbrain, real time RT–PCR, Sonic hedgehog.

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In higher vertebrates, most dopaminergic (DA) neurones are located in the *substantia nigra* and the ventral tegmental area in the mesencephalon (MES). They form the nigrostriatal and the mesocorticolimbic pathways, involved in motor control, cognition and emotion (Perrone-Capano and di Porzio 1996). Midbrain DA pathways are implicated in psychiatric and neurological disorders, including Parkinson's disease, where embryonic DA neurone grafts have a beneficial effect (Dunnett *et al.* 2001).

Transcription factors, such as the bicoid-related Ptx3 (Smidt *et al.* 1997; Nunes *et al.* 2003) and the nuclear orphan receptor Nurr1 (Law *et al.* 1992; Zetterstrom *et al.* 1996) play a pivotal role in MES DA neurone differentiation. Mice lacking Ptx3 fail to develop DA neurones of the *substantia nigra* (Nunes *et al.* 2003), whereas in *nurr1* null mice all midbrain DA precursors fail to mature (Zetterstrom *et al.* 1997; Saucedo-Cardenas *et al.* 1998; Le *et al.* 1999a; Wallen *et al.* 1999). Nurr1 can regulate key genes involved in DA neurotransmission, including tyrosine hydroxylase (*TH*), vesicular monoamine transporter 2 (*VMAT*), the DA transporter (*DAT*; Sakurada *et al.* 1999; Iwawaki *et al.* 2000; Sacchetti *et al.* 2001), and *Ret*, which encodes for the coreceptor for the glial cell line-derived neurotrophic factor (GDNF) family associated to the GDNF family receptor alpha (GFRαs; Wallen *et al.* 2001; Airaksinen and Saarma 2002).

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Abbreviations used: bFGF, basic fibroblast growth factor; DA, dopamine; DAT, dopamine trasporter; DIV, days *in vitro*; E, embryonic age; ERK, signal regulated kinases 1/2; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFRα, 1–2, GDNF family receptor α1–2; HPRT, hypoxantine-phosphoribosyl-transferase; MES, mesencephalon; PBS, phosphate-buffered saline; PKA, protein kainase A; PVDF, polyvinylidene difluoride; Ret, tyrosine kinase receptor Ret; RT–PCR, reverse trascription–polimerase chain reaction; SHH, sonic hedgehog; TH, tyrosine hydroxilase; VMAT, vesicular monoamine transmitter transporter 2.

The differentiation of embryonic stem cell-derived DA neurones *in vitro* is accompanied by activation of *nurr1* gene (Carvey *et al.* 2001; Haas and Wree 2002) and the exogenous expression of Nurr1 into stem cells enhances differentiation and maturation into DA neurones (Wagner *et al.* 1999; Chung *et al.* 2002; Kim *et al.* 2002, 2003a). In addition, Nurr1 regulates dopamine synthesis and storage in MN9D dopamine cells (Hermanson *et al.* 2003).

The expression of Nurr1 is maintained in differentiated MES DA neurones (Saucedo-Cardenas and Conneely 1996; Zetterstrom *et al.* 1996b). Although its function in mature DA neurones has not been determined, it appears to be important for control of normal DA neurotransmission, as shown by reduced DA levels in the adult brain and enhanced stress-induced locomotor behaviour in heterozygous mice for the Nurr1 allele (Eells *et al.* 2002). These mice also show increased vulnerability to the DA neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Le *et al.* 1999b). Over-expression of *nurr1* modifies susceptibility of mouse neural stem cells to neurotoxins (Lee *et al.* 2002). Interestingly, polymorphism in the intron six of *nurr1* gene in humans (also known as *NOT* or NR4A2) is associated with Parkinson's disease (Xu *et al.* 2002; Zheng *et al.* 2003). In addition, missense mutations in *nurr1* gene have been found in cases of schizophrenia and manic–depressive disorder (Buervenich *et al.* 2000; Chen *et al.* 2001).

Modulation of nurr1 mRNA has been reported in adult MES in D2 receptor-deficient mice (Tseng *et al.* 2000) and cocaine abusers (Bannon *et al.* 2002), as well as in cell lines (Law *et al.* 1992; Kovalovsky *et al.* 2002; Satoh and Kuroda 2002) and peripheral neurone primary cultures (Brosenitsch and Katz 2001).

Despite these interesting findings, little is known about the mechanisms that regulate *nurr1* gene expression in developing DA neurones. To ascertain how the expression of this gene is regulated during MES development, we studied its expression pattern during rat ontogeny and in cultured embryonic MES neurones under different experimental conditions.

Materials and methods

Animals and dissections

Timed pregnant Sprague–Dawley rats (Charles River Breeding Laboratories, Milan Italy) were killed in accordance with Society for Neuroscience guidelines and Italian law. Embryonic age (E) was determined by considering the day of insemination (as confirmed by vaginal plug) as day E0. Embryos from 12 to 20 (± 0.5) days of gestation were quickly removed and placed in phosphate-buffered saline (PBS) without calcium and magnesium and supplemented with 33 mM glucose. The ventral mesencephalon from various prenatal and post-natal ages were carefully dissected under a stereoscope in sterile conditions and processed for RNA and protein isolation or for cell cultures.

Cell cultures

Cells were dissociated from embryonic rat ventral MES and cultured as previously described (di Porzio *et al.* 1980). Briefly, the tissues were dissected from E12–E13 embryos and dissociated using mechanical trituration with a fire-polished Pasteur pipette in culture medium (see below) and 0.01% pancreatic deoxyribonuclease (Sigma, Milan, Italy), centrifuged 10 min at 800 *g*, suspended in plating medium and counted. Tissues from E14 embryos were enzymatically dissociated by incubation for 30 min at 37°C in a solution (Warthington, 20 U/mL) in Earle's balanced salts containing 1 mM EDTA, 1 mM cysteine and 0.01% pancreatic deoxyribonuclease. After addition of 1 mg/mL of bovine serum albumin (fraction V, Sigma) and 1 mg/mL ovomucoid (Sigma) the cell suspension was centrifuged 10 min at 800 g, suspended in plating medium with a fire polished Pasteur pipette and counted (Fiszman *et al.* 1991).

Standard culture conditions

Cells were plated at a density of 125 000/cm^2 (24-multiwell plates, Costar, Milan, Italy), in dishes coated with 15 μg/mL of poly-D-Lysine (Sigma). Cells were grown in neurobasal medium (Invitrogen, Milan, Italy), L-glutammine (0.5 mM, Sigma), 7.5% fetal bovine serum (HyClone Laboratories Inc., Milan, Italy), 5% horse serum (Euroclone, Milan, Italy); 5 μM cytosine β-D-arabinofuranoside (AraC, Sigma) was added to the cultures after 3 days *in vitro* to inhibit non-neuronal cell proliferation.

Expanded cultures

Cells were dissociated from E12 rat ventral MES as described above and plated at a density of 18 000/cm^2, as above. Cells were grown as above in absence of serum with the addition of B27 supplement (Invitrogen), bFGF 20 ng/mL, Sigma and the N-terminal fragment of the Sonic hedgehog (SHH) protein (50 ng/mL). Half of the medium was changed every 3 days; bFGF and SHH were withdrawn at various days *in vitro* (DIV), as indicated. AraC was never added. In some experiments, SHH activity was blocked by anti-SHH antibodies (5E1, Developmental Studies Hybridoma Bank, Iowa City, IA, USA).

Cultures were depolarized by adding to the growth medium 56 mM KCl and 10 mM $CaCl_2$ for 2 h, or as indicated. Alternatively 10 μM veratrine hydrocloride (Sigma) was used, with or without tetrodotoxin (5 μM, Sigma). Depolarization experiments were repeated using at least 10 different preparations of expanded MES DA cultures.

The drugs used were: signal regulated kinases 1/2 (ERK) inhibitor U0126 (Calbiochem, Milan, Italy), the protein kinase A (PKA) inhibitor H89 (Calbiochem) at 10 μM in dimethylsulphoxide (DMSO, Sigma); calcium channels blocker ω-conotoxin (1 μM, Sigma) and nimodipine (1.5 μM, Sigma); calcium was depleted from intracellular stores by thapsigargin (1 μM, Sigma), and extracellular calcium was blocked by 200 μM EGTA (Sigma). The DA, norepinephrine and serotonin membrane transporters were inhibited by GBR-12909 (100 μM, Sigma), reboxetine (10 nM, Pharmacia Upjohn, Milan, Italy) and fluoxetine hydrochloride (1 μM, Sigma), respectively; protein synthesis was inhibited by cycloheximide (50 μM, Sigma, 30 min pre-incubation followed by 2 h incubation with or without KCl). All drugs were added to the incubation medium after a 10 min pre-incubation (unless otherwise specified).

When drugs were dissolved in DMSO, control cultures were also treated with the solvent alone.

[^{3}H]-DA uptake was performed as previously described (Prochiantz *et al.* 1979). Briefly, cultures were washed in PBS containing 33 mM glucose, 1 mM $CaCl_2$ and $MgCl_2$ and incubated in the same buffer containing 50 nM [7,8-^{3}H]-DA (S.A. 40–60 Ci/mmol, Amersham Biosciences, Milan, Italy) at 37°C for 15 min, in the presence or absence of the amine uptake inhibitors or at 0°C (to obtain blank values). After three washes with cold PBS, cells were dissolved in 0.2 mL of Triton X-100 (0.2%) and 0.2 N NaOH, then neutralized with equimolar concentrations of HCl and counted in 5 mL Biodegradable counting scintillant (Amersham) in a Beckman liquid scintillation counter. The uptake was calculated by subtracting blank values from the amount of radioactivity accumulated at 37°C.

Cultures were always carried at least in triplicate sister samples for each experimental point analysed and were processed separately.

RT–PCR analyses

RNA was extracted from pools (five to 18 embryos) of embryonic rat ventral midbrain of the same age, from single post-natal (P) tissues or from single cultures, using the Tri-Reagent isolation system (Sigma) and following the manufacturer's instructions. The analyses were always carried in triplicate obtained from different pools, tissue samples or cultures for each experimental point analysed and were processed separately. The yield and the integrity of RNA were determined by spectrophotometrical measurement of A_{260} and agarose gel electrophoresis, respectively. RT–PCR analyses were as previously described (Perrone-Capano *et al.* 1994; Pernas-Alonso *et al.* 1999). Briefly, total RNA was treated with DNA free kit (Ambion Inc., Milan, Italy) to prevent false results by DNA contamination. Two micrograms of RNA were reverse transcribed using random hexanucleotides as primers (New England Biolabs Inc., Milan, Italy, 6 μM) and 200 U of moloney-murine leukaemia virus reverse transcriptase (Ambion). One-twentieth of the reverse-transcribed cDNA was amplified in a 25 μL reaction mixture containing AmpliTaq Gold DNA polymerase buffer (Applied Biosystem, Milan, Italy), 0.2 mM dNTPs (Finnzymes OY, Espoo, Finland), 0.4 μM each primer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystem) and 1 μCi [^{32}P]dCTP (3000 Ci/mmol, Amersham).

Different sets of primer pairs were used in the same reaction tube to co-amplify cDNA, together with primers for the hypoxantine-phosphoribosyl-transferase (*HPRT*), a constantly expressed gene during CNS development, used as internal standard (Steel and Buckley 1993). After a first denaturing step at 95°C for 8 min, PCR amplification was performed for 27 cycles organized as follows: 95°C for 0.5 min; 56°C– 58°C for 0.5 min; 72°C for 0.5 min and was followed by a final extension step (72°C for 5 min). The number of cycles was experimentally chosen in order to fall into the exponential phase of the amplification reaction. Forward and reverse primers (MWG-Biotech, Ebersberg, Germany) used are listed in Table 1, together with the length of amplified fragments. The specificity of PCR primers was determined by performing BLAST searches against the databases. Non-reverse-transcribed RNA templates and mock controls were always run in PCR reactions and never gave amplification products. Identity of each amplified

Table 1 Primers nucleotide sequence (5′-3′) used for RT–PCR experiments

Ret	F. CGGCTGCATGAGAATGACTGGA	494 bp	Nestin	F. CGCTCGGGAGTGTCGCTTAGA	273 bp
	R. CAAAGCCTCCAGCACATACTTC			R. GCCTCCAGCAGAGTCCTGTAT	
DAT	F. CGTGGGACCAATGTCTTCAGTG	498 bp	NET	F. TGCTGCTCTGCCTGATGGTC	541 bp
	R. ATGGTGAAGGAGGAGAAGAAGT			R. AGCCCCAGAGCCAGGAGCAT	
EAAT-1	F. TTGGTTCGAGGACACAGTGAG	504 bp	NFL	F. ACCTCTCCGCCGCTCTCAAG	613 bp
	R. CAGGCCCACGATTTTGTATTC			R. TCTCCTCGACCTCTGTCTGCTCT	
EP10	F. GCTGGAAGGCATGGAAGGTT	242 bp	NFM	F. GAAATGGAAGAAACCCTCACA	474 bp
	R. AGCCCCATCACCGTAGCAAC			R. CCGGCCTTGGCCTCTGGTTTTGG	
GAD65	F. GGCTCTGGCTTTTGGTCCTTC	437 bp	Nurr1	F. CGCGTCGCAGTTGCTTGACAC	449 bp
	R. TGCCAATTCCCAATTATACTCTTGA			R. CGCGTCGCAGTTGCTTGACAC	
GAD67	F. GACCGACTTCTCCAACCTGTT	464 bp	Ptx3	F. GCAACTGGCCGCCCAAGG	83 bp
	R. TCCCATCACCATCTTTATTTG			R. AGGCCCCACGTTGACCGA	
GFAP	F. GCTGGAGGTGGAGAGGGACA	456 bp	SERT	F. AGGAAGATCTGCCCGATTTTC	487 bp
	R. TGGCGGCGATAGTCGTTAGC			R. CTGCCAGTTGGGTTTCAAGTA	
GFRα1	F. CGCTGCCACTCCTGGATTTG	512 bp	TH	F. TGTCACGTCCCCAAGGTTCAT	276 bp
	R. GGTGCAGGGGGTGATGTAGG			R. GGGCAGGCCGGGTCTCTAAGT	
GFRα2	F. GGGAACATGGAAGAAGAGTGT	278 bp	TrpH	F. TTCCAGGAGAATCATGTGAGC	273 bp
	R. CACATGCTTAACTCTTTGGAG			R. TTCGGATCCATACAACAGCA	
HPRT	F. CCTGCTGGATTACATTAAAGCACTG	370 bp	VMAT	F. ATCCAGACCACCAGACCAGAG	616 bp
	R. CCTGAAGTACTCATTATAGTCAAGG			R. CCCCATCCAAGAGCACCAAGG	

The table shows the forward (F) and reverse (R) primer sequence with length of amplified fragment (bp) used in RT–PCR for the following genes: tyrosine kinase receptor Ret (*Ret*), dopamine transporter (*DAT*), neuronal glutamate transporter (*EAAT-1*), glutamic acid decarboxylase *GAD65*, *GAD67* and its embryonic form *EP10*, glial fibrilary acidic protein (*GFAP*), GDNF family receptor α 1–2 (*GFRα1 and GFRα2*); hypoxantine-phosphoribosyl-transferase (*HPRT*), *nestin*, noradrenaline transporter (*NET*), light neurofilament (*NFL*), medium neurofilament (*NFM*), Nurr1, the bicoid-related homeobox gene *Ptx3*, serotonine transporter (*SERT*), tyrosine hydroxilase (*TH*), tryptophan hydroxylase (*TrpH*), vesicular monoamine transmitter transporter 2 (*VMAT*).

Table 2 Primers nucleotide sequence (5'-3') used for real time RT–PCR experiments

HPRT	F. AGTCCCAGCGTCGTGATTAG	160 bp
	R. CCATCTCCTTCATGACATCTCG	
Nurr1	F. CAACTACAGCACAGGCTACGA	98 bp
	R. GCATCTGAATGTCTTCTACCTTAATG	
TH	F. CCTTTGACCCAGACACAGCA	121 bp
	R. ATACGAGAGGCATAGTTCCTGAG	

The table shows the forward (F) and reverse (R) primer sequence with length of amplified fragment (bp) used in real time RT–PCR for the following genes: hypoxanthine-phosphoribosyl-transferase (*HPRT*), *nurr1*, tyrosine hydroxilase (*TH*)

fragment was confirmed by size (see Table 1) and by digestion with restriction enzymes or by sequence analysis.

The [^{32}P]-labelled amplified products were separated by electrophoresis in 1.5% agarose gel, dried and exposed to a PhosphorImager screen. Quantitation was achieved by integrating the volume areas of each fragment obtained from scanning the screens with a PhosphorImager apparatus (Molecular Dynamics, Sunnyvale, CA, USA), equipped with an ImageQuant software. The ratio between the yield of each amplified product and that of co-amplified HPRT allowed a relative estimate of the mRNA levels (Pernas-Alonso *et al.* 1999). Triplicate samples allowed statistical analysis (see below). In some experiments, RT–PCR results were also confirmed by real-time PCR, using QuantiTect SYBR Green PCR Kit (Quiagen, Milan, Italy) and a Biorad iCycler iQ multicolor real time PCR detection system. Primers used were as in Table 2. Primers were designed according to manufacturer instruction, in order to obtain amplified fragments of comparable length (around 100 bp). The amplified products were measured with the intercalating dye SYBR Green. mRNA levels were calculated according to the threshold cycle numbers and standardized vs. the housekeeping gene *HPRT* obtained for every sample in parallel assays. A melting curve was obtained for each PCR product after each run, to confirm that the SYBR green signal corresponded to a unique and specific amplified product.

Immunocytochemistry

Mesencephalic cell cultures were fixed for 30 min at RT, in 4% paraformaldehyde in PBS, followed by three washes in PBS, permeabilized for 15 min in PBS containing 0.1% Triton X-100 (unless indicated otherwise) and 10% normal goat serum (NGS) and incubated for 2 h at RT or overnight at 4°C in the primary antibodies diluted in PBS containing 10% NGS. The following antibodies were used at the indicated dilutions: monoclonal (mAb) anti-TH (1 : 200, Chemicon, Milan, Italy); rabbit anti-TH (1 : 100, Chemicon); mAb antiglial fibrillary acidic protein (GFAP; 1 : 400, Sigma); mAb antinestin (Rat-401; 1 : 100, Developmental Studies Hybridoma Bank, Iowa City, IA, USA); mAb antiglutamic acid decarboxylase (GAD-6; 1 : 100, Developmental Studies Hybridoma Bank); rabbit antimedium neurofilament subunit (NFM; 1 : 200, Chemicon). After rinsing in PBS, mesencephalic cell cultures were stained according to standard avidin–biotin immunocytochemistry procedures (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) using peroxidase substrate kit (DiaAminoBenzidine) or were incubated in fluorescent-labelled secondary antibodies (Texas red goat anti-rabbit, Molecular Probes Inc, Eugene, OR, USA; goat anti-mouse fluorescein-conjugated, Chemicon) in PBS containing 5% NGS. Control cells were incubated in the same solutions without primary antibodies and subsequently processed as above. Positive cells were counted in each well of a lab-tek chamber slide (Nunc, VWR International, Milan, Italy) using an eye piece grid. Three culture wells were analysed in each experiment for every experimental condition.

Western blots

For western blot analyses, control and depolarized expanded MES DA cultures were lysed in 150 mM NaCl, 10 mM Tris–Cl pH 8, 0,4 mM EDTA, 1% Triton X-100 containing a cocktail of protease inhibitors (Roche, Milan, Italy). Protein content of each sample was determined by the Bio-Rad assay kit (Bio-Rad Laboratories S.r.l., Milan, Italy). Sixty micrograms/lane of proteins were separated on 10% SDS–polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride (PVDF) filters (Amersham) in transfer buffer (25 mM Trizma, 193 mM glycine, 20% methanol, Sigma). Filters were soaked for 20 min in transfer buffer and were blocked for 3 h in blocking buffer (10 mM Tris–HCl pH 8, 150 mM NaCl, 5% -non-fat dry milk, Bio-Rad). Membranes were probed for 1 h at room temperature with following antibodies: Nurr1 (Chemicon, Milan, Italy), ERK1 (Santa Cruz Biotechnology Inc., Milan, Italy) and phosho-p44/42 MAP kinase (P-ERK1/2, Cell Signalling Technology, Milan, Italy). After washing (five times for 5 min) with TTBS (Tris–HCl 10 mM pH 8.0, NaCl 200 mM, Tween-20 0,1%) and once with TBS, immunoblots were incubated with goat anti-rabbit IgG antibody (Amersham, in blocking buffer, 1 h at room temperature) and washed as above. The reaction was detected with ECL procedure (Amersham).

SHH purification

Escherichia coli strain BL21 was transformated with a plasmid pGEX-N (gift of P. Beachy), which express the N-terminal fragment of mouse SHH, and induced with IPTG. Cells were lysed and cleared lysates were bound to glutathione-conjugated sepharose beads (Pharmacia). The bound fusion protein was cleaved with a mass of thrombin (Sigma) equivalent to 1% the mass of fusion protein in 10 mM Tris–HCl (pH.7.5), 150 mM NaCl, 2.5mM $CaCl_2$. The protein released from the beads was batch adsorbed to antithrombin–agarose (Sigma). SHH was purified from this supernatant by adsorption to heparin–agarose (Pharmacia) in PBS containing 1 mM dithiothretol (DTT; Sigma) followed by elution in PBS containing 550 mM NaCl, 1 mM DTT, 10% glycerine (Sigma). The concentration of SHH was determined by Bradford assay (Bio-Rad). SHH product was detected as a single band of 22 kDa by SDS–polyacrilamide gel stained with Coomassie blue, indicating a purity greater than 95% . The purified protein was aliquoted and stored at –80°C until use.

Statistical analysis

At least triplicate samples were used in all the experiment described, thus allowing statistical analysis. Analysis of variance was carried out, followed by post hoc comparison (ANOVA Scheffè *F*-test). Data were expressed as mean ± SEM.

Results

nurr1 gene expression during midbrain development: *in vivo* and *in vitro* studies

By using semiquantitative RT–PCR reaction assays, we analysed the pattern of *nurr1* gene expression during ontogeny of the rat ventral mesencephalon. Nurr1 transcripts were clearly detectable from the first time-point examined (E12), when also *TH* gene expression was detectable. *nurr1* mRNA levels showed a sharp significant increase at E13. The high levels of nurr1 mRNA persisted for about 2 days, until E15, when they reached a value at least 15-fold higher than at E12. After this point, Nurr1 transcripts rapidly decreased to levels similar to those observed at earlier stages and remained constant throughout late embryonic and post-natal development and in adult life (Fig. 1a). Other genes expressed by DA neurones during MES ontogeny, such as *DAT*, *TH*, *VMAT*, have different patterns of expression (Perrone-Capano *et al.* 1994).

In order to ascertain whether the *nurr1* gene expression pattern during development was dependent from persistent external influences or was cell autonomous, we established rat primary cultures from E12, E13 and E14 MES, grown in standard conditions (i.e. at high density and in the presence of serum). We examined *nurr1* gene expression profile at various DIV. A significant peak of nurr1 mRNA was observed at 3 DIV in E12 cultures, at 2 DIV in E13 cultures and at 1 DIV in E14 cultures, followed by a lower level of expression thereafter (Fig. 1b). This pattern of expression was specific for *nurr1*, as other genes expressed by DA neurones in primary cultures (*TH*, *DAT*, *VMAT*) have different profiles, as previously shown (Perrone-Capano and di Porzio 1996). These data show that the expression of *nurr1* gene is developmentally regulated during the maturation of MES DA neurones, both *in vivo* and *in vitro*, and suggest that it might be modulated by epigenetic factors.

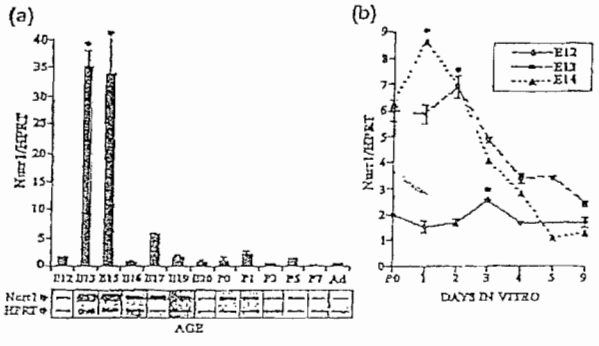

Fig. 1 Pattern of *nurr1* gene expression during ontogeny of the rat mesencephalon (a) and in MES primary cultures (b). The diagrams show the relative quantitation (mean ± SE) of the amplified products compared to that of the hypoxanthine phosphoribosyl transferase (HPRT, internal standard). Data are expressed as ratio Nurr1/HPRT at various embryonic (E) and post-natal (P) ages and in the adult (Ad) or at various days *in vitro* (DIV) in E12 (●), E13 (■) and E14 (▲) MES cultures grown in standard conditions (see Materials and methods). The gel in the inset shows an example of the co-amplified products of Nurr1 and HPRT at any given age studied. Asterisks represent $p < 0.01$ when compared to the E12 value (a) or to the other DIV in the same culture (b) (ANOVA, Scheffé *F*-test).

Although primary cultures have proven a useful tool to study DA neurone differentiation, their generation require large amount of embryonic tissues. Expanded cultures from embryonic MES are a valid alternative to generate new DA neuroblasts *in vitro* and induce their differentiation, starting from a small amount of MES embryonic cells. The controlled *in vitro* generation of dopaminergic neurones is an ideal tool to investigate the function of new genes recently implicated in the differentiation of dopaminergic neurones (Studer *et al.* 1998).

We thus used expanded cultures generated from ventral MES from E12 rat, when a large number of DA precursors should be present. In order to induce neuroblast proliferation *in vitro*, we administred bFGF (20 ng/mL) to these cultures. We observed that addition of bFGF from the beginning of the culture was mandatory to ensure the proliferation of E12 MES cells plated at low density (18 000 cells/cm^2) in serum-free medium (Bouvier and Mytilineou 1995; Volpicelli *et al.* 2004). In order to increase dopaminergic differentiation, the cultures were treated with the amino-terminal recombinant fragment of SHH (50 ng/mL), an inductive signal produced by the floor plate and notocord necessary in the specification events of the DA phenotype (Hynes *et al.* 1995; Puelles *et al.* 2003). These expanded cells can be cultured for long time and display all the main differentiative features of MES DA neurones. We have tested various combinations of addition/withdrawal of bFGF and SHH (Volpicelli *et al.* 2003). For the experiments described below we have chosen the conditions that gave the greater number of TH$^+$ cells, i.e. when SHH was added to the medium from the time of plating, together with bFGF, and both factors were withdrawn after 6 DIV of stimulation; these 'expanded MES DA cultures' were always analysed after 9 DIV. At this time *in vitro*, the number of TH$^+$ cells was increased about 20-fold when compared to 3 DIV and about fourfold when compared to 6 DIV. At the end of the expansion procedure, the number of DA neurones generated *in vitro* from 18 000 MES cells in the expanded cultures was comparable to the number of DA neurones present in primary cultures generated from about 250 000 MES cells/plate. To verify that the increase in the number of TH$^+$ neurones was selectively induced by the recombinant SHH added to the cultures, neutralizing antibodies were used to block its activity. As shown in Fig. 2(a), a saturating dose

of SHH (50 ng/mL) promoted a 30% increase in the number of TH^+ neurones, whereas the same dose of SHH in the presence of a five fold molar excess of anti-SHH antibodies completely inhibited this response.

The expanded MES DA cultures contained neural precursors, as shown by the expression of the nestin gene and differentiated neurones expressing neurofilament subunits. Immunocytochemistry showed that at least 90% of the expanded cells were nestin-positive, and that at least 70% were NFM-positive. Co-localization of nestin and NFM was observed in about 30% of the cells. Cells expressing glial markers, such as the GFAP, were extremely rare (on average one or two GFAP-positive cells/well at 9 DIV). As previously shown, glioblasts appear dormant as serum addition increased *GFAP* gene expression (Volpicelli *et al.* 2003).

In addition, expanded MES DA cultures expressed all the 'dopaminergic' genes analysed, such as *TH*, *VMAT*, *DAT*, *Ret*, *GFRα1*, *GFRα2* and *nurr1* (Fig. 2b).

In the expanded MES cultures, DA neurones maturation occurred, as shown by the appearance of specific high-affinity DA uptake. The latter is driven by DAT, whose function (as well as the turn on of the encoding gene) appears at later stages of DA development when compared to other dopaminergic markers, both *in vivo* and *in vitro* (Perrone Capano and di Porzio 2000). In order to verify that the DA uptake was specifically carried out by DA neurones and to exclude the presence of other aminergic neurones in the expanded MES DA cultures, specific uptake inhibitors were used. As shown in Fig. 2(c), [^{3}H]-DA uptake was completely abolished by a selective uptake inhibitor in dopaminergic neurones, GBR-12909 (Baldo and Kelley 1991); on the contrary it was not affected by a selective inhibitor of amine uptake in serotonergic neurones, fluoxetine, nor by a selective inhibitor of uptake in noradrenergic neurones, reboxetine. These data indicated that virtually all TH^+-positive cells detected in the expanded MES cultures were DA neurones and that other

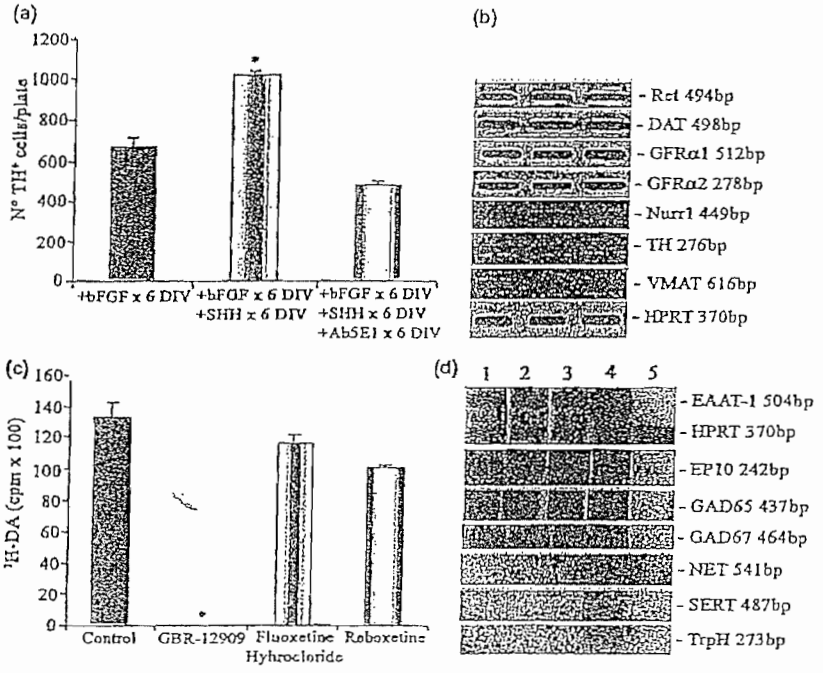

Fig. 2 Characterization of expanded MES DA cultures. (a) Number of TH-positive cells in 9 days *in vitro* (DIV) cultures treated for the first 6 DIV with bFGF (20 ng/mL) with or without SHH (50 ng/mL) and the antibody 5E1. (b) RT–PCR analyses of genes involved in midbrain neurone development and function from triplicate cultures at 9 DIV. The size of the amplified products (ethidium bromide stained) is shown on the right in base pairs (bp). (c) [^{3}H]-dopamine uptake in control cultures at 9 DIV or treated with the selective DA transporter inhibitor GBR-12909 (100 μM), the selective serotonin transporter fluoxetine (1 μM) and the selective noradrenaline transporter reboxetine (10 nM). (d) RT–PCR analyses of neuronal glutamate transporter (*EAAT-1*), glutamic acid decarboxylase *GAD65*, *GAD67* and its embryonic form *EP10*, noradrenaline transporter (*NET*), serotonine transporter (*SERT*), tryptophan hydroxylase (*TrpH*). Lanes 1–3 show the amplified products in triplicate expanded MES DA cultures at 9 DIV, lane 4 shows the fragments in rat control tissues (frontal cortex for *EAAT-1*; pons for *NET*, *SERT* and *TrpH*; E15 MES for *GAD65*, *GAD67* and *EP10*), lane 5 shows the fragments in adult heart (negative control). The size of the amplified products (ethidium bromide-stained) is shown on the right in base pairs (bp). The bars represent the mean ± SE of triplicate sister samples for each experimental point. Asterisks represent $p < 0.0$ when compared to control cultures (ANOVA, Scheffè *F*-test).

aminergic neurones were virtually absent. In order to verify this assumption, we performed RT–PCR experiments to detect expression of serotonergic (serotonin transporter, tryptophan hydroxylase) and noradrenergic (noradrenalin transporter) genes. As expected, there was no expression of any of these genes in MES expanded cultures, whereas their transcripts were detected in control tissues (Fig. 2d).

In order to further characterize the neuronal population present in the expanded MES cultures, the presence of GABAergic neurones was assessed by GAD immunocytochemistry (about 300 positive cells/well) and RT–PCR for *GAD65*, *GAD67* and its embryonic form *EP10*. Immunocytochemical co-localization of GAD and TH was never observed. RT–PCR for the neurone-specific glutamate transporter EAAT-1 demonstrated the presence of glutamatergic neurones (Fig. 2d).

Depolarization stimulates *nurr1* gene expression in embryonic DA neurones

nurr1 gene expression can be induced by electrical stimulation in the adult CNS (Pena de Ortiz and Jamieson 1996; Xing *et al.* 1997; Crispino *et al.* 1998) and in cultured cells (Law *et al.* 1992; Brosenitsch and Katz 2001; Kovalovsky *et al.* 2002). In order to test the hypothesis that membrane potential might influence *nurr1* mRNA levels also in embryonic MES DA cultures, we conducted membrane depolarization experiments. Nurr1 mRNA was strongly increased (three- to sevenfold) in E13 MES cells cultured in standard conditions (F. Volpicelli *et al.*, unpublished observations) as well as in expanded MES DA cultures after treatment with high K^+ (2 h, 56 mM KCl), as assayed by semiquantitative RT–PCR (Fig. 3a) and confirmed by real-time PCR experiments (Table 3). To verify that the observed effect was due to membrane depolarization, expanded MES DA cultures were treated for 2 h with veratrine, which causes voltage-gated sodium channels to remain open. As expected, veratrine treatment also enhanced *nurr1* mRNA levels and its effect was completely inhibited by tetrodotoxin, which specifically blocks voltage-gated sodium channels (Fig. 3a). Depolarization-induced increase of *nurr1* mRNA was equally observed after 2 h treatment of the cultures with 25 mM K^+, but not by 10 mM K^+ (data not shown).

The time-course of *nurr1* mRNA increase following depolarization was assessed in expanded MES DA cultures treated with 56 mM K^+ for various length of time. As shown in Fig. 3(b), 5 min of elevated K^+ treatment were not sufficient to induce any change in *nurr1* mRNA, whereas elevation of *nurr1* mRNA – although not statistically

Table 3 Real time RT–PCR on control and depolarized expanded MES DA cultures

	Control	Depol.
Nurr1/HPRT	0.16 ± 0.05	0.75 ± 0048 *
TH/HPRT	0.37 ± 0029	0.32 ± 0.03

All values are reported as ratio of the threshold cycle numbers and standardized vs. the housekeeping gene hypoxantine-phosphoribosyltransferase (*HPRT*). Each value represents the mean of three independent experiments ± SEM. *Asterisks represent $p < 0.01$ when compared to control cultures (ANOVA, Scheffè *F*-test). Quantitative real time RT–PCR was used to confirm the levels of *nurr1* and tyrosine hydroxylase (*TH*) mRNA in 9-day-old expanded MES DA cultures treated with 56 mM KCl for 2 h and in untreated control cultures.

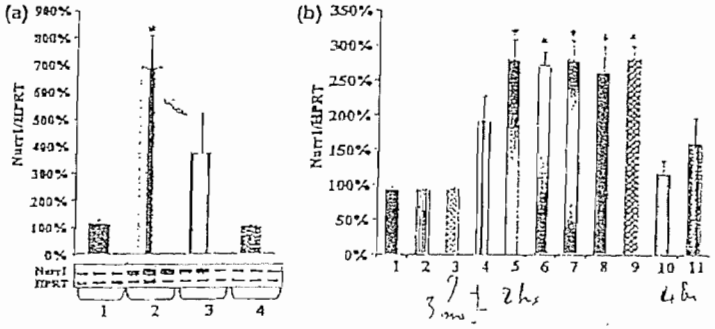

Fig. 3 Depolarization-induced *nurr1* gene expression in expanded MES DA cultures. The diagrams show the relative quantitation (mean ± SE) of the Nurr1 amplified product compared to that of the hypoxanthine phosphoribosyl transferase (HPRT, internal standard). Data are expressed as ratio Nurr1/HPRT in expanded MES DA cultures at 9 DIV. (a) 1, control cultures; 2, cultures treated for 2 h with K^+ (56 mM); 3, cultures treated for 2 h with veratrine (10 μM); 4, cultures treated for 2 h with veratrine (10 μM)+-tetrodoxin (5 μM, TTX). The gel in the inset shows triplicate samples for each experimental condition of the coamplified Nurr1 and HPRT fragments. (b) 1, control cultures; 2, control cultures + cycloeximide (50 μM, 2 h incubation); cultures depolarized with K^+ (56 mM) in the following conditions: 3, for 5 min; 4, for 1 h; 5, for 2 h; 6, for 2 h without $CaCl_2$; 7, for 2 h without external $CaCl_2$ + EGTA (200 μM); 8, for 2 h without external $CaCl_2$ + EGTA (200 μM) + thapsigargin (1 μM); 9, for 2 h with cycloheximide (50 μM); 10, for 4 h; 11, for 2 h +2 h without K^+. Asterisks represent $p < 0.01$ when compared to control cultures (ANOVA, Scheffè *F*-test).

significant – was detectable after 1 h of depolarization and reached significant higher values, when compared to untreated cultures, after 2 h of K^+ treatment. Prolonged K^+ depolarization, up to 4 h, not only did not cause any significant further increase in *nurr1* mRNA but instead restored the initial levels. *nurr1* mRNA levels returned to control values within 2 h after the end of the depolarization (Fig. 3b). Thus, in cultured MES DA neurones, *nurr1* gene expression responded to membrane depolarization with a rapid increase in *nurr1* mRNA followed by an as much rapid decrease. This rapid induction occurs also in the presence of cycloheximide, which blocks *de novo* prothein synthesis (Fig. 3b), this result confirms the immediate early gene nature of *nurr1*. Differently, depolarization did not modify the expression of other genes analysed (namely, *TH*, *DAT*, *Ret*, *Ptx3*, *GFRα1* and *GFRα2*). Thus, only *nurr1*, among the 'DA' genes analysed, responded within 2 h to this type of stimulation.

Signal transduction

In order to dissect the molecular mechanisms that link membrane depolarization with *nurr1* gene expression, we first evaluated the potential involvement of L-type and N-type voltage-activated calcium channels, which mediate specific activity-dependent neuronal gene expression by raising intracellular calcium levels (Finkbeiner and Greenberg 1998). We find that neither nimodipine nor ω-conotoxin, inhibitors of L- and N-type channels, respectively, were able to affect the induction of *nurr1* mRNA in response to K^+-induced membrane depolarization.

We then evaluated the involvement of calcium in mediating depolarization-dependent *nurr1* gene expression. Therefore, depolarization experiments were conducted in the presence of EGTA to chelate extracellular calcium or also in the presence of thapsigargin, to deplete intracellular calcium stores. As shown in Fig. 3(b), both treatments were unable to block the hyperexpression of *nurr1* mRNA, indicating that in expanded MES DA cultures, K^+ membrane depolarization induced *nurr1* gene expression by calcium-independent mechanisms.

We found that, in expanded MES DA cultures, K^+ depolarization caused a robust ERK phosphorylation, which showed a peak after 5 min and was still evident, although to a less extent, after 2 h of depolarization (Fig. 4a). However, K^+ depolarization was still able to induce *nurr1* gene expression also in the presence of the MEK inhibitor U0126 (Fig. 4b), whereas ERK phosphorylation was abolished (Fig. 4a).

We finally performed K^+ depolarization on expanded MES DA cultures, in the presence of the PKA inhibitor H89. Also this treatment did not significantly alter Nurr1 induction (Fig. 4b). In control experiments, the inhibitors U0126 and H89 were effective in blocking phosphorilation of ERK and CREB, respectively, following acute NGF and forskolin stimulation in PC12 cells (data not shown).

To ascertain whether the observed upregulation of Nurr1 transcripts corresponded to an increase in the Nurr1 protein, we performed western blot experiments with anti-Nurr1 antibodies using protein extracts from expanded MES DA cultures, untreated and K^+ depolarized. As expected, the intensity of the 60 KDa band stained by the Nurr1 antibody, was greater in depolarized than in control cultures (Fig. 4c).

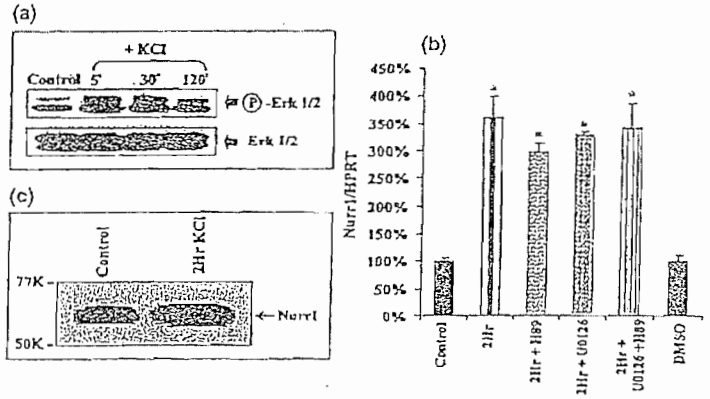

Fig. 4 Nurr1 mRNA increase is ERK signalling-independent. (a) Depolarization-induced ERK phosphorylation: Phospho ERK (P-ERK 1/2) and ERK (ERK 1/2) levels were analysed by western blots using antiphosho-p44/42 MAP kinase (P-ERK1/2) and anti-ERK1 in cultures untreated (control) and depolarized for various length of time. (b) Effect of selective inhibitors on *nurr1* gene expression: cultures untreated (control) and depolarized for 2 h (2 h) were treated with the PKA inhibitor H89, the ERK inhibitor U0126 or both, or with their solvent alone (DMSO). (c) Nurr1 protein is increased in cultures following a 2-h depolarization (2 h) when compared to control cultures, as analysed by western blot using an anti-Nurr1 antibody. On the left are shown the molecular weights.

Discussion

Recent studies have identified a number of genes critically involved in midbrain DA development, including morphogens, such as FGF8 and SHH, and transcription factors, like Lmx1b, Ptx3 and Nurr1 (Smidt *et al.* 1997, 2000; Zetterstrom *et al.* 1997; Wallen *et al.* 1999; Nunes *et al.* 2003). In this study, we have analysed the pattern of *nurr1* gene expression in developing MES DA neurones *in vivo* and *in vitro* and we show that it is developmentally regulated and plastic.

During rat MES ontogeny, a sharp peak of *nurr1* gene expression takes place between E13 and E15, when most DA neurones differentiate (Perrone-Capano and di Porzio 2000). In this developmental window, the levels of Nurr1 transcripts are at least 15-fold higher when compared to E12 or to late embryonic or post-natal periods. Therefore, *nurr1* shows a peculiar pattern of expression when compared to other MES DA genes such as *TH* or *DAT*. The latter progressively increase from the time of onset until they reach a plateau (Perrone-Capano *et al.* 1994), although using *in situ* hybridization approach, increased TH mRNA has also been observed during post-natal development (Solberg *et al.* 1992). It is well established that multistep regulatory mechanisms for *TH* gene expression occur during midbrain DA neurone development and that the TH promoter possesses consesus domains for a number of transcriptional elements that can regulate *TH* gene expression (Lebel *et al.* 2001; Matsushita *et al.* 2002).

Thus, high levels of Nurr1 seem required at crucial embryonic developmental stages for the proper DA determination in midbrain. Consistent with this hypothesis, Nurr1 is necessary for the generation of MES DA cells (Zetterstrom *et al.* 1997; Le *et al.* 1999a) and plays a critical role in their maturation, migration, striatal innervation and survival (Saucedo-Cardenas *et al.* 1998; Wallen *et al.* 1999), and that Nurr1 overexpression can generate functional DA neurones from CNS precursors (Kim *et al.* 2003a).

How Nurr1 expression is regulated and which are the control signals, remain still elusive. We addressed the question whether *nurr1* gene expression pattern is cell autonomous or dependent from extracellular signals, conducting *in vitro* experiments on MES DA neurones. Our data show that *nurr1* mRNA *in vitro* follows a pattern of expression similar to that observed *in vivo* during development, independently from the embryonic developmental stages. It is worth noticing that the magnitude of the peak of *nurr1* transcripts is higher *in vivo* than *in vitro*, suggesting that the developmental increase of *nurr1* gene expression may depend, at least partially, from cues intrinsic to MES DA neurones. However, still unknown epigenetic factors, presumably absent in MES primary cultures, could enhance this effect. Thus, *nurr1* gene expression seems plastic *in vivo* as well as *in vitro*, as highlighted by *nurr1* mRNA increase in our cultures, when depolarized. Because Nurr1 in developing and mature midbrain co-localizes with TH^+ and DAT^+ neurones (Zetterstrom *et al.* 1997; Wallen *et al.* 2001; Bannon *et al.* 2002), our data suggest that electrical activity may physiologically modulate Nurr1 in developing DA neurones. Indeed it is known that *nurr1* gene expression, as other immediate early genes, can be rapidly induced by electrical stimulation *in vivo* and *in vitro* (Pena de Ortiz and Jamieson 1996; Xing *et al.* 1997; Crispino *et al.* 1998; Broseuitsch and Katz 2001).

Studies on DA neurone development *in vitro* are limited by the relative low number of DA neurones in primary cultures. We thus have adopted a method to expand neuroblasts derived from MES in primary cultures by bFGF stimulation (Bouvier and Mytilineou 1995; Studer *et al.* 1998) and enriched these cultures in DA cells by the action of SHH. We show that withdrawal of bFGF is necessary to enhance DA differentiation and that, under these conditions, SHH plays a specific inductive role on dispersed DA precursors, extending the results obtained in explant cultures, by ectopic SHH grafts, and in stem cells (Hynes and Rosenthal 1999; Lee *et al.* 2000; Kim *et al.* 2003b). It is plausible that most DA precursours in E11-12 ventral midbrain were already induced by SHH. However, it is not surprising that SHH could further increase DA differentiation in the expanded cultures, which is blocked by specific antibodies and accounts for about 30% of the DA neurones differentiated at 9 DIV. We suggest that SHH exerts its effects *in vitro* on the still dividing bFGF-stimulated neural precursors. Indeed, the number of TH^+ cells is higher when SHH is added at the time of plating, when compared to its addition after 6 DIV (Volpicelli *et al.* 2003). DA neurones in the expanded cultures show important features of MES DA neurones, including DA gene expression (*nurr1*, *Ptx3*, *TH*, *VMAT*, *Ret*, *DAT*) and DA high-affinity uptake. In the expanded MES DA cultures, DA affinity uptake is highly specific, indicating that in these cultures only DA neurones differentiate among aminergic neurones. This conclusion is supported by the absence of serotonergic and noradrenergic markers. However, the expanded cultures contain also GABAergic and glutamatergic neurones, but are virtually deprived of glial cells, as assessed by RT–PCR and immunocytochemistry. These expanded MES DA cultures provide a useful source of MES DA neurones as MES DA precursors are expanded up to 20-fold. They could thus provide a new tool for functional reconstitution experiments in animal models of Parkinson's disease and in regenerative approaches (Dunnett *et al.* 2001). Indeed, the question whether DA neurones can be functionally used in nigrostriatal grafts, independently from their anatomical and functional origin, is still open as the interactions between embryonic and adult neurones in the nigrostriatal pathway are highly selective. For instance, only DA neurones obtained from MES, but not from other CNS regions, are

able to restore function when grafted in mouse hosts with lesioned nigrostriatal system (Zuddas *et al.* 1991). Moreover, Nurr1 overexpressing naive CNS precursors, which *in vitro* show many features of DA cells, do not yield any functional improvements when grafted in Parkinsonian rats and show reduced *in vivo* survival and differentiation, compared with E12 precursor-derived DA cells (Kim *et al.* 2003a). This supports the view that new approaches are needed to generate large number of DA neurones of MES origin, as it occurs in the expanded MES DA cultures.

Interestingly, in MES DA cultures, depolarization-dependent Nurr1 upregulation is rapid and sustained, occurring within 2 h. It is paralleled by an increase in the corresponding protein, suggesting that it can modulate transcription of a cascade of target genes. In our conditions, no variation in known DA genes, putative targets of Nurr1, including *TH*, *DAT* and *Ret*, is observed. Similarly, other mRNAs such as GFRα1 and GFRα2, recently shown to be modulated by depolarization in chick peripheral nervous system (PNS) neurones (Doxakis *et al.* 2000), do not vary following K^+ stimulation. It is possible that other genes or experimental paradigms or longer times may be required to see modulation of known or unknown genes, triggered by Nurr1.

Which could be the physiological role of depolarization-induced Nurr1 activation? It is attractive to speculate that neural activity could play a role in selectively regulating the expression of a transcription factor critical for the development of MES DA neurones, at crucial stages during embryogenesis. Indeed, the peak of *nurr1* gene expression occurring during MES ontogeny precedes the onset of activation of the other genes selectively expressed by MES DA neurones, such as *TH* and *DAT* (Zetterstrom *et al.* 1997; Perrone-Capano and di Porzio 2000). On the other hand, it is well known that activity-dependent gene expression plays a critical role in diverse neural functions, including differentiation (Buonanno and Fields 1999), neurite growth and patterning (Wong and Ghosh 2002) and neuronal survival (Ghosh *et al.* 1994).

Our data showing that *nurr1* gene expression is maintained in the adult MES at a detectable level support the view that modulation of Nurr1 by neural activity could occur also in the adult midbrain. Indeed, maintainance of *nurr1* gene expression in adult MES has been reported also by other laboratories (Zetterstrom *et al.* 1996, 1997; Bannon *et al.* 2002). Modulation of Nurr1 in adult MES could be of relevant clinical interest for neurological diseases related to dysfunction of DA systems, on the light of recent findings showing that:

1. *nurr1* gene dosage regulates DA levels in adult brain (Eells *et al.* 2002)
2. Nurr1 can have a neuroprotective role on MES DA neurones (Le *et al.* 1999b)
3. Nurr1 is downregulated in the midbrain of cocaine abusers (Bannon *et al.* 2002)
4. Polymorphism in the intron six of *nurr1* gene in humans is associated with Parkinson's disease (Xu *et al.* 2002)

In addition, missense mutations in *nurr1* gene have been found in cases of schizophrenia and manic–depressive disorder (Buervenich *et al.* 2000; Chen *et al.* 2001).

Understanding the intracellular signaling pathways driving Nurr1 overexpression following depolarization can give important cues on the molecular mechanisms that underlay this phenomenon and, consequently, the regulation of *nurr1* gene expression. In addition this information can drive new pharmacological approaches for the treatment of diseases involving the MES DA system. Interestingly, Nurr1 induction by prolonged depolarization appeared to be independent on the entry of extracellular Ca^{2+} via either L-or N-type voltage-dependent Ca^{2+} channels, as well as on MEK or PKA activity. Although our experiments cannot rule out a participation of intracellular Ca^{2+} in depolarization-induced Nurr1 induction, both calcium-dependent and -independent mechanisms have been described for Nurr1 induction in endocrine (Kovalovsky *et al.* 2002) and neuronal cells (Brosenitsch and Katz 2001). Thus different intracellular pathways may lead to Nurr1 activation depending on the cell context studied and/or the developmental maturation; alternatively, more pathways might be simultaneously required for Nurr1 induction in our MES DA neurones; therefore, blockade of each of them independently may not be sufficient to prevent Nurr1 increase following depolarization. Interestingly, in dissociated primary sensory neurones, chronic membrane depolarization and patterned electrical stimulation appear to trigger distinct intracellular pathways (Brosenitsch and Katz 2001). Additional studies are therefore required to verify whether growth or trophic factor(s) released by depolarization in the cultures participate in Nurr1 induction in our experimental model.

In summary, our data show that *nurr1* gene is highly expressed in midbrain DA neurones in a sharp temporal window, suggesting that high levels of Nurr1 may be required for the proper DA differentiation during midbrain ontogeny. In addition we show that SHH direct DA differentiation in proliferating DA precursors *in vitro*, providing a potential optimal source of expanded MES DA neurones. To our knowledge, this is the first study describing depolarization-induced *nurr1* gene expression in primary cultures from embryonic midbrain, the CNS region where the function of Nurr1 is a key player in the development of DA neurones. Thus modulation of Nurr1 in MES could be a mechanism to regulate development and maintenance of DA neurones.

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Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn

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Summary

Mutations in the human and mouse *PTF1A/Ptf1a* genes result in permanent diabetes mellitus and cerebellar agenesis. We show that Ptf1a is present in precursors to GABAergic neurons in spinal cord dorsal horn as well as the cerebellum. A null mutation in *Ptf1a* reveals its requirement for the dorsal horn GABAergic neurons. Specifically, Ptf1a is required for the generation of early-born (dI4, E10.5) and late-born (dIL^A, E12.5) dorsal interneuron populations identified by homeodomain factors Lhx1/5 and Pax2. Furthermore, in the absence of Ptf1a, the dI4 dorsal interneurons trans-fate to dI5 (Lmx1b$^+$), and the dIL^A to dIL^B (Lmx1b$^+$;Tlx3$^+$). This mis-specification of neurons results in a complete loss of inhibitory GABAergic neurons and an increase in the excitatory glutamatergic neurons in the dorsal horn of the spinal cord by E16.5. Thus, Ptf1a function is essential for GABAergic over glutamatergic neuronal cell fates in the developing spinal cord, and provides an important genetic link between inhibitory and excitatory interneuron development.

Key words: Spinal cord development, Dorsal horn inhibitory neurons, BHLH transcription factor, Mouse

Introduction

Truncation of the human *PTF1A* gene leads to permanent neonatal diabetes mellitus and cerebellar hypoplasia (Sellick et al., 2004), while a null mutation of *Ptf1a* (*Ptf1-p48*) in mouse results in neonatal lethality with pancreatic and cerebellar agenesis (Hoshino et al., 2005; Krapp et al., 1998; Rose et al., 2001; Sellick et al., 2004). *Ptf1a* encodes a basic helix-loop-helix (bHLH) transcription factor most closely related to the Twist subclass of bHLH genes (Obata et al., 2001). It was first identified as one of three subunits of the PTF1 transcription factor complex required for expression of pancreatic digestive enzyme genes (Cockell et al., 1989). As a bHLH factor, Ptf1a is notable in that it not only heterodimerizes with the E-protein, E47, but it also complexes with Rbpsuh (RBP-Jk, CBF1), an intercellular mediator of Notch signaling, and thus, may directly impact Notch signaling pathways (Beres et al., 2005; Obata et al., 2001). This is a unique characteristic for a bHLH factor, and provides added complexity to the molecular models proposed for neural specific bHLH factors and Rbpsuh function in nervous system development (Artavanis-Tsakonas et al., 1999; Bertrand et al., 2002; Bray and Furriols, 2001).

In addition to expression in the pancreas and developing cerebellum, Ptf1a is present in the dorsal neural tube of early stage embryos (Obata et al., 2001), an embryonic structure that gives rise to the dorsal horn of the spinal cord in the mature animal. The spinal cord dorsal horn largely consists of excitatory (glutamatergic) and inhibitory (GABAergic) neurons that modulate somatosensory inputs from the periphery, including pain, temperature and mechanoception. Distinct neuronal subtypes in the developing dorsal neural tube have been defined by the timing of their birth, and key differences in the expression of homeodomain (HD) transcription factors (Jessell, 2000). The requirement for bHLH transcription factors, Math1 (Atoh1 – Mouse Genome Informatics), Ngn1, Ngn2, Mash1 (Ascl1 – Mouse Genome Informatics) and Olig3, for the formation of specific neuronal subtypes defined by the HD factors has been demonstrated (Bermingham et al., 2001; Gowan et al., 2001; Helms et al., 2005; Müller et al., 2005), but their roles in specifying neurotransmitter identity have not been reported. Rather, the HD genes are involved in generating glutamatergic versus GABAergic neurons in the dorsal horn. *Tlx1* and *Tlx3* have been shown to be post-mitotic selector genes for the glutamatergic transmitter phenotype (Cheng et al., 2004). In the dorsal horn of *Tlx1/3* double mutants, glutamatergic neurons are reduced while GABAergic neurons are increased. By contrast, no selector gene has been described for the GABAergic phenotype, although HD factors Lbx1 and Pax2 play roles in generating GABAergic neurons in the dorsal horn (Cheng et al., 2005; Cheng et al., 2004; Gross et al., 2002; Müller et al., 2002).

Here we report that, in the neural tube, Ptf1a is required for

dorsal neural tube Pax2 expression and suppression of Tlx3, which leads to the formation of GABAergic neurons while suppressing the formation of glutamatergic neurons. Thus, Ptf1a and Tlx1/3 act as opposing switches for the generation of neurons with specific neurotransmitter phenotypes, revealing genetic interactions that link the development of these two classes of interneurons in the spinal cord. A disruption in the balance between inhibitory and excitatory neuronal activity of the magnitude that occurs in the absence of Ptf1a function has profound consequences for the organism, and may explain the respiratory difficulties and cerebral excitability seen in humans with a mutation in this gene (Hoveyda et al., 1999; Sellick et al., 2004).

Materials and methods

Animals

The *Ptf1a*^{Cre} mutant mice have been previously described; these mice have Cre-recombinase in place of the *Ptf1a* protein-coding region (Kawaguchi et al., 2002). The reporter strains *R26R-stop-YFP* (Srinivas et al., 2001) and *R26R-stop-LacZ* (Soriano, 1999) (Gt(ROSA)26Sor^{tm1Sor}) were used to visualize cells and their progeny that were exposed to Cre recombinase activity. Genotyping was done using PCR with primers 5′-AACCAGGCCCAGAAGGTTAT-3′ and 5′-TCAAAGGGTGGTTCGTTCTC-3′ for wild-type *Ptf1a* locus, and with 5′-GCATAACCAGTGAAACAGCATTGCTG-3′ and 5′-GGACATGTTCAGGGATCGCCAGGCG-3′ for *Cre* in the mutant allele. R26R mice were genotyped as described (Soriano, 1999).

X-gal staining

Embryos were fixed for 1 hour in 4% formaldehyde in 0.1 mol/l sodium phosphate buffer pH 7.4 at room temperature and washed three times in 0.1 mol/l sodium phosphate buffer pH 7.4 for 30 minutes each. Embryos were incubated at 30°C for 24 hours in X-gal staining solution (PBS/5 mmol/l potassium ferricyanide, 5 mmol/l potassium ferrocyanide, 2 mmol/l $MgCl_2$, 1 mg/ml X-gal), washed three times in 0.1 mol/l sodium phosphate buffer pH 7.4 and whole-mount images were taken; then the embryos were sunk in 30% sucrose in 0.1 mol/l sodium phosphate buffer pH 7.4 overnight at 4°C, embedded in OCT, and cryosectioned at 40 μm. Adult mice were perfused with 4% formaldehyde in 0.1 mol/l sodium phosphate buffer pH 7.4 under standard conditions. Brains and spinal cords were fixed for an additional 2 hours at 4°C and processed as above for cryosection at 20 μm. Sections were incubated in X-gal solution, rinsed and counterstained with Nuclear Fast Red or Nissl.

Immunofluorescence and mRNA in-situ hybridization

Appropriately staged embryos were dissected in ice-cold 0.1 mol/l sodium phosphate buffer pH 7.4 and fixed for 2 hours at 4°C in 4% formaldehyde in 0.1 mol/l sodium phosphate buffer pH 7.4. Embryos were processed as above for cryosection at 30 μm.

Immunofluorescence was performed using the following primary antibodies: Mouse anti-BrdU (Becton Dickinson), guinea pig anti-VGLUT2 (Chemicon), mouse anti-Lhx1/5 (4F2) (Developmental Studies Hybridoma Bank), rabbit anti-Ptf1a (Li and Edlund, 2001), mouse anti-Mash1 (Lo et al., 1991), rabbit anti-GFP (Molecular Probes), chicken anti-GFP (Chemicon), guinea pig anti-Lmx1b (Müller et al., 2002), mouse anti-GAD67 (Sigma), rabbit anti-Islet1/2 (Tsuchida et al., 1994), rabbit anti-Pax2 (Zymed), rabbit anti-Tlx3 (gift from T. Müller and C. Birchmeier), and mouse anti-GABA (Sigma). For BrdU experiments, BrdU (200 μg/g body weight) was injected into pregnant mothers for 1 hour before sacrifice. Double immunofluorescence of Ptf1a and BrdU was performed sequentially with Ptf1a antibodies followed by treatment of the sections with 2N HCl for 15 minutes, 0.1 mol/l sodium borate pH 8.5 for 20 minutes, and incubation with primary BrdU. Specific neuronal cell types were counted using confocal (Bio-Rad MRC 1024) images from a minimum of three different animals on three or more sections. mRNA in-situ hybridization was carried out as described (Birren et al., 1993; Cheng et al., 2004). Antisense probes were made from plasmids provided by Q. Ma (Cheng et al., 2004). All sections shown are from the forelimb level.

Results

Ptf1a is present in the developing nervous system

We used recombination-based lineage tracing in vivo to characterize regions of the nervous system derived from *Ptf1a*-expressing cells. The mouse strain *Ptf1a*$^{Cre/+}$, which has the *Ptf1a* protein-coding region replaced by that of Cre recombinase (Kawaguchi et al., 2002), was crossed with the Cre reporter strain *R26R-stop-lacZ*$^{+/-}$. The *R26R-stop-lacZ*$^{+/-}$ allele provides a permanent lineage marker for the cells with Cre recombinase activity (Soriano, 1999). β-Gal staining in a *Ptf1a*$^{Cre/+}$;*R26R-stop-lacZ*$^{+/-}$ embryo at embryonic day (E) 11.5, and dissected brain and spinal cord at E16.5, illustrate that before these stages *Ptf1a* is expressed in the neural tube, and the *Ptf1a* lineage extends from the hindbrain caudally to the tail (Fig. 1A,B). β-Gal staining was also detected in the pancreas at E11.5 (Fig. 1A, arrow). Within the neural tube, β-gal staining was restricted to dorsal regions (Fig. 1A, inset; B, arrowhead), and in restricted regions in the ventral diencephalon (Fig. 1B, inset) that include the preoptic nucleus and ventral hypothalamus. To identify the cell types in the adult nervous system that are derived from *Ptf1a*-expressing cells, we examined β-gal staining in adult brains and spinal cords from these mice. A majority of stained cells in the spinal cord reside in the dorsal horn primarily in laminae I-IV, with a few scattered cells found in ventral laminae (Fig. 1C,D). In the cerebellum, *Ptf1a*-expressing cells gave rise to multiple GABAergic cell types, including at least a subset of Purkinje, stellate, basket and Golgi cells (Hoshino et al., 2005) (Fig. 1E). A population of cells in the ventral hypothalamus was also detected (Fig. 1F). The requirement for Ptf1a for the development of GABAergic neurons in the cerebellum has recently been reported (Hoshino et al., 2005).

Ptf1a is restricted to post-mitotic cells within the ventricular zone of the dorsal neural tube in an overlapping pattern with Mash1

The β-gal staining in the dorsal neural tube resembled the expression pattern of another bHLH transcription factor, Mash1 (Gowan et al., 2001; Helms et al., 2005). To characterize the expression pattern of *Ptf1a* in more detail in this region, we used double label immunofluorescence with rabbit anti-Ptf1a (Li and Edlund, 2001) and mouse anti-Mash1 (Lo et al., 1991), or mouse anti-BrdU in BrdU-pulsed embryos. At E10.5, Ptf1a was detected within the central portion of the dorsal Mash1 domain (Fig. 1G). In this region, Mash1 levels were low relative to levels in adjacent dorsal and ventral regions (Fig. 1G). Because Mash1 is present in ventricular zone cells adjacent to dI3-dI5 neurons (Helms et al., 2005), this pattern suggests that Ptf1a may be in the dI4 precursor domain. Within the domain common to both factors, Ptf1a and Mash1 co-labeled a subset of cells located on the ventricular side (Fig. 1G, inset). The pattern of Ptf1a is dynamic, and by E11.5 the

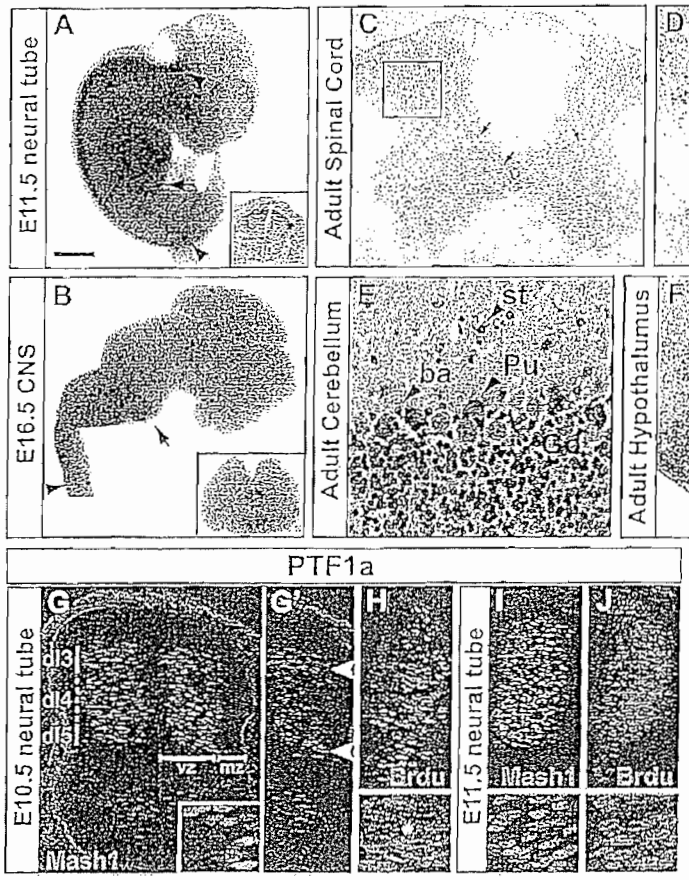


Fig. 1. Ptf1a is in precursors to multiple neural tissues and overlaps with Mash1 in the dorsal neural tube. The Ptf1a lineage was visualized by β-gal staining of *Ptf1a*$^{Cre/+}$;*R26R-stop-lacZ*$^{+/-}$ mice. (A) E11.5 whole mount, showing β-gal staining in the dorsal neural tube (inset) from the mid-hindbrain to the tail (arrowheads). Staining in the pancreas is indicated by the arrow. (B) brain and spinal cord dissected from an E16.5 embryo, stained for β-gal, showing expression in cerebellum (arrow) and dorsal spinal cord (arrowhead). Ventral diencephalon also reveals cells from the Ptf1a lineage (inset). (C,D) Adult cervical spinal cord, showing β-gal stained cells primarily in laminae I-IV. The boxed area in C is shown at higher magnification in D. Arrows (C) highlight labeled cells found ventral to boxed area. (E) Adult cerebellum with β-gal-stained Purkinje, stellate, basket and Golgi cells. (F) Coronal section of the adult brain reveals β-gal-stained cells in the ventral hypothalamus. Tissue is counterstained with Nuclear Fast Red (C,D,F) and Nissl (E). Immunofluorescence on transverse neural tube sections of E10.5 (G,H) and E11.5 (I,J) wild-type mice. (G,I) Ptf1a (red), Mash1 (green) and their co-labeling (yellow) in a subset of cells. (H,J) Ptf1a (red) and BrdU incorporation (green) are largely non-overlapping. The asterisk in H indicates a rare BrdU-incorporating Ptf1a cell. Scale bar: 1 mm in A; 50 μm in D-J; 12.5 μm in the insets. ba, basket cells; dI3-dI5, dorsal interneurons 3-5; Go, Golgi cells; MZ, marginal zone; Pu, Purkinje cells; st, stellate cells; VZ, ventricular zone.

dorsal and ventral boundaries of Ptf1a became identical with those of Mash1 (Fig. 1I). At this stage of neural tube development, the dorsal ventricular zone gives rise to the two late-born neurons, dIL^{A} and dIL^{B}. Ptf1a marks cells that appear to have exited the cell cycle, as they rarely co-label with BrdU incorporation at either E10.5 or 11.5 (Fig. 1H,J). This cell-cycle status of Ptf1a cells contrasts to the Mash1 population, in which a significant proportion of cells are still dividing (Helms et al., 2005). Taken together, Ptf1a is largely restricted to post-mitotic cells in the ventricular zone of the dorsal neural tube in a pattern that suggests it may be in the precursors to dI4, dIL^{A} and/or dIL^{B} neurons.

Ptf1a is required to generate dI4 and to suppress dI5 dorsal interneurons

Embryos null for Ptf1a (*Ptf1a*$^{Cre/Cre}$) were examined at E10.5 for markers that distinguish interneuron populations dI2-dI6. These early neuronal populations are defined by several criteria that include birth date (before E11) and the expression of HD transcription factors (Helms and Johnson, 2003) (Fig. 2I). In the absence of Ptf1a (*Ptf1a*$^{Cre/Cre}$), the dI4 population was absent, as demonstrated by the loss of Pax2;Lhx1/5 double positive cells (Fig. 2A,B; arrow). Conversely, dI5 neurons, marked by Lmx1b, expanded dorsally into the domain normally containing dI4 neurons (Fig. 2A-D; arrow). No change in cell number was observed in the dI2 (Lhx1/5) and dI3 (Isl1) populations (Fig. 2B,D; see counts in Fig. 2J). No change in cell death was observed using TUNEL or Caspase3 immunocytochemistry at this stage (data not shown). Thus, Ptf1a is essential for the generation of dI4 interneurons and the suppression of dI5 (Fig. 2K). This is by contrast to recent studies showing Mash1 is required for dI5 while suppressing dI4 neurons (Helms et al., 2005).

In the *Ptf1a* null mutant embryos, the number of cells increased in the dI5 cells complemented the number of dI4 neurons lost (Fig. 2J). These results suggest that in the absence of Ptf1a, the dI4 cells trans-fate into dI5. To test this directly, we crossed the *Ptf1a*$^{Cre/+}$ mice to the *R26R-stop-YFP* Cre reporter strain, which will express YFP in cells with Cre recombinase and in all descendants of these cells (Srinivas et al., 2001). In *Ptf1a*$^{Cre/+}$;*R26R-stop-YFP*$^{+/-}$ mice, the dI4 marker Lhx1/5 co-localized with YFP, demonstrating that dI4 neurons are derived from Ptf1a precursor cells, and thus, the loss of dI4 neurons in the Ptf1a null embryos is cell-autonomous (Fig. 2E,F). In these embryos, YFP did not co-label with the dI5 marker Lmx1b (Fig. 2G). By contrast, in *Ptf1a*$^{Cre/Cre}$;*R26R-stop-YFP*$^{+/-}$ embryos, the dI4 neurons were lost and YFP now co-localized with Lmx1b (dI5) (Fig. 2G,H). Thus, in the absence of Ptf1a, the cell generates a dI5 neuron rather than a dI4.

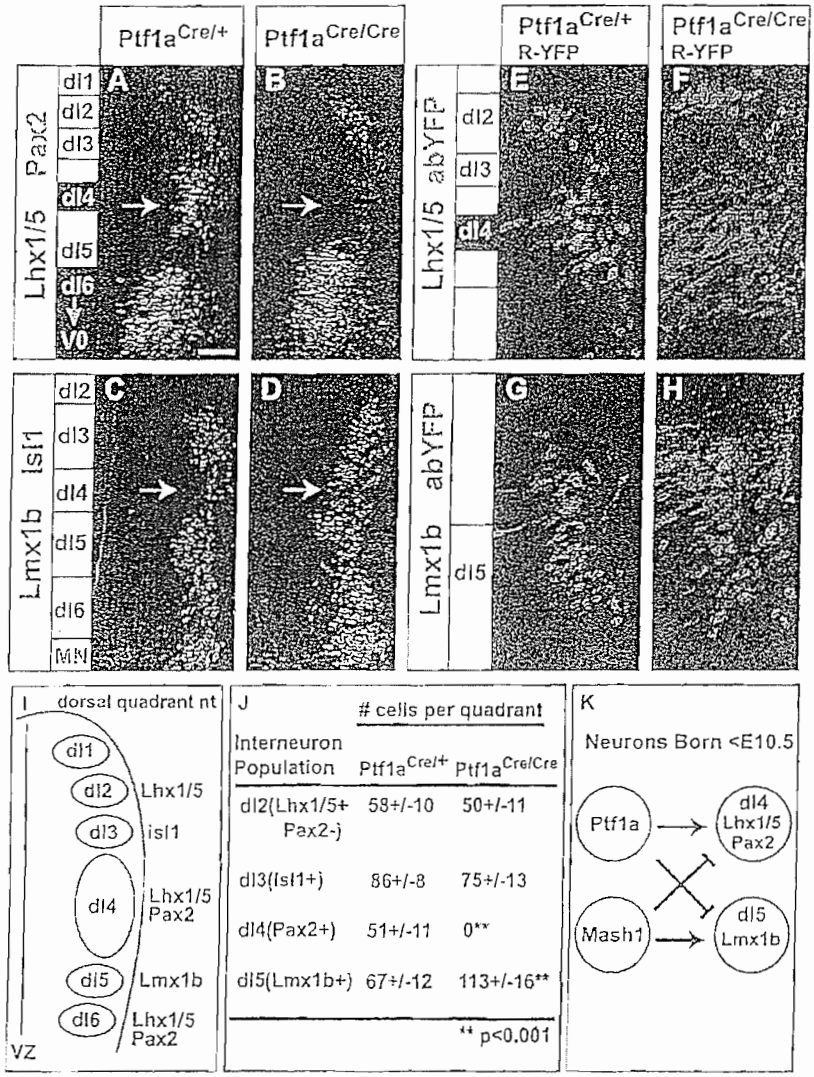
At all stages examined, YFP expression was higher in the *Ptf1a* mutant than in the *Ptf1a* heterozygous embryos. This is

at least partly due to the presence of two Cre alleles in *Ptf1a*$^{Cre/Cre}$ versus one allele in *Ptf1a*$^{Cre/+}$. However, it could also reflect a component of a negative feedback loop in the regulation of the *Ptf1a* locus.

Ptf1a is required to generate late-born dIL^A and to suppress dIL^B interneurons

A second round of neurogenesis occurs in the developing spinal cord between E11 and E13 to form the dIL^A and dIL^B populations of dorsal interneurons (Gross et al., 2002; Müller et al., 2002). The *Ptf1a* mutants were examined at E12.5 with Pax2 and Lhx1/5, which mark dIL^A, and Lmx1b and Tlx3, which mark dIL^B, to determine if these two late-born populations require Ptf1a. In the absence of Ptf1a, Pax2 was completely lost and Lhx1/5 was dramatically reduced, specifically in the dorsal half of the neural tube, revealing a loss of dIL^A neurons (Fig. 3A,B). The number of cells expressing Lmx1b or Tlx3 (dIL^B) was significantly increased in the absence of Ptf1a, while cells expressing Isl1 were unaffected (Fig. 3C,D,I,J; see Fig. 3K for cell counts). No increase in cell death was detected using TUNEL or Caspase3 immunocytochemistry at this stage (data not shown). These results demonstrate that Ptf1a is required for the formation of dIL^A neurons and normally suppresses the formation of dIL^B neurons.

Although not as complete as the dI4/dI5 switch at E10.5, there was a concomitant increase in dIL^B neurons when dIL^A neurons were lost, suggesting that there is a fate switch from dIL^A to dIL^B in *Ptf1a* mutant embryos. To visualize the switch in cell fate, we again utilized *Ptf1a*^{Cre};*R26R-stop-YFP* embryos. In *Ptf1a*$^{Cre/+}$;*R26R-stop-YFP*$^{+/-}$ embryos, Lhx1/5 (dIL^A) co-localized with YFP, demonstrating that the loss of dIL^A in the *Ptf1a* null is a cell-autonomous effect (Fig. 3E,E′,F,F′). The dIL^B markers Lmx1b and Tlx3 were not co-expressed with YFP in embryos heterozygous for *Ptf1a* (Fig. 3G,G′,I). However, in *Ptf1a*$^{Cre/Cre}$;*R26R-stop-YFP*$^{+/-}$ embryos, there was a dramatic increase in the number of YFP/Lmx1b and YFP/Tlx3 double positive cells, consistent with a cell fate switch from dIL^A to dIL^B (Fig. 3H,H′,J). Interpretation of this result is tempered by the increase in YFP expression in the *Ptf1a* mutant (Fig. 3F,H), as stated earlier. Also, the increase in the markers for dIL^B in the mutant cannot completely account for the number of dIL^A cells lost. This could reflect differences in temporal characteristics or detection efficiency of the individual markers. Regardless, the overlap in YFP and dIL^B markers in the mutant, combined with Ptf1a being largely restricted to post-mitotic cells, the increase in the number of dIL^B, and the lack of apoptosis, together suggest that Ptf1a



J

Interneuron Population	# cells per quadrant Ptf1a$^{Cre/+}$	Ptf1a$^{Cre/Cre}$
dI2(Lhx1/5+ Pax2-)	58+/-10	50+/-11
dI3(Isl1+)	86+/-8	75+/-13
dI4(Pax2+)	51+/-11	0**
dI5(Lmx1b+)	67+/-12	113+/-16**

** p<0.001

Fig. 2. dI4 are trans-fated to dI5 interneurons in Ptf1a-deficient embryos. Immunofluorescence on neural tube transverse sections of *Ptf1a*$^{Cre/+}$ (A,C) and *Ptf1a*$^{cre/cre}$ (B,D) mouse E10.5 embryos. (A,B) dI4 neurons marked in yellow by co-labeling of Lhx1/5 (red) and Pax2 (green) are lost in the *Ptf1a* null (arrows). (C,D) Lmx1b (red) labeling dI5 is expanded in *Ptf1a* null embryos, while Isl1 (green) labeling dI3 are not affected. (E-H) Anti-GFP antibody was used to detect YFP in E10.5 *Ptf1a*^{Cre};*R26R-stop-YFP* embryos. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. YFP (green) co-localizes with the Lhx1/5 (dI4;red), but fails to do so in *Ptf1* null embryos (compare E and F). (G) Lmx1b (dI5;red) does not co-localize with YFP in the presence of Ptf1a, but they do co-localize in the null (H). The ventricle is to the left in all panels, as shown in a schematic representation of the markers used to distinguish individual neuronal populations (I). (J) Cell counts for each neuronal population. (K) Summary diagram showing that Ptf1a is required for dI4 and suppression of dI5 interneurons (see Results). Scale bar: 50 μm in A-D; 25 μm in E-H.

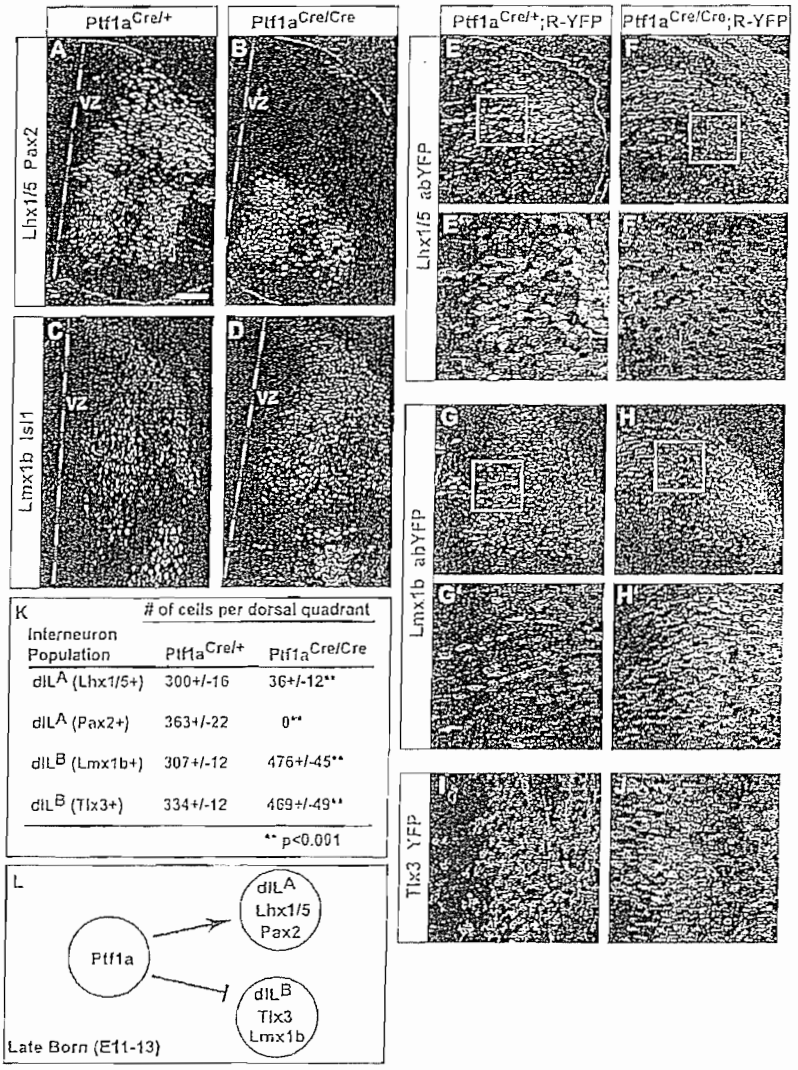


Fig. 3. dIL^A trans-fate to dIL^B interneurons in Ptf1a null embryos. Immunofluorescence on neural tube transverse sections of *Ptf1a*$^{Cre/+}$ (A,C) and *Ptf1a*$^{Cre/Cre}$ (B,D) mouse E12.5 embryos. (A,B) dIL^A neurons, marked by Lhx1/5 (red) and Pax2 (green), are lost specifically in the dorsal neural tube, but not the ventral neural tube in the *Ptf1a* null (dashed line indicates the position of the ventricle). (C,D) dIL^B neurons, marked by Lmx1b (red) increase in the *Ptf1a* null, but there is no change in the number of Isl1 (green) cells. (E-J) Anti-GFP antibody was used to detect YFP at E12.5 in *Ptf1a*^{Cre}*;R26R-stop-YFP* embryos. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. (E-F′) In the presence of Ptf1a, YFP (green) co-localizes (yellow) with the dIL^A marker, Lhx1/5 (red), but it does not do so in *Ptf1a* null embryos. (G-H′) Lmx1b (dIL^B; red) and (I-J) Tlx3 (dIL^B; red) rarely co-localizes with YFP in embryos with Ptf1a, while substantially more Lmx1b/YFP and Tlx3/YFP double positive cells appear in *Ptf1a*-deficient embryos (yellow). The boxed regions in E-H are shown at higher magnification in E′-H′. (K) Cell counts of specific neuronal populations. (L) Summary diagram showing that Ptf1a is required for the dIL^A interneuron cell fate, while suppressing dIL^B. Scale bar: 50 μm in A-H; 25 μm in E′-H′,I,J.

serves as a switch between the dIL^A and dIL^B interneuron subtypes (Fig. 3L).

Ptf1a is required for the formation of GABAergic neurons in the dorsal horn

In the absence of Pax2, expression of the GABAergic marker gene *Gad1* (encoding glutamic acid decarboxylase, GAD67) is lost in the dorsal horn of E13 mouse embryos (Cheng et al., 2004). As the *Ptf1a* null embryos completely lose expression of Pax2 in the dorsal neural tube, we predicted that the absence of Ptf1a would also result in loss of GABAergic neurons. To test this hypothesis, *Ptf1a* mutants were analyzed at E16.5 for expression of GABAergic markers. In-situ hybridization with a *Gad1* probe demonstrated that there was a complete loss of *Gad1* in the dorsal horn in the absence of Ptf1a, while the most ventral expression appeared largely unaffected (Fig. 4A,B). The protein encoded by *Gad1*, GAD67, was also absent in the dorsal horn of Ptf1a-deficient embryos (Fig. 4F), as was GABA, the neurotransmitter itself that defines a GABAergic interneuron (Fig. 4H).

Although the level of Ptf1a decreased by E16.5, we were able to use *Ptf1a*^{Cre}*;R26R-stop-YFP* embryos to map the fate of *Ptf1a*-expressing cells into E16.5 dorsal horns to verify that the loss of GABAergic neurons is cell-autonomous. The vast majority of YFP in *Ptf1a*$^{Cre/+}$*;R26R-stop-YFP*$^{+/-}$ embryos was restricted to the dorsal spinal cord at E16.5 (Fig. 4C). Co-localization of YFP with GAD67 and GABA indicated that the loss of GABAergic neurons in Ptf1a-deficient embryos is at least in part a cell-autonomous effect as expected (Fig. 4E,G). These co-localization experiments are not as clear as the analysis with the transcription factor markers at earlier stages, because the neurotransmitter proteins tend to localize in the distal processes of the neurons, while the YFP is mainly cytoplasmic with some signal reaching distal processes. In Fig. 4E, arrows indicate regions outside the cell body where YFP and GAD67 co-localized. GABA is easier to detect in cell bodies, and thus the overlap with YFP is clearer (Fig. 4G, arrows). It is also important to note that the in-vivo recombination system used here to trace the lineage of Ptf1a cells is not 100% efficient and is not expected to indicate every Ptf1a descendent. Regardless, this analysis together with experiments described in Fig. 3 demonstrate that Ptf1a is in cells fated to become GABAergic neurons of the dorsal horn and that it is essential for this neuronal subtype to form.

YFP in *Ptf1a*$^{Cre/Cre}$*;R26R-stop-YFP*$^{+/-}$ embryos, which are deficient in Ptf1a, was also largely restricted to the dorsal horn, but it was detected in more cells and encompassed a broader medial-lateral area compared with heterozygous embryos (Fig.

4C,D). Besides suggesting that Ptf1a may normally be in a negative autoregulatory loop, the aberrant location of YFP-labeled cells on the lateral edges that appear to stream ventrally are consistent with mis-specification of neuronal subtype in the mutant.

Ptf1a suppresses glutamatergic neuronal differentiation in the dorsal horn

In the absence of Ptf1a there was a neuronal subtype switch from dI4/dIL^{A} to dI5/dIL^{B} (Figs 2, 3). dIL^{B} neurons form the glutamatergic neurons in the dorsal horn (Cheng et al., 2004). To determine if the aberrantly formed dIL^{B} neurons continue to mature with glutamatergic characteristics, we examined *Ptf1a* mutant embryos for vesicular glutamate transporter2 (VGLUT2) and glutamate receptor (GluR2/3). mRNA in-situ hybridization and immunofluorescence demonstrated an increase of VGLUT2 and GluR2/3 in the dorsal horn of *Ptf1a*$^{Cre/Cre}$ embryos at E16.5, when compared with *Ptf1a*$^{Cre/+}$ embryos (Fig. 5A-D, and data not shown). This increase was clearly indicated by an increase in VGLUT2 in the more superficial laminae, as indicated by the arrows in Fig. 5C,D. Likewise, an increase in the density of Tlx3-expressing cells was also seen at E16.5 (Fig. 5G,H), consistent with the importance of Tlx1/3 in the generation of dorsal horn glutamatergic neurons (Cheng et al., 2004). By contrast, no increase was detected in the glycinergic neuronal marker GlyT2 by mRNA in-situ hybridization (data not shown).

Just as at the earlier embryonic stages, we examined *Ptf1a*$^{Cre/+}$*;R26R-stop-YFP*$^{+/-}$ and *Ptf1a*$^{Cre/Cre}$*;R26R-stop-YFP*$^{+/-}$ embryos for co-localization of VGLUT2 with YFP to address the question of a neurotransmitter fate switch of the *Ptf1a* mutant cells. Significant co-expression of VGLUT2 with YFP was observed only in the *Ptf1a* null embryos (Fig. 5E,F). As with the GABAergic markers, the co-localization of VGLUT2 with YFP has caveats due to the enrichment of VGLUT2 in distal processes. However, taken together with the loss of Pax2 expression and increase in Tlx3, these results demonstrate that Ptf1a functions as a switch; it is required for the generation of GABAergic neurons and suppresses generation of glutamatergic neurons in the dorsal horn of the spinal cord (Fig. 5I).

Discussion

GABAergic neurons in the dorsal horn produce presynaptic inhibition of primary sensory afferents, and thus represent a major gatekeeper for the strength of sensory input to the spinal cord (Rudomin and Schmidt, 1999). The imbalance of inhibitory and excitatory neuronal activity can result in sensory disorders such as hyper- or hypo-algesia; thus, how these different types of neurons form is fundamental to understanding the development of neuronal circuits that modulate responses to sensory input. The functional importance of these local circuit inhibitory neurons is exemplified in disorders such as those seen in peripheral nerve injury, in which unregulated pain responses (allodynia) are associated with the loss of inhibitory activity of GABAergic neurons (Ibuki et al., 1997; Wiesenfeld-Hallin et al., 1997). We demonstrate the requirement of the bHLH transcription factor, Ptf1a, for the overwhelming majority of GABAergic neurons present in the dorsal spinal cord.

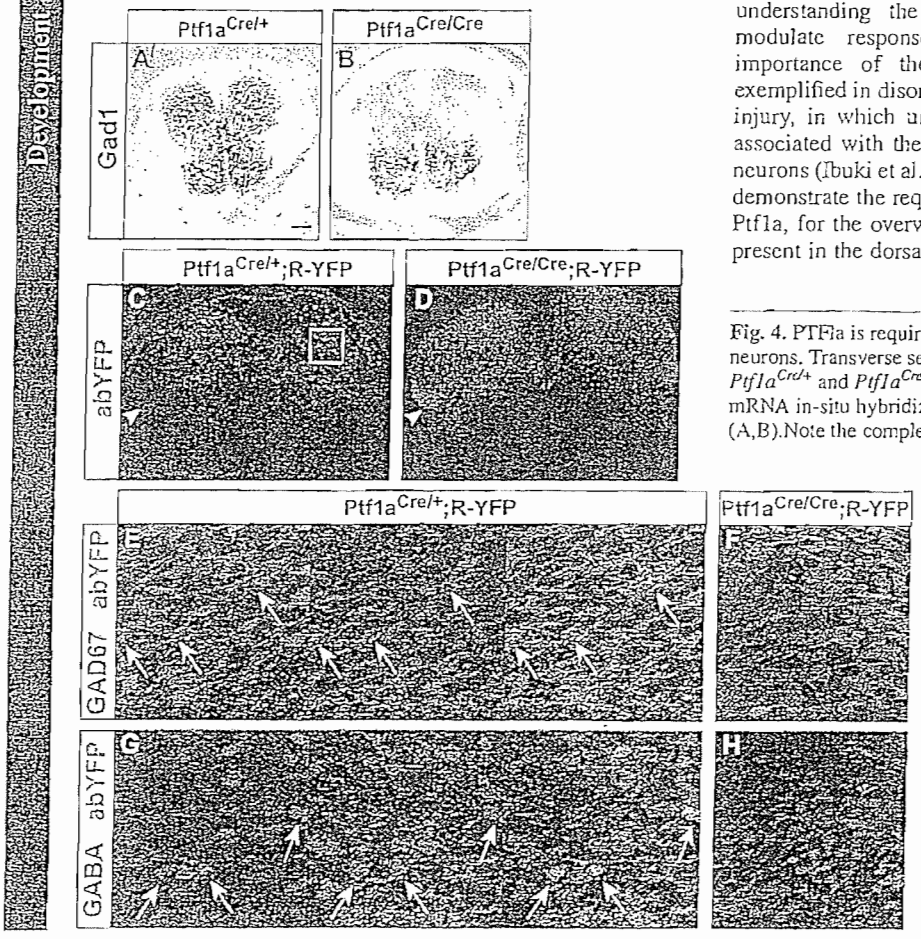


Fig. 4. PTFla is required for generation of dorsal horn GABAergic neurons. Transverse sections through spinal cord cervical regions of *Ptf1a*$^{Cre/+}$ and *Ptf1a*$^{Cre/Cre}$ mouse E16.5 embryos were processed for mRNA in-situ hybridization with the GABAergic marker gene Gad1 (A,B).Note the complete absence of Gad1 in the dorsal regions in the absence of Ptf1a. Loss of ventral Gad1 was not consistently observed. (C-H) Anti-GFP antibody was used to detect YFP in *Ptf1a*^{Cre}*;R26R-stop-YFP* E16.5 embryos. YFP acts as a lineage marker for cells that have expressed the *Ptf1a* locus. (C) The lineage reporter YFP is detected largely in the dorsal regions in wild type. (D) By contrast, in the mutant, YFP is detected at higher levels in more cells and with a different organization (arrowheads). The boxed area in C is the approximate area shown in E-H. Immunofluorescence for GAD67 (E) and the neurotransmitter GABA (G) co-localize with YFP in embryos heterozygous for *Ptf1a* (arrows) but not in embryos lacking *Ptf1a* (F,H). Panels E-H are all from dorsal horn regions. Scale bar: 110 μm in A,B; 13 μm in E-H.

GABAergic and glutamatergic neurons appear to be alternative fate choices in the dorsal neural tube, and their development is genetically linked through the function of transcription factors such as Ptf1a, Tlx1/3, Lbx1 and Pax2 (Cheng et al., 2005; Cheng et al., 2004; Gross et al., 2002; Müller et al., 2002). Recently it was proposed that the HD transcription factors Tlx1 and Tlx3 are selector proteins biasing choice of glutamatergic over GABAergic cell fates (Cheng et al., 2004). This may at least be in part due to their inhibition of *Pax2* expression, as Pax2 is required for the formation of GABAergic neurons in the dorsal horn, and in the Tlx1/3 double mutant, Pax2 is dramatically increased (Cheng et al., 2004). Pax2 does not appear to have selector function, as there is no concomitant increase in glutamatergic neurons in *Pax2* null embryos (Cheng et al., 2004). Another HD factor, Lbx1, is also required for generating the correct numbers of GABAergic neurons (Cheng et al., 2005; Gross et al., 2002; Müller et al., 2002). Recently, the suppression of Lbx1 activity by Tlx3 has been suggested as a mechanism for selecting dorsal horn glutamatergic cell fate (Cheng et al., 2005). Results presented here demonstrate that Ptf1a is largely in post-mitotic cells in the dorsal neural tube. Therefore, Ptf1a has selector function opposite to Tlx1/3; it is required for the generation of dI4 and dIL^A fates, which mature into GABAergic neurons, and it suppresses the alternative fates, dI5 and dIL^B, which form glutamatergic neurons (Fig. 5I). The function of Ptf1a in switching cell fates in the dorsal spinal cord is similar to the role attributed to Ptf1a in pancreatic development. Inactivation of Ptf1a switches progenitor cells from pancreatic lineages to duodenal lineages (Kawaguchi et al., 2002).

Efforts to unravel the transcription factor code for the specification of spinal cord neurons have revealed a complex interplay of spatial and temporal control between bHLH and HD transcriptions factors (Caspary and Anderson, 2003; Lee and Pfaff, 2001). bHLH transcription factors present in the ventricular zone form the basis for the code for dI1 to dI5 in the dorsal neural tube. Math1 and Ngn1/2 specify the two most dorsal interneuron populations, dI1 and dI2 (Bermingham et al., 2001; Gowan et al., 2001). Mash1 specifies dI3 and dI5 neurons (Helms et al., 2005), while Ptf1a is required for dI4 (Fig. 2). The function of Mash1 and Ptf1a in dI3-dI5 is consistent with their expression patterns. Ptf1a is present in the central region of the Mash1 domain, a region that contains cells with the lowest Mash1 expression. Indeed, using an in-vivo recombination-based lineage tracing paradigm, Mash1 efficiently traces to dI3 and dI5, but not to dI4 neurons (Helms et al., 2005), while Ptf1a traced to dI4 (Fig. 2). Further refinement of the code is seen with co-expression of bHLH factors such as Mash1 and Olig3 (Müller et al., 2005), which specify the dI3 fate, while Mash1 alone, or with another factor such as the HD factor Lbx1, specify dI5 (Helms et al., 2005). The fate switch of the dI4 to dI5 rather than dI3 in the Ptf1a null may reflect the presence of Mash1 and Lbx1 in these cells, as the presence of Lbx1 suppresses the dI3 marker Isl1 (Gross

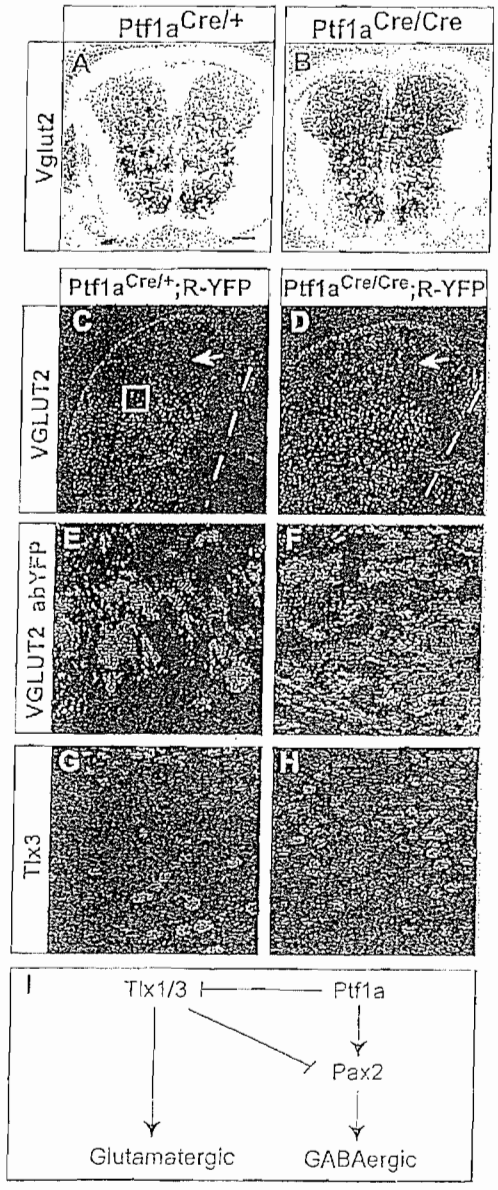


Fig. 5. PTFla is required for suppression of dorsal horn glutamatergic neurons. Transverse sections through spinal cord cervical regions of *Ptf1a$^{Cre/+}$* and *Ptf1a$^{Cre/Cre}$* mouse E16.5 embryos were processed for mRNA in-situ hybridization for the glutamatergic marker Vglut2 gene (A,B) or for immunofluorescence for the protein VGLUT2 (C,D). Note the increase in Vglut2 specifically in the dorsal regions in the absence of Ptf1a. The arrows in C,D indicate superficial laminae that have substantial increase in VGLUT2 in the mutant. The dashed line in C,D indicates the midline. (E,F) Anti-GFP antibody was used to detect YFP in *Ptf1a^{Cre};R26R-stop-YFP* E16.5 embryos. VGLUT2 and YFP seldom co-localize in embryos containing Ptf1a (E); however, co-localization of VGLUT2 and YFP is detected in distal processes in the *Ptf1a* null (F). The density of Tlx3+ cells is increased in dorsal regions in the mutant (G,H). (E-H) are all from dorsal horn regions indicated by the box in C. (I) A model for the role of Ptf1a in GABAergic and glutamatergic neurons in the dorsal horn. Ptf1a acts in two ways: determination of GABAergic neurons by inducing Pax2, which is required for the expression of the GABAergic phenotype in these cells, and suppressing Tlx3, which is required for specifying glutamatergic neurons by inducing glutamatergic specific genes and suppressing Pax2 (Cheng et al., 2004). Scale bar: 110 μm in A,B; 50 μm in C,D; 13 μm in E-H.

et al., 2002; Müller et al., 2002). HD factors in addition to Lbx1, including Msx3 and Gsh1/2, also influence the transcription factor code, probably through regulation of bHLH expression (Kriks et al., 2005; Liu et al., 2004). Taken together, it is clear that neural-specific bHLH factors, combined with HD factors, play an important role in obtaining the neuronal diversity seen in the mature spinal cord.

Ptf1a is a Twist-like bHLH factor that can heterodimerize with E-proteins such as E47, and bind e-box containing DNA (Beres et al., 2005; Obata et al., 2001). However, it is unique in the bHLH family in that it also directly interacts with Rbpsuh, a transcriptional effector of the Notch signaling pathway, to bind a combined e-box;T/C box containing DNA sequence (Beres et al., 2005; Obata et al., 2001). Evidence from Beres et al. (Beres et al., 2005) suggests that the interaction site of Rbpsuh with Ptf1a overlaps that of Rbpsuh with Notch intracellular domain. Thus, the timer, Ptf1a;E-protein:Rbpsuh, reveals a Notch-independent Rbpsuh mechanism of action. These different protein-protein and protein-DNA interactions suggest a variety of mechanisms of action for Ptf1a, including many of those mechanisms previously described for selector gene function (Mann and Carroll, 2002). For example, similar to Twist function, heterodimerization with E-proteins could sequester this shared partner from other class A bHLH factors such as Mash1. Depending on the transcriptional activity of these different heterodimers, the consequence could be either an increase or decrease in specific target expression (Obata et al., 2001). Furthermore, by forming a trimer with Rbpsuh and E-protein (Beres et al., 2005), this complex not only has altered DNA target recognition, but may also directly impact Notch signaling by competing for use of the Rbpsuh subunit. Combining these different protein-protein interactions together with complex arrangement of cis-elements on transcriptional targets, makes Ptf1a a crucial component for the intricate interactions required for generating the appropriate number of specific neuronal cell-types.

Overexpression of Ptf1a, or a DNA-binding.mutant of Ptf1a, in the chick neural tube resulted in excess $Lhx1/5^{+}$ cells (dI2-like) (data not shown). This phenotype is probably due to sequestering E-protein, as co-expressing E47 with Ptf1a suppressed this phenotype (data not shown). The possible competing roles for Ptf1a in different complexes, such as the heterodimer with E-proteins versus the heterotrimer with E-protein and RbpsuH, make overexpression paradigms difficult to interpret. Even so, mis-expression of Ptf1a in the dorsal telencephalon conferred a GABAergic phenotype to the newly forming neurons (Hoshino et al., 2005), supporting a role for Ptf1a in determining GABAergic neuronal cell fates.

Ptf1a is not required for all GABAergic neurons in the nervous system. Its expression is restricted to a subset of cells in the developing diencephalon, hindbrain and spinal cord. In *Ptf1a* mutant embryos, cells expressing GABAergic markers remain, as is seen in the ventral spinal cord (Fig. 4B). Two other bHLH transcription factors, Mash1 and Heslike (also known as Helt), have been shown to mediate GABAergic fate in the telencephalon (Fode et al., 2000; Miyoshi et al., 2004). Mash1 has overlapping expression with Ptf1a in the dorsal neural tube but it is Ptf1a and not Mash1 that is required for the GABAergic phenotype in the spinal cord. Thus, different bHLH factors are required for GABAergic neurons in different regions of the nervous system. It remains to be determined whether Ptf1a codes for shared characteristics in GABAergic neurons in the dorsal horn of the spinal cord and in the cerebellum, in addition to the neurotransmitter phenotype, that are distinct from those in GABAergic neurons derived from Mash1-expressing cells in the telencephalon.

Dramatic phenotypes have been detected in the development of the pancreas (Kawaguchi et al., 2002), the cerebellum (Sellick et al., 2004) and the dorsal spinal cord (this manuscript). In pancreas, there is almost a complete loss of islet and exocrine tissue. The requirement for Ptf1a for generation of multiple GABAergic interneurons in the cerebellum and deep cerebellar nuclei, including Purkinje, stellate, basket and Golgi cells, is responsible for the cerebellar agenesis (Hoshino et al., 2005). Phenotypes in these two tissues also occur in humans with a truncation in the *PTF1A* gene (Sellick et al., 2004). As shown here, the *Ptf1a* null mouse develops a dorsal spinal cord with nearly a complete loss of inhibitory GABAergic neurons with an increase in glutamatergic neurons. This excess in excitatory neurons unopposed by inhibitory neurons may explain the irregular respiratory patterns and increased cerebral excitability in human patients with mutations in *Ptf1a* (Hoveyda et al., 1999). Given the absolute requirement for Ptf1a for the formation of the cell types studied so far, it is likely that additional neurons in the brainstem and the ventral hypothalamus, two other domains of *Ptf1a* expression, are mis-specified in the mutant as well.

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Islet1 and Islet2 have equivalent abilities to promote motoneuron formation and to specify motoneuron subtype identity

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The expression of LIM homeobox genes *islet1* and *islet2* is tightly regulated during development of zebrafish primary motoneurons. All primary motoneurons express *islet1* around the time they exit the cell cycle. By the time primary motoneurons undergo axogenesis, specific subtypes express *islet1*, whereas other subtypes express *islet2*, suggesting that these two genes have different functions. Here, we show that Islet1 is required for formation of zebrafish primary motoneurons; in the absence of Islet1, primary motoneurons are missing and there is an apparent increase in some types of ventral interneurons. We also provide evidence that Islet2 can substitute for Islet1 during primary motoneuron formation. Surprisingly, our results demonstrate that despite the motoneuron subtype-specific expression patterns of Islet1 and Islet2, the differences between the Islet1 and Islet2 proteins are not important for specification of the different primary motoneuron subtypes. Thus, primary motoneuron subtypes are likely to be specified by factors that act in parallel to or upstream of *islet1* and *islet2*.

KEY WORDS: Primary motoneuron, Secondary motoneuron, LIM homeodomain, Interneuron, Spinal motoneuron, pMN domain, Zebrafish

INTRODUCTION

Vertebrate motoneurons innervate muscles in an exquisitely precise pattern. Studies of motoneurons have been instrumental in revealing the processes by which progenitor cells generate different types of neurons, e.g. motoneurons or interneurons, as well as the processes by which each type of neuron adopts a specific subtype identity (Curtiss and Heilig, 1998; Edlund and Jessell, 1999; Eisen, 1999; Jurata et al., 2000; Lee and Pfaff, 2001; Shirasaki and Pfaff, 2002; Sockanathan, 2003; Tanabe and Jessell, 1996). The subtype identity of a motoneuron is defined by its axonal projection out of the central nervous system (CNS) and the specific muscle it innervates (Eisen, 1999; Pfaff and Kintner, 1998; Tosney et al., 1995). Although many proteins have been implicated in motoneuron formation and subtype specification, the precise roles of some of these proteins remain unresolved. In this paper, we focus on the roles of two related proteins, Islet1 and Islet2, in formation and specification of subtype identity of spinal motoneurons in zebrafish.

Zebrafish have two types of spinal motoneurons, primary motoneurons and secondary motoneurons (Myers, 1985), both of which are derived from the spinal cord motoneuron progenitor (pMN) domain (Kimmel et al., 1994; Park et al., 2004). Primary motoneurons (PMNs) are born early in development, around the end of gastrulation. Each PMN is individually identifiable based on its cell body position, axonal trajectory and the muscle region it innervates, providing the opportunity to study vertebrate motoneuron formation at the single cell level (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). Here, we focus on two of the PMNs, MiP, which has a dorsally projecting axon, and CaP, which has a ventrally projecting axon. In contrast to PMNs, secondary motoneurons (SMNs) arise later in development and are more numerous than PMNs (Myers, 1985; Myers et al., 1986). Although some SMNs project dorsally and others project ventrally (Myers et al., 1986; Westerfield et al., 1986), it is currently unclear whether SMNs also develop individually identifiable subtypes. Because PMNs have thus far only been described in anamniote vertebrates such as fish and frogs (Eisen, 1994), it is thought that SMNs more closely resemble the motoneurons described in amniote vertebrates (Kimmel and Westerfield, 1990).

LIM homeodomain (LIM-HD) protein family members are expressed by all vertebrate motoneurons studied to date and play a prominent role in several aspects of motoneuron development, including initial specification and adoption of a particular subtype identity (Curtiss and Heilig, 1998; Eisen, 1999; Jurata et al., 2000; Lee and Pfaff, 2001; Pfaff and Kintner, 1998; Shirasaki and Pfaff, 2002; Sockanathan, 2003; Tanabe and Jessell, 1996). LIM-HD proteins have two N-terminal protein binding LIM domains and one C-terminal DNA binding homeodomain (Bach, 2000; Curtiss and Heilig, 1998). In mouse and chick, the LIM-HD protein Islet1 appears to be pan-motoneuronal around the time motoneurons exit the cell cycle (Ericson et al., 1992; Tsuchida et al., 1994). Slightly later, motoneurons express a related LIM-HD protein, Islet2 (Thaler et al., 2004; Tsuchida et al., 1994). Studies in mouse demonstrated that Islet1 is required for motoneuron formation; in the absence of Islet1, there is widespread cell death in the ventral spinal cord (Pfaff et al., 1996). By contrast, Islet2 is only required for formation of visceral motoneurons, although it is expressed at least transiently in all mouse spinal motoneurons (Thaler et al., 2004).

As in other vertebrates, zebrafish motoneurons express Islet1 and Islet2 (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993; Tokumoto et al., 1995). SMNs express both of these proteins, but it is still unclear whether they are co-expressed or are in distinct SMN populations (Appel et al., 1995; Inoue et al., 1994). Unlike SMNs, the expression patterns of *islet1* and *islet2* have been studied in great detail in PMNs (Fig. 1A). *islet1* is expressed in all PMNs around the time they exit the cell cycle (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993; Tokumoto et al., 1995). MiPs transiently downregulate and then reinitiate *islet1* expression prior to axogenesis; these cells do not express *islet2* (Appel et al., 1995). By

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contrast, prior to axogenesis, CaPs initiate expression of *islet2* and then downregulate expression of *islet1* (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993; Tokumoto et al., 1995). The end result of this dynamic pattern of *islet* gene expression is that by the time of axon extension, MiPs express exclusively *islet1* and CaPs express exclusively *islet2*. This expression pattern leads to the hypothesis that differential expression of Islet proteins specifies PMN subtype. Consistent with this idea, previous work suggested that Islet2 is required for CaP formation (Segawa et al., 2001). However, the role of Islet1 in formation of zebrafish PMNs and MiP subtype specification has not been previously explored.

We used morpholino antisense oligonucleotides (Nasevicius and Ekker, 2000) and mRNA misexpression to investigate the roles of Islet1 and Islet2 in formation of zebrafish motoneurons. Here, we provide evidence that in zebrafish, as in mouse (Pfaff et al., 1996), Islet1 is required for PMN and SMN formation. However, instead of apparently dying like mouse motoneurons that lack Islet1 (Pfaff et al., 1996), zebrafish PMNs appear to change fate and develop as interneurons in the absence of Islet1. Surprisingly, despite the highly regulated expression patterns of Islet1 and Islet2 (Fig. 1A), our results suggest that these proteins have redundant functions. We provide evidence that Islet2 can substitute for Islet1 early on (during motoneuron formation) and later on (during specification of MiP subtype identity). Our results are consistent with a model in which upstream factors that regulate the differential expression of Islet proteins or factors that act in parallel to them establish the differences between PMN subtypes.

MATERIALS AND METHODS

Embryos

Zebrafish (*Danio rerio*) embryos were collected from natural crosses of wild-type (AB) adults, raised at 28.5°C, and staged by hours post fertilization at 28.5°C (hpf) and gross morphology (Kimmel et al., 1995).

RNA in situ hybridization

RNA in situ hybridization was performed as described by Appel et al (Appel et al., 1995). RNA probes include *islet1* and *islet2* (Appel et al., 1995).

Immunohistochemistry and TUNEL labeling

The following primary antibodies (Abs) were used: monoclonal anti-Islet (Korzh et al., 1993), which recognizes Islet1 and Islet2 proteins (1:200; 39.4D5 Developmental Studies Hybridoma Bank); polyclonal anti-GABA (1:1000, Sigma); monoclonal znl (1:200) (Trevarrow et al., 1990); monoclonal znp1 (1:1000) (Melancon et al., 1997; Trevarrow et al., 1990); polyclonal anti-Lhx4 (1:500; S.A.H. and J.S.E., unpublished); and polyclonal anti-Lhx3 (1:500; S.A.H. and J.S.E., unpublished). The following secondary antibodies from Molecular Probes were used: goat anti-mouse Alexa-488 (1:1000), goat anti-mouse IgG_1 Alexa-488 (1:500), goat anti-mouse IgG_{2a} Alexa-488 (1:500), goat anti-mouse IgG_{2b} Alexa-546 (1:500), goat anti-rabbit Alexa-546 (1:1000) and goat anti-rabbit Alexa-488 (1:1000). Goat anti-mouse Cy5 (1:200) from Jackson Laboratories was also used. Embryos were fixed for 3.5-4.0 hours in 4% paraformaldehyde (PFA) and 1×Fix Buffer (Westerfield, 1995) at 4°C; blocked in 1×PBS, 5% NGS, 4 mg/ml BSA, 0.5% Triton X-100 for 1 hour at room temperature; incubated in primary antibody diluted in block overnight at 4°C; washed at room temperature for 1.5 hours in PBS + 0.1% Tween-20; incubated in secondary antibody diluted in block for 4 hours at room temperature; and then washed for 1.5 hours at room temperature in PBS + 0.1% Tween-20. Embryos were stored in 4% paraformaldehyde until analyzed.

For triple labeling with Islet Ab, Lhx4 Ab and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling), embryos were first labeled with Islet and Lhx4 Abs. Embryos were then post-fixed for 20 minutes at room temperature in 4% PFA. After post-fixation, embryos were washed three times for 5 minutes with PBST (1×PBS + 0.1% Tween-20). Proteinase K (10 mg/ml) diluted 1:5000 in water was used to permeabilize embryos for 1.5 minutes at room temperature. Embryos were fixed for 15 minutes at room temperature in 4% PFA and washed out of fix with PBST. Embryos were incubated for 1 hour in the dark on ice followed by one hour in the dark at 37°C in TUNEL solution (Roche). After incubation, embryos were washed four times for 10 minutes in PBST; the last wash was overnight at 4°C.

Microscopy

Images of embryos were captured on a Zeiss Axioplan compound microscope equipped with a Zeiss Axiocam, or on a Zeiss Pascal confocal microscope. Adobe Photoshop was used to adjust brightness and contrast of images.

RNA and morpholino injections

islet1 RNA (Appel et al., 1995; Inoue et al., 1994; Tokumoto et al., 1995) and *islet2* RNA (Appel et al., 1995; Tokumoto et al., 1995) were transcribed using the mMessage Machine (Ambion) according to instructions. One-cell stage embryos were injected with several nanoliters of 56 ng/μl *islet1* RNA or 61 ng/μl *islet2* RNA for overexpression experiments. *islet1* RNA was reduced to 28 ng/μl for rescue experiments, but *islet2* RNA remained at 61 ng/μl.

To create embryos with reduced Islet1, two splice-blocking morpholinos were designed by Gene Tools (Corvallis, Oregon) to the splice donor sites after the second and third exons of *islet1*. *islet1E2* began at position 208 of *islet1* cDNA at the end of exon 2 (5′-TTAATCTGCGTTACCTGATGTA-GTC-3′) and ended in the second intron of *islet1* genomic DNA. *islet1E3* began at position 472 of *islet1* cDNA at the end of exon 3 (5′-GAATGCAATGCCTACCTGCCATTTG-3′) and ended in the third intron of *islet1* genomic DNA. The sequence for *islet1E2* MO differs from the corresponding sequence in *islet2* genomic DNA at the end of exon 2 (5′-GATTACGTACGGTACGAGCAACTAT-3′) by 13 bp. The end of exon 3 in *islet2* genomic DNA sequence (5′-ATCCCAGGTAGTAGTAAAAATA-ATA-3′) is different from *islet1E3* MO sequence by 18 bp. Several nanoliters of 1 mg/ml *islet1E2* and 1 mg/ml *islet1E3* were co-injected into one-cell stage embryos as described previously (Lewis and Eisen, 2001). Embryos looked generally healthy and had little or no Islet1 protein remaining. The same phenotype was observed when an *islet1* translation blocking MO beginning at position –25 in the 5′UTR (5′-CCCATGTCAAGAAAGTA-AGGCGGTG-3′) was injected into one-cell stage embryos.

To create embryos with reduced Islet2, a translation blocking morpholino was designed by Gene Tools to the translation start site. *islet2* MO began at position –2 of *islet2* 5′UTR (5′-GGATGCGGTAGAATATCCACCATAC-3′) and was tagged with fluorescein. Several nanoliters of 5 mg/ml were injected into one-cell stage embryos as describe previously (Lewis and Eisen, 2001). A second morpholino also designed to the translation start site of *islet2* gave the same phenotype as the first *islet2* MO. The second *islet2* MO began at position –9 of the *islet2* 5′UTR (5′-GTAGAAT-ATCCACCATACAGGAGGG-3′). Several nanoliters of 1 mg/ml *islet1E2*, 1 mg/ml *islet1E3* and 5 mg/ml *islet2* MOs were co-injected into one-cell stage embryos to eliminate both Islet1 and Islet2 proteins.

Quantitation

We quantified the efficiency of our splice-blocking MOs by counting the number of cell nuclei labeled with *islet1* RNA adjacent to somites 8-11 and calculating the percentage of cells with nuclear *islet1* RNA labeling in *islet1* MO-injected versus control (uninjected) embryos at 20-21 hpf. We also counted the number of cells in the pMN domain adjacent to somites 8-11 labeled with Islet Ab at 28 hpf and calculated the percentage of Islet-positive cells in *islet1* MO-injected embryos versus control embryos.

PMN axons stained with znl and znp1 Abs were counted in embryos at 24 or 28 hpf. Axons were counted as belonging to MiPs if they extended caudal and dorsal to the CaP cell body. Axons were counted as long CaP axons if they projected ventrally of the horizontal myoseptum. Axons were counted as short CaP axons if they exited the spinal cord, but did not project ventral of the horizontal myoseptum. To represent the number of axons in uninjected control versus injected embryos, we calculated the percentage of axons remaining in segments 8-12.

Islet Ab-labeled cells in the pMN domain adjacent to somites 8-10 were counted at 72 hpf from confocal microscopy images. We calculated the percentage of Islet-positive cells in MO-injected embryos in comparison with controls.

To count interneurons, we stained embryos with GABA Ab at 24 and 28 hpf and imaged them by confocal microscopy as described above. Cells were counted as in the KA" position if they were in the medioventral spinal cord directly lateral to the floor plate. Cells were counted as in the V-K position if they were within three cell diameters dorsal of the floor plate. The average number of interneurons in the neural tube adjacent to segments 8-11 was compared between control and injected embryos.

To examine cell death, the number of cells co-expressing TUNEL and Lhx4 was counted in the ventral spinal cord adjacent to somites 8-11.

RESULTS

Islet1 protein is required for motoneuron formation

We tested the role of Islet1 in motoneuron formation by injecting one-cell stage embryos with morpholino antisense oligonucleotides (MOs) designed to block the splice donor sites at the ends of the second and third exons of *islet1* genomic DNA. *islet1* mRNA was localized in the nucleus of PMNs in *islet1* MO-injected embryos (Fig. 1B,C), indicating that the MO blocked *islet1* splicing (Yan et al., 2002). We also stained embryos at 28 hpf with an Islet Ab that recognizes both Islet1 and Islet2 proteins (Korzh et al., 1993; Thor et al., 1991). Islet Ab staining was absent from *islet1* MO-injected embryos, indicating that Islet1 and Islet2 proteins were both significantly reduced (Fig. 1D,E). The reduction in Islet2 protein could result from *islet1* MOs affecting the splicing of *islet2* RNA. However, this is extremely unlikely because the splice site sequences are very different (see Materials and methods). In addition, *islet2* mRNA was absent from *islet1* MO-injected embryos (Fig. 2A,B). This contrasts with the nuclear localization of *islet1* mRNA in these embryos. These results are consistent with the idea that our *islet1* MOs are specific for *islet1*, and raise the possibility that Islet1 regulates *islet2* expression or that PMNs are not specified in the absence of Islet1 and thus do not express later PMN markers, such as *islet2*.

To examine the role of Islet1 in PMN formation, we looked for defects in motor axon outgrowth at 24 and 28 hpf in *islet1* MO-injected embryos using znl and znp1 Abs, which recognize motoneurons (Melancon et al., 1997; Trevarrow et al., 1990). We assayed the number of motor axons in segments 8-12 and found that both dorsally projecting MiP and ventrally projecting CaP axons were significantly reduced in *islet1* MO-injected embryos (Fig. 2D,E; Table 1). *islet1* MO-injected embryos had a few truncated CaP axons, something never seen in control embryos (Table 1; data not shown). However, the number of truncated CaP axons in *islet1* MO-injected embryos was significantly fewer than the number of normal CaP axons in control embryos. We examined the specificity of our *islet1* MOs by co-injection of *islet1* RNA and *islet1* MO, and found that PMN axons were restored and appeared normal (Fig. 2F; Table 1). Thus, we conclude that Islet1 is required for zebrafish PMN formation. Interestingly, we also found that, in most cases, injection of *islet1* RNA did not restore *islet2* mRNA expression (Fig. 2C). These data suggest that that Islet1 does not regulate *islet2* mRNA expression and support the hypothesis that in the absence of Islet1 PMNs are not specified, and thus do not express later markers, such as *islet2*.

Having found that Islet1 is required for formation of zebrafish PMNs, we asked whether it is also required for formation of SMNs. We assessed the role of Islet1 protein on SMN formation at 72 hpf by staining embryos with Neurolin Ab, which recognizes SMNs but not PMNs (Fashena and Westerfield, 1999). Essentially all SMN dorsally projecting and ventrally projecting axons were absent from embryos injected with *islet1* MO (Fig. 3A,B), and the number of Islet-positive cells in the ventral spinal cord was decreased by 52% (Fig. 3A′,B′). The presence of Islet-positive cells in the ventral spinal cord of *islet1* MO-injected embryos might result from reduction of MO efficacy as the embryos developed over several days. Alternatively, it might be due to the presence of Islet2 protein. We did two experiments to distinguish between these possibilities. First, we injected one-cell stage embryos with a MO designed to block the *islet2* translation start site (*islet2* MO) and found that this caused a 10% reduction in the number of Islet-positive cells in the ventral spinal cord (Fig. 3A,A′,C,C′), an almost total loss of dorsally projecting SMN axons

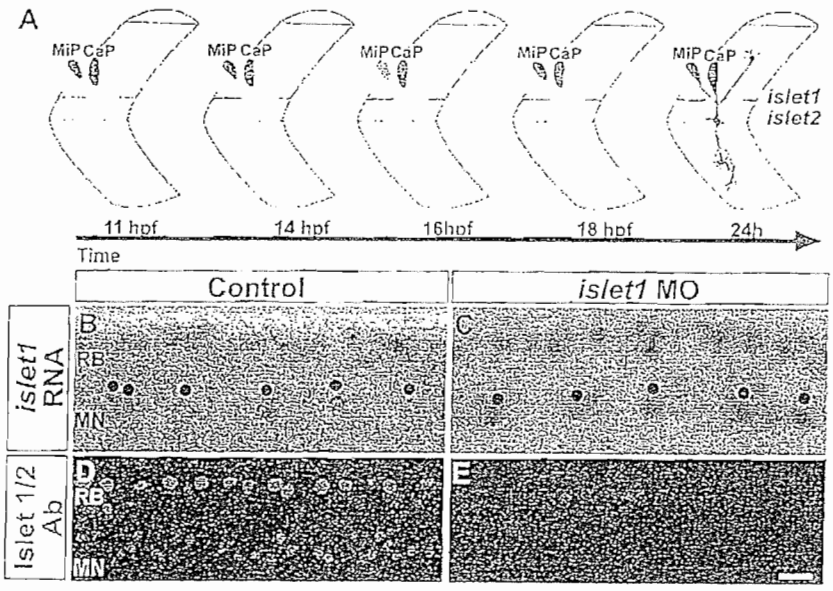


Fig. 1. Islet1 regulates Islet2 expression. In this and subsequent figures, all photographs show 8-12 segment region of whole-mount embryos with rostral towards the left and dorsal towards the top, unless otherwise noted. (A) Schematic showing expression of *islet1* (blue) and *islet2* (red) in CaP and MiP between 11 and 24 hpf. Blue and red stripes indicate co-expression of *islet1* and *islet2*; grey indicates downregulation of *islet1*. (B) At 20 hpf, *islet1* RNA is expressed in dorsal Rohon-Beard sensory neurons (RB) and MiPs in control embryos. MN designates the row containing MiPs; individual MiPs are marked by black dots. In the segment farthest to the left, both MiP and RoP are indicated by black dots. RoPs are PMNs that express *islet1* later than MiPs (Appel et al., 1995), and thus are absent from most of our figures. (C) *islet1* MO-injected embryos express *islet1* in RBs and MiPs, but the RNA is nuclear instead of cytoplasmic as it is in controls (47% of PMNs have nuclear *islet1* RNA staining in *islet1* MO-injected embryos when compared with 1% in controls*; *n*=7 *islet1* MO-injected embryos; *n*=8 control embryos); black dots indicate MiPs. (D) At 28 hpf, Islet Ab (red) labels RBs and motoneurons (MN) in control embryos. (E) Islet Ab labeling is absent from *islet1* MO-injected embryos (92% fewer cells labeled with Islet Ab in the pMN domain*; *n*=22 *islet1* MO-injected embryos; *n*=26 control embryos). *$P<0.001$. Scale bar: 20 μm.

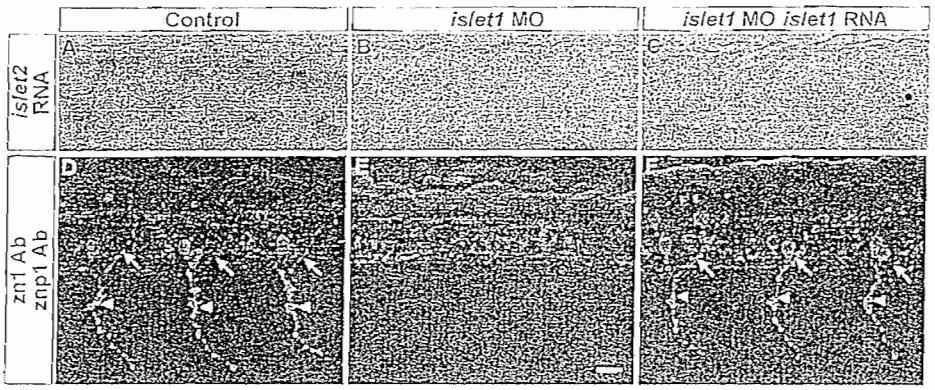


Fig. 2. Islet1 is required for PMN formation. (A-C) 20 hpf embryos stained with *islet2* riboprobe. Control embryos (A) express *islet2* in RBs (dorsally located cells) and CaPs (ventrally located cells). *islet1* MO-injected embryos (B) lack *islet2* expression. *islet1* MO-injected embryos co-injected with *islet1* RNA (C) also lack most *islet2* expression; one *islet2*-positive PMN is indicated by a black dot. (D-F) 28 hpf embryos stained with zn1 and znp1 Abs (green). Control embryos (D) have dorsally projecting MiP axons (arrows) and ventrally projecting CaP axons (arrowheads). *islet1* MO-injected embryos (E) lack both MiP and CaP axons. Co-injection of *islet1* MO and *islet1* RNA (F) restored both MiP and CaP axons. Scale bar: 20 μm.

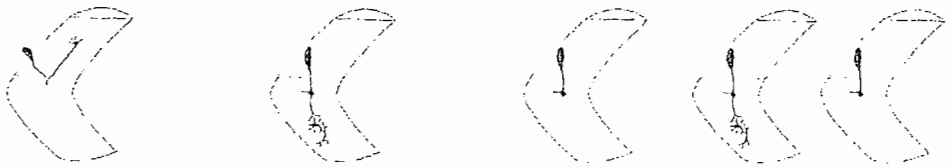
and a partial loss and disorganization of ventrally projecting SMN axons (Fig. 3C). Second, we co-injected *islet1* and *islet2* MOs and found that this caused a nearly complete loss of Islet-positive cells from the ventral spinal cord (Fig. 3D,D′). *islet1* MO-injected embryos had many more Islet-positive cells in the ventral spinal cord than did embryos injected with both *islet1* and *islet2* MOs; however, the severe reduction of SMN axon projections out of the spinal cord was similar in both cases (Fig. 3B,D). These data provide evidence that the Islet Ab labeling of SMN cell bodies in *islet1* MO-injected embryos was due to the presence of Islet2 protein. They also support the idea that Islet1 protein is required for SMN formation and Islet2 protein is required for proper SMN axon outgrowth.

Islet1 is required to specify PMN fate

In mouse, loss of Islet1 leads to absence of motoneurons and widespread cell death in the ventral spinal cord (Pfaff et al., 1996). By contrast, our analysis of *islet1* RNA expression in *islet1* MO-injected embryos revealed that PMN cell bodies were still present in the absence of Islet1 (Fig. 1B,C). To confirm this, we examined cell death in the ventral spinal cord at 28 hpf by co-labeling embryos with TUNEL and Lhx4 Ab, which labels PMNs as well as some other neurons in the ventral spinal cord (S.A.H. and J.S.E., unpublished). The number of Lhx4-positive cells was the same in *islet1* MO-injected and control embryos, and we observed no increase in the number of cells positive for TUNEL in experimental embryos, indicating that PMNs did not die in the absence of Islet1 (Table 2).

Although PMN cell bodies were present in the absence of Islet1, these cells did not project axons out of the spinal cord, thus they did not meet the criteria to be defined as motoneurons. The zebrafish pMN domain generates several types of interneurons in addition to PMNs and SMNs (Kimmel et al., 1994; Park et al., 2004), raising the possibility that PMNs might develop as interneurons in the absence of Islet1. We tested this hypothesis by examining co-expression of Lhx3 and zn1. In control embryos, PMNs are the

Table 1. Islet1 is required for PMN axon outgrowth



	MiP axons		CaP axons		Short CaP axons		Total CaP axons	
	24 hpf	28 hpf	24 hpf	28 hpf	24 hpf	28 hpf	24 hpf	28 hpf
Control	74.8% (*n*=127)	99.1% (*n*=320)	100% (*n*=127)	100% (*n*=320)	0% (*n*=127)	0% (*n*=320)	100% (*n*=127)	100% (*n*=320)
islet1 MO	11.1%* (*n*=270)	12.2%* (*n*=450)	14.8%* (*n*=270)	3.8%* (*n*=450)	35.9%* (*n*=270)	12.7%* (*n*=450)	50.7%* (*n*=270)	16.4%* (*n*=450)
islet1 MO *islet1* RNA	N/A	79.6% (*n*=250)	N/A	69.2% (*n*=250)	N/A	21.6% (*n*=250)	N/A	90.8% (*n*=250)
islet1 MO *islet2* RNA	N/A	57.8% (*n*=185)	N/A	71.9% (*n*=185)	N/A	6.5% (*n*=185)	N/A	78.4% (*n*=185)

Percentage of spinal segments with axons at axial level 8-12.
*$P<0.00001$ in comparison with controls.
n, number of segments.
N/A, not available.

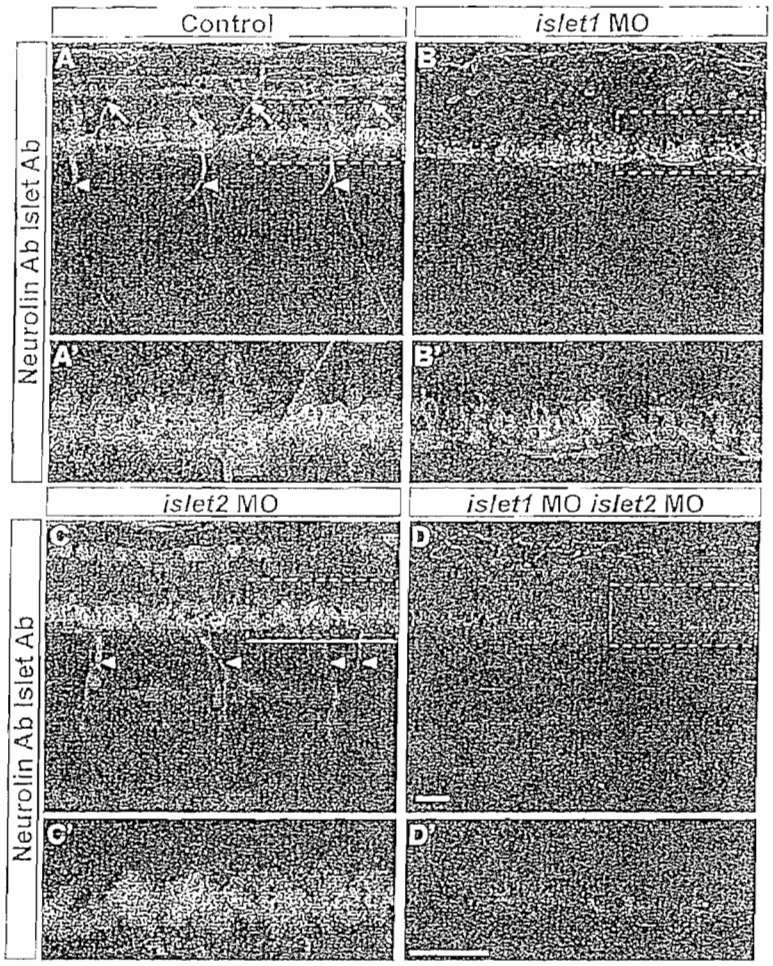


Fig. 3. Islet proteins are required for SMN formation. (A-D) 72 hpf embryos stained with Neurolin (green) and Islet (red) Abs. For each panel, one segment (outlined) is magnified and shown below (A'-D'). Control embryos (A) had dorsally projecting (arrows) and ventrally projecting (arrowheads) Neurolin-positive SMNs and many Islet-positive cells. *islet1* MO-injected embryos (B) lacked SMN axons and had 52% fewer Islet-positive cells (*P<0.01, 18 segments of three embryos). *islet2* MO-injected embryos (C) had 10% fewer SMN cell bodies (*P<0.01, 24 segments of four embryos). These embryos entirely lacked dorsally projecting SMN axons and ventrally projecting SMN axons (arrowheads) were disorganized. The cells labeled with Islet Ab outside the neural tube next to the ventrally projecting axons are most likely dorsal root ganglion cells that are out of position. Embryos co-injected with *islet1* and *islet2* MOs (D) lacked all SMNs and 99.5% of Islet-positive cells were absent (*P<0.01, 18 segments of three embryos), although Neurolin-positive floor plate was still present. Scale bars: 20 μm.

predominant cell type co-labeled by Lhx3 and znl antibodies (Fig. 4A); however, both of these antigens are also expressed by VeLD interneurons (Appel et al., 1995) that are derived from the pMN domain (Park et al., 2004). *islet1* MO-injected and control embryos had approximately the same number and distribution of cells co-labeled with Lhx3 and znl Abs. In control embryos most Lhx3$^+$, znl$^+$ cells projected axons out of the spinal cord (Fig. 4A), consistent with their PMN identity. By contrast, in *islet1* MO-injected embryos Lhx3$^+$, znl$^+$ cells did not project axons out of the spinal cord, instead projecting axons within the spinal cord (Fig. 4B), similar to interneurons, and consistent with the hypothesis that in the absence of Islet1, PMNs developed as interneurons.

To further test the idea that in the absence of Islet1 PMNs develop as interneurons, we stained embryos with an antibody to the neurotransmitter GABA which is expressed in several types of pMN domain-derived interneurons, including VeLD, KA′ and KA″ (Bernhardt et al., 1992; Park et al., 2004). We counted the number of cells in the VeLD, KA′ and KA″ positions in the spinal segments adjacent to somites 8-11. Cells were considered to be in the KA″ position if they were located immediately lateral to the floor plate. The number of cells in the KA″ position was similar in control and *islet1* MO-injected embryos (Fig. 4C,D; Table 3). Cells in the VeLD and KA′ positions were counted together because, although both cell types are located dorsal to KA″ neurons (Park et al., 2004) and within three cell diameters dorsal of the floor plate, they are hard to distinguish without additional markers. Thus, we refer to cells in this location as V-K. There were significantly more cells in the V-K position of *islet1* MO-injected embryos than in controls (Fig. 4C,D; Table 3). Interestingly, although an average of 24 PMNs were lost from the spinal cord adjacent to somites 8-11 of *islet1* MO-injected

Table 2. PMNs do not die in *islet1* MO-injected embryos

	Number of Lhx4$^+$ cells		Number of Lhx4$^+$/TUNEL$^+$ cells	
	20 hpf	28 hpf	20 hpf	28 hpf
Control	49.1±9.5 (n=27)	99.3±12.3 (n=15)	0 (n=27)	0 (n=15)
islet1 MO	51.4±10.5* (n=28)	93.2±15.5* (n=11)	0 (n=28)	0 (n=11)

Number of Lhx4$^+$ cells in the ventral spinal cord at axial level 8-12.
Values given ±s.d.
*P>0.05; n, number of embryos.

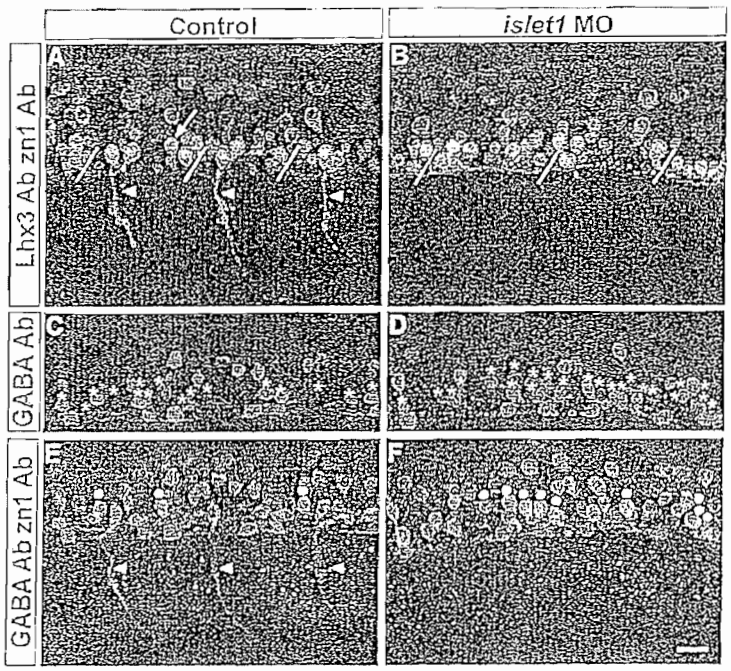


Fig. 4. Islet1 is required to inhibit interneuron formation. (A,B) Embryos co-stained with Lhx3 (red) and zn1 (green) Abs. (A) In control embryos, zn1 and Lhx3 were co-expressed in motoneurons that projected axons (arrowheads) out of the spinal cord and in VeLD interneurons (one is indicated by an arrow; slanted lines denote somite boundaries). (B) *islet1* MO-injected embryos had cells that co-expressed Lhx3 and zn1, but they did not project axons out of the spinal cord, and instead had axons that projected caudally within the spinal cord. (C,D) Embryos stained with GABA Ab. (C) In control embryos, GABA Ab reveals KA″, KA′, VeLD and other (unidentified) interneurons; cells in the V-K position are marked by asterisks. (D) Cells in the V-K position (asterisks) are more numerous in *islet1* MO-injected embryos. (E,F) The same embryos shown in C and D, but here showing co-labeling with GABA (red) and zn1 (green) Abs. Dots indicate cells co-expressing GABA and zn1. (E) In control embryos, only a few cells co-express these markers. Arrowheads in E indicate CaP axons. (F) In *islet1* MO-injected embryos there are many more cells that co-express zn1 and GABA. All embryos shown in this figure are at 28 hpf. Scale bar: 20 μm.

embryos, there were only an average of 11 extra GABA-positive cells in the V-K position (Table 3), suggesting that some PMNs did not develop into GABA-expressing interneurons. To determine whether this was the case, we co-labeled embryos with GABA and zn1 Abs. At 28 hpf these antigens co-localized in a few cells in the V-K position in control embryos (Fig. 4E). *islet1* MO-injected embryos had more cells in the V-K position that co-expressed GABA and zn1 (Fig. 4F). However, some of the zn1-positive interneuron-like cells did not co-express GABA (Fig. 4F), suggesting that some PMNs developed into a type of interneuron distinct from VeLDs or KA′ neurons. Alternatively, these cells may have adopted a hybrid identity in which they developed interneuron-like axonal projections, without expressing interneuron markers. Without interneuron type-specific markers, we are unable to distinguish between these possibilities. Together these data provide evidence that *islet1* MO-injected embryos have more pMN domain-derived interneurons than control embryos, and suggest that these supernumerary cells are PMNs that have become interneurons in the absence of Islet1.

islet1 RNA misexpression induces supernumerary motoneurons and inhibits interneuron formation

Our studies using *islet1* MO showed that Islet1 is required to promote PMN formation and to inhibit interneuron formation. To test whether *islet1* is sufficient to induce PMNs, we misexpressed *islet1* RNA at the one-cell stage and examined the number of PMNs at 28 hpf. Zn1 and znp1 Ab labeling showed that *islet1* RNA-injected embryos had supernumerary PMN cell bodies and thicker ventral motor nerves (Fig. 5A,B). Some *islet1* RNA-injected embryos had supernumerary *islet2*-positive cells in the ventral neural tube (Fig. 5C,D), consistent with the hypothesis that some of the supernumerary PMNs were CaPs whose axons contributed to the thicker ventral motor nerve. Interestingly, the supernumerary PMN cell bodies were not scattered throughout the spinal cord, but were localized in the normal dorsoventral position of PMNs, suggesting that the ability of Islet1 to specify PMN development is limited to cells derived from the pMN domain.

Table 3. Islet1 is required to inhibit interneuron formation

	V-K position		KA″ position	
	24 hpf	28 hpf	24 hpf	28 hpf
Control	15.1±4.7 (*n*=41)	20.7±3.6 (*n*=31)	9.2±2.4 (*n*=32)	13±1.7 (*n*=35)
islet1 MO	26.0±4.6* (*n*=22)	31.7±4.5* (*n*=20)	9.8±2.8‡ (*n*=20)	12.6±2.3‡ (*n*=16)
islet1 RNA	12.5±4.5† (*n*=27)	14.2±4.3* (*n*=22)	9.6±2.4‡ (*n*=18)	13.8±2.5‡ (*n*=21)

Number of GABA-positive interneurons at axial level 8-11.
Values given±s.d.
*P<0.00001; †P<0.05; ‡not significant; *n*, number of embryos.
Red cells in diagram represent the interneuron population counted in columns below picture.

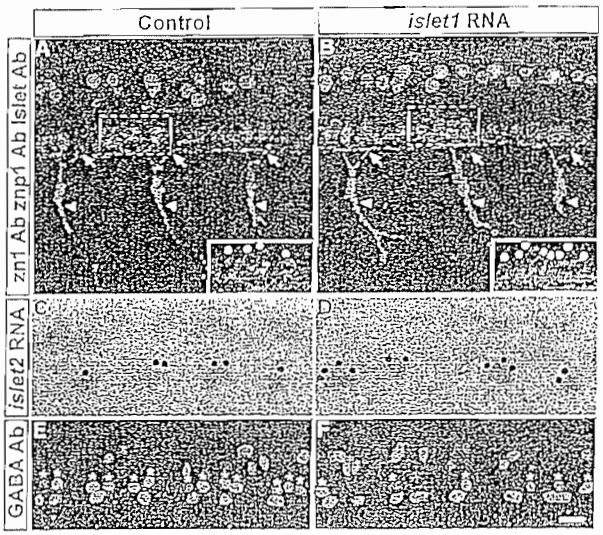


Fig. 5. Misexpression of *islet1* RNA induces PMN formation and inhibits interneuron formation. (A,B) 28 hpf embryos labeled with zn1 and znp1 (green) and Islet (red) Abs. Control embryos (A) have MiP axons (arrows), CaP axons (arrowheads) and several PMN cell bodies (inset; zn1 Ab; dots). Misexpression of *islet1* RNA (B) causes a thicker ventral motor nerve (arrowheads) and more zn1-positive cell bodies (inset, dots). (C,D) 20 hpf embryos labeled with *islet2* riboprobe. *islet2* is expressed in one or two PMNs per segment (black dots) in control embryos (C); the second cell is VaP, a duplicate CaP that is sometimes present and typically dies (Eisen et al., 1990). *islet2* is expressed in two to four PMNs per segment in embryos misexpressing *islet1* RNA (D). (E,F) 28 hpf embryos labeled with GABA Ab. The number of cells in the V-K position (asterisks) is decreased in embryos misexpressing *islet1* RNA (F) when compared with controls (E). Scale bar: 20 μm.

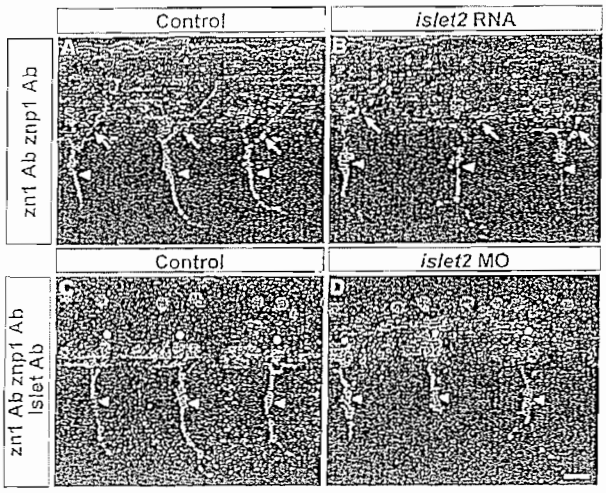


Fig. 6. Formation of CaP subtype identity is independent of Islet2. (A,B) Embryos labeled with zn1 and znp1 Abs (green). Arrows indicate MiP axons, arrowheads indicate CaP axons. At 28 hof, control embryos (A) have both CaP and MiP axons. Embryos misexpressing *islet2* RNA (B) also have normal MiP and CaP axons. (C,D) Embryos labeled with zn1 and znp1 (green), and Islet (red) Abs. Arrowheads indicate CaP axons. CaP cell bodies (dots) co-label with Islet and zn1 Abs and project axons ventrally at 28 hpf in control embryos (C). *islet2* MO-injected embryos (D) lack Islet staining in CaP cell bodies and have abnormal CaP axons. Scale bar: 20 μm.

To learn whether the supernumerary PMNs in *islet1* RNA-injected embryos correlated with a loss of ventral interneurons, we stained *islet1* RNA-injected embryos with GABA Ab and counted the number of pMN domain-derived interneurons. There was a significant reduction of cells in the V-K position in *islet1* RNA-injected embryos, but the number of cells in the KA″ position was unchanged from controls (Fig. 5E,F; Table 3). Thus, *islet1* RNA misexpression inhibited interneuron formation and promoted PMN formation, but this effect appeared limited to a subset of interneurons derived from the pMN domain. These results support our hypothesis that Islet1 is required to promote PMN fate and inhibit interneuron fate. However, they contrast with results from mouse in which *islet1* misexpression did not induce motoneuron formation (Thaler et al., 2002).

CaP subtype specification is independent of Islet2

The specific expression of Islet2 in CaPs, but not in MiPs, led us to hypothesize that Islet2 is required for CaP subtype identity. CaPs transiently co-express *islet1* and *islet2*, but they downregulate expression of *islet1* prior to axogenesis (Appel et al., 1995). Recent characterization of narrow somite mutants revealed that PMNs that maintain co-expression of Islet1 and Islet2 develop a CaP axon trajectory (Lewis and Eisen, 2004), suggesting that Islet2 is sufficient to cause a PMN to become a CaP, even when Islet1 is not downregulated. Therefore, we asked whether misexpression of Islet2 could turn MiPs into CaPs. We found that misexpression of *islet2* RNA had no effect on formation of dorsally projecting MiP axons (Fig. 6A,B). Thus, MiPs maintained their subtype identity despite co-expressing Islet1 and Islet2, providing evidence that Islet2 is not sufficient to turn a MiP into a CaP.

To further test whether Islet2 is required for CaP subtype identity, we injected embryos with *islet2* MO to block Islet2 protein formation. As a control, we stained embryos with Islet Ab and looked for loss of Islet protein in CaPs. After 15 hpf, CaPs express Islet2, but not Islet1 (Appel et al., 1995); therefore, we were able to use Islet Ab staining after 15 hpf to assay loss of Islet2 in CaPs. Islet protein was absent from CaP cell bodies in *islet2* MO-injected embryos, indicating the MO was able to knock down Islet2 protein (Fig. 6C,D). *islet2* MO-injected embryos had some CaPs with truncated axons and some with abnormally branched axons; however, many CaPs were normal. These results suggest that Islet2 is not required for formation of the ventral axon that defines the CaP subtype identity, but that it is involved in later aspects of CaP axon pathfinding. Together with our finding that *islet1* RNA can rescue all PMNs in *islet1* MO-injected embryos even when *islet2* mRNA expression is not induced (Fig. 2A-C), this result suggests that Islet1 alone is sufficient for specification of CaP subtype identity.

Islet2 can promote motoneuron formation and substitute for Islet1 in MiP formation

Our observation that Islet1 alone was sufficient for specification of CaP subtype identity prompted us to ask whether Islet2 was similarly sufficient to specify CaP. We tested whether Islet2 could promote CaP formation in the absence of Islet1 by misexpressing *islet2* RNA in *islet1* MO-injected embryos and labeling them with zn1 and znp1 Abs. We found that CaP formation was restored in these embryos (Fig. 7A,B; Table1), revealing that, similar to Islet1, Islet2 is sufficient to specify CaP subtype identity. These results also reveal that Islet2 can substitute for Islet1 in motoneuron formation.

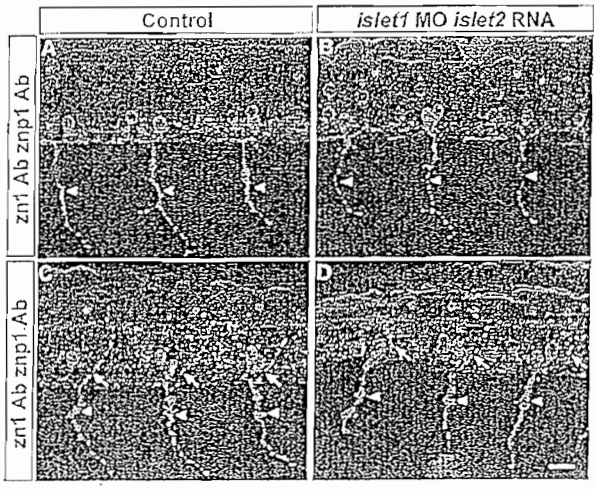


Fig. 7. Islet1 and Islet2 can function redundantly in PMN formation. (A-D) 28 hpf embryos stained with znl and znp1 (green) Abs. CaP axons are indicated by arrowheads; MiP axons are indicated by arrows. Control embryos (A) have normal CaP axons. Embryos co-injected with *islet2* RNA and *islet1* MO (B) have normal CaP axons. Control embryos (C) have dorsally projecting MiP axons. MiP axons are present in embryos co-injected with *islet1* MO and *islet2* RNA (D). Scale bar: 20 μm.

Our results suggest that the differences between the Islet1 and Islet2 proteins are not important for CaP formation. Therefore, we asked whether the differences between these proteins mattered for formation of other PMN subtypes. MiPs never express Islet2, thus we investigated whether Islet2 could substitute for Islet1 in MiP formation. We misexpressed *islet2* RNA in *islet1* MO-injected embryos and found that this restored normal MiP development (Fig. 7C,D; Table1). Our results reveal that Islet1 or Islet2 is sufficient to specify both CaP and MiP subtype identity. Thus, the differences between these proteins cannot be what controls PMN subtype specification. These results do not support our original hypothesis, that the differences between the Islet1 and Islet2 proteins are responsible for the differences between the MiP and CaP subtypes. Instead, they suggest that PMN subtype specification depends on upstream factors that regulate the differential expression of *islet1* and *islet2* in MiP and CaP, or on factors that act in parallel with Islet1 and/or Islet2.

DISCUSSION

We report three key findings. First zebrafish Islet1 protein is required not only to promote PMN formation, but also to inhibit interneuron formation. Second, despite distinct expression patterns, Islet2 can substitute for Islet1 to promote PMN formation. Finally, PMN subtype specification is independent of the differences between the Islet1 and Islet2 proteins.

Islet1 promotes motoneuron formation at the expense of interneuron formation

Zebrafish Islet1 is required for both SMN and PMN formation, and appears to mediate a switch between motoneuron and interneuron fates in the pMN domain. This apparently contrasts with the reported role of Islet1 in mouse, to promote motoneuron survival (Pfaff et al., 1996). However, several additional studies raise the possibility that in mouse and chick, Islet1 may also inhibit interneuron formation. For example, transplanting neural tubes from Islet1-deficient mice into chicks prevents the death of nascent motoneurons. These surviving cells express interneuron markers (Thaler et al., 2004), although it is unclear whether they project motoneuron-like axons out of the spinal cord or interneuron-like axons within the spinal cord. Similarly, mouse embryos with a targeted deletion of the Mnx family member Hb9 initially express Islet1 in nascent motoneurons, allowing these cells to develop as motoneurons and extend axons out of the spinal cord. However, Islet1 expression is very quickly extinguished in these mice, and motoneurons express interneuron markers (Arber et al., 1999; Thaler et al., 1999), suggesting that both Hb9 and Islet1 may participate in inhibiting interneuron formation. Together, these results support the idea that in mouse and chick, as in zebrafish, Islet1 may play a role in inhibiting interneuron formation.

Whether Islet1 normally mediates a decision between motoneuron and interneuron fates may depend whether these cells are derived from the same progenitor population. Although lineage studies in chick using recombinant retroviruses provided evidence that an individual spinal cord progenitor cell can generate both motoneurons and interneurons (Leber et al., 1990), more recent studies in both chick and mouse have advocated the idea that motoneurons arise from the pMN domain, whereas interneurons are generated from adjacent p3 and p2 domains, as well as from other domains that are more distal from the pMN domain (Briscoe and Ericson, 2001; Briscoe et al., 2000). In mouse and chick, pMN domain-derived motoneurons co-express Lhx3 and Islet1, whereas V2 interneurons, which are derived from the p2 progenitor domain situated just dorsal to the pMN domain, express Lhx3 but not Islet1 (Ericson et al., 1992; Sharma et al., 1998; Tanabe et al., 1998). Studies in the chick spinal cord show that misexpression of Islet1 alone has no effect on motoneuron formation, whereas misexpression of Lhx3 alone promotes V2 interneuron formation (Tanabe et al., 1998; Thaler et al., 2002). Misexpression of both Lhx3 and Islet1 causes cells to become motoneurons, even when they do not originate from the pMN domain (Thaler et al., 2002). As in mouse and chick, zebrafish PMNs co-express Islet1 and Lhx3, whereas VeLD interneurons express Lhx3 but not Islet1 (Appel et al., 1995). However, in contrast to mouse and chick, PMNs and VeLD interneurons are both derived from the pMN domain (Park et al., 2004). Clonal analysis in zebrafish reveals that a single ventral neural tube progenitor in the pMN domain can generate PMNs, interneurons, or both PMNs and interneurons; however, there is no consistent lineage relationship among these cell types (Kimmel et al., 1994; Park et al., 2004). Zebrafish lacking Islet1 lack PMNs, but have a normal number of $Lhx3^+$ pMN domain cells, consistent with the idea that loss of Islet1 results in pMN domain-derived cells that express only Lhx3 and therefore develop as interneurons. In contrast to chick, misexpression of zebrafish Islet1 alone leads to formation of supernumerary PMNs. However, these cells only form in the normal PMN position, suggesting that $Lhx3^+$ cells within the pMN domain become PMNs when they co-express Islet1. Thus, we suspect that in zebrafish the fate decision between PMNs and interneurons is determined by the interaction of transcription factors, such as Islet1, that are motoneuron-specific within the pMN domain and Lhx3, which is expressed by both motoneurons and interneurons. This is similar to what Thaler and colleagues proposed happens in chick (Thaler et al., 2002), except that in zebrafish the fate decision appears to occur between cell types generated within the same progenitor domain, whereas in chick it appears to occur between cell types generated in adjacent progenitor domains. If our interpretation is correct, then

Islet1 may only normally mediate a switch between motoneuron and interneuron fates in cells that co-express Lhx3 and are derived from the same progenitor population.

An outstanding question that remains to be addressed is the identity of the supernumerary interneurons that form in zebrafish in the absence of Islet1. The pMN domain generates at least four types of interneurons: VeLD, KA′, KA″ and CiD (Park et al., 2004). Unfortunately, we currently have few markers other than cell morphology to distinguish these cells (Lewis and Eisen, 2003). Using GABA as a marker for VeLD, KA′ and KA″ interneurons, we found that the number of cells in the V-K position increased in *islet1* MO-injected embryos, whereas the number of cells in the KA″ position was unchanged. Interestingly, the number of GABA-positive, supernumerary interneurons in *islet1* MO-injected embryos was about half the number of PMNs that were lost, raising the possibility that some PMNs were only partially transformed into interneurons, and changed their axon trajectory without expressing GABA. Alternatively, there might be an increase in another type of pMN domain-derived interneuron that was not detectable with our markers, or there could have been an increase in several types of interneurons, only some of which express GABA. We are unable to distinguish among these possibilities with the available interneuron markers. Thus, it is crucial to identify cell-type specific markers for pMN domain derivatives to further assess the fates of these cells under different conditions.

Islet1 and Islet2 are functionally redundant

Previous studies have suggested that Islet1 and Islet2 may have redundant functions during motoneuron formation; however, this has not previously been tested. Thaler and colleagues (Thaler et al., 2004) proposed that the level of Islet protein, not the specific type of Islet protein, determines whether a cell becomes a visceral motoneuron. We have tested directly whether Islet1 and Islet2 have redundant functions by co-injecting embryos with *islet1* MO and *islet2* RNA to learn whether Islet2 can substitute for Islet1 during motoneuron formation in zebrafish. We found that Islet2, like Islet1, could promote motoneuron formation, consistent with the hypothesis that the differences between Islet1 and Islet2 proteins are unimportant for motoneuron formation.

CaP and MiP subtype specification is independent of the differences between the Islet1 and Islet2 proteins

What is most surprising is that our results provide evidence that, despite the exquisite and dynamic regulation of expression of *islet1* and *islet2* in zebrafish PMNs (Appel et al., 1995; Inoue et al., 1994; Korzh et al., 1993; Tokumoto et al., 1995), the differences between these proteins are not important in establishing the differences between the different PMN subtypes. Islet2 is expressed only in CaPs, yet our data suggest that either Islet1 or Islet2 is sufficient for specification of CaP subtype identity. Previous studies from our laboratory suggested that Islet2 expression might force PMNs to develop as CaPs, because in some mutants, PMNs expressing both Islet1 and Islet2 formed CaP axon projections and not MiP axon projections (Lewis and Eisen, 2001). Thus, it was surprising that misexpression of *islet2* RNA did not prevent formation of MiP dorsal projections, indicating that in the context of wild-type embryos, Islet2 is insufficient to inhibit MiP development. We also found that knockdown of Islet2 protein resulted in only minor defects in CaP axon outgrowth. These results contrast with a previous study showing that expression of a dominant negative Islet2 LIM domain caused severe defects in CaP projections and in some cases caused CaPs to develop into interneurons (Segawa et al., 2001). However, the same study found that Islet2 knockdown using MOs resulted in a much less severe effect on CaPs that appears to be very similar to what we have described. One possible way to reconcile these results is to imagine that the dominant-negative Islet2 LIM domain interfered with some, but not all Islet1 functions, consistent with the finding of Thaler and colleagues (Thaler et al., 2002) that LIM domains of different LIM-HD proteins can have overlapping and non-overlapping functions. If this were the case, it could significantly lower the efficacy of both Islet2 and Islet1 proteins, resulting in insufficient Islet function to repress interneuron formation. This would then be similar to the result we got from knocking down Islet1 alone, and fits well with the model that the overall levels of Islet protein are important in motoneuron formation (Thaler et al., 2004). Together, these results lead to the surprising conclusion that Islet2 is not required for CaP subtype identity, despite its specific expression in CaP motoneurons.

Islet1 expression is maintained in MiPs but not in CaPs; therefore, we hypothesized that this late expression of Islet1 is required for MiP subtype identity. However, when we substituted Islet2 for Islet1 in embryos co-injected with *islet2* RNA and *islet1* MOs, MiPs formed normal, dorsally projecting axons. These results do not support our original hypothesis, but instead indicate that Islet1 protein is not required for MiP subtype specification if another Islet protein is available.

There have been previous reports that highly related proteins can substitute for one another, despite their distinct expression patterns (Geng et al., 1999; Hanks et al., 1995; Hirth et al., 2001; Wang and Jaenisch, 1997; Wang et al., 1996). Sequence analysis of zebrafish Islet1 and Islet2 proteins indicate they are highly related [98% identity in the DNA-binding homeodomain and 92% or 70% identity in the first and second LIM domains, respectively; Tokumoto et al. (Tokumoto et al., 1995)]. Our data show that Islet1 and Islet2 are also able to substitute for one another functionally during motoneuron formation, suggesting that the regulation of *islet1* and *islet2* transcript expression, rather than the distinct sequences of the proteins they encode, establishes their specific functions. Therefore, transcription factors expressed very early in motoneuron development are likely determinants of PMN subtype identity. *islet1* is the earliest reported gene expressed in PMNs following expression of so-called patterning genes, such as *olig2* (Park et al., 2002) and *nkx6.1* (Cheesman et al., 2004), that are expressed in pMN domain progenitor cells as well as in post-mitotic PMNs (Cheesman et al., 2004; Park et al., 2002). Thus, it will be important to determine whether any of the known patterning genes, or patterning genes yet to be discovered, plays a role in PMN subtype specification by regulating *islet* expression.

Islet proteins function in motoneuron development in other taxa

Islet1 appears to be expressed in all vertebrate motoneurons and in every instance in which it has been examined, it seems to be necessary for their formation. By contrast, the single *islet* gene of the fruit fly, *Drosophila melanogaster*, is expressed only in a subset of motoneurons that project their axons ventrally (Thor and Thomas, 1997) and thus cannot be required to confer 'motoneuron-ness' (Thor and Thomas, 2002). Similar to zebrafish, in the absence of Islet function Islet-expressing fruit fly motoneurons are present, but their axonal projections are aberrant. In most cases, the cells still send axons into the periphery, but they fail to make appropriate neuromuscular connections. Consistent with this, overexpression of

Islet also causes some motoneurons to project to inappropriate muscles. Interestingly, however, two Islet-positive fruit fly motoneurons apparently do not project axons into the periphery in the absence of Islet function, but instead project axons within the CNS, in essence acting as though they have become interneurons, similar to what we have reported for zebrafish PMNs in the absence of Islet1. No *islet* homolog has been reported in the nematode worm, *Caenorhabditis elegans*. However, three related LIM-HD genes, *lin-11* (Hobert and Ruvkun, 1998), *lim-6* (Hobert et al., 1999) and *lim-4* (Tsalik et al., 2003), function in aspects of development of specific *C. elegans* motoneurons: axon pathfinding in the case of *lin-11*, neurotransmitter receptor expression in the case of *lim-4* and axon pathfinding and neurotransmitter synthesis in the case of *lim-6*. Thus far, mouse is the only species in which a LIM-HD protein, in this case Islet1, appears required for motoneuron survival (Pfaff et al., 1996). Other LIM-HD proteins are expressed in tetrapod vertebrate motoneurons (Sharma et al., 1998; Tsuchida et al., 1994), but like the LIM-HD proteins of flies, worms and zebrafish, these all seem to function in later aspects of motoneuron development, especially axon pathfinding and neurotransmitter choice. Thus, it will be important to study motoneuron development and LIM-HD protein function in other species to fully understand how Islet1 function has changed over time.

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Molecular analysis of neural crest formation

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Abstract

Neural crest cells arise within the ectoderm during neurulation and give rise to most of the peripheral nervous system. Following neural tube closure, they come to lie within the dorsal neural tube from which they emerge and subsequently migrate extensively to numerous and characteristic sites. There, they differentiate into neurons and glia of the peripheral nervous system, cartilage and bone of the face, melanocytes and various other cell types. Fate mapping experiments have demonstrated that the neural crest arises at the juncture between presumptive epidermis and neural plate. However, injection of lineage tracer into individual cells reveals that single neural fold cells are not committed to a neural crest fate; rather these cells can form all ectodermal derivatives (epidermis, neural tube, neural crest). Inductive interactions between the neural and non-neural ectoderm can generate neural crest cells, suggesting that signals travel through the epidermis to generate neural crest cells prior to neural tube closure. Induction of the neural crest appears to be a multiphasic process and involves a combination of an early Wnt signal together with later functions of BMP signaling pathways. We have used a variety of molecular screens to isolate molecular constituents involved in neural crest formation. We have identified a secreted factor, Noelin-1, which is expressed in the prospective avian neural plate and may play a role making the neural tube competent to form neural crest. Noelin-1 mRNA is expressed in a graded pattern in the closing neural tube, with highest expression in the neural folds and no detectable expression at the ventral midline. Its expression precedes that of Slug, a zinc finger transcription factor that represents the earliest known neural crest marker gene. Over-expression of Noelin-1 using recombinant retroviruses causes an excess of neural crest emigration and prolongs the time that the neural tube is competent to generate and regenerate neural crest cells. These results support an important role for Noelin-1 in rendering the neural tube competent to respond to inductive cues to generate neural crest.

Keywords: Neural Crest; BMP; Wnt; Neural development; Neural plate; Ectoderm

1. Introduction

The vertebrate nervous system primarily arises from the neural plate, with the remaining contribution coming from the sensory epidermal placodes. During neurulation, the neural plate thickens and invaginates to form the neural tube. Presumptive neural crest cells lie at the border region between the neural plate and non-neural ectoderm, coming to occupy the dorsal portion of the neuroepithelium after neural tube closure (Fig. 1). At the time of neurulation, a divergence has been thought to occur between the central and peripheral nervous systems. While the former arises from the neural tube proper, the latter derives from neural crest cells at the junction of the neural plate and the lateral ectoderm.

Following neural tube closure, neural crest cells lie within the dorsal portion of the neural tube and begin to emigrate shortly thereafter (Fig. 1). Initiation of neural crest cell migration proceeds in a head-to-tailward (rostrocaudal) wave. After emigration, these cells move in a highly patterned fashion through neighboring tissues. They localize in diverse but characteristic sites within the embryo and give rise to many diverse derivatives, including sensory and sympathetic ganglia, melanocytes and cartilage of the face.

2. Cell lineage analysis of neural tube and neural folds

Shortly after neural tube closure, all neuroepithelial cells appear morphologically similar. Those destined to form neural crest cells reside within the dorsal portion of the neural tube. When individual precursors within the dorsal neural tube or migrating along neural crest pathways are labeled with a lineage tracer, they

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contribute to form multiple neural crest derivatives as diverse as sensory and sympathetic ganglion cells [3,4,6]. In addition to neural crest derivatives, they also give rise to progeny in the dorsal neural tube such as roof plate cells and commissural neurons (Fig. 2).

The finding that both neural crest and neural tube derivatives often arise from a single precursor in the dorsal neural tube suggests that the precursor cells can contribute to both central and peripheral nervous system derivatives. Thus, neural crest cells are not a segregated population within the neural tube. Rather, there appears to be a common lineage for neural crest and neural tube cells, when lineage analyses are performed shortly after neural tube closure.

Similar types of lineage analysis carried out at an early stage—in the neural folds prior to neural tube closure—demonstrate that neural folds cells can give rise not only to neural crest and neural tube cells but also to cells of the non-neural ectoderm [10]. This suggests that cells in the neural crest forming zone retain the capacity to give rise to other ectodermal derivatives (i.e. epidermis and neural tube). This population is likely to represent a stem cell population with the ability to form multiple ectodermal derivatives.

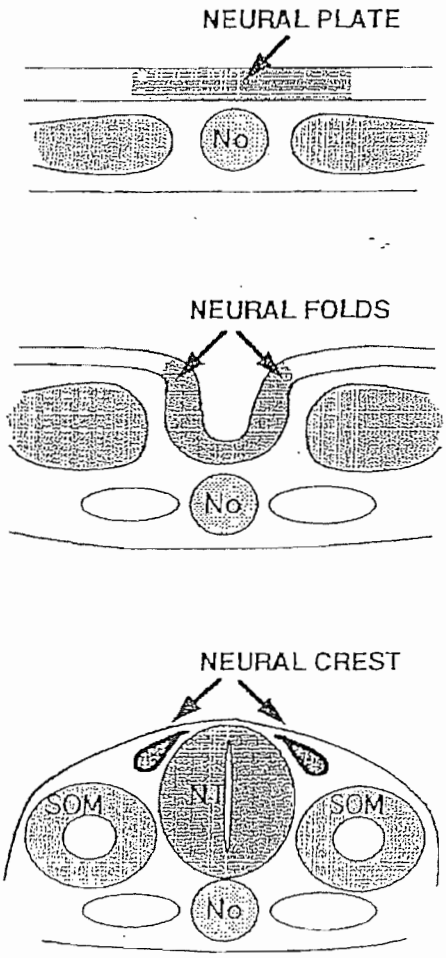

Fig. 1. Schematic diagram illustrating the process of neurulation in the trunk region during which neural crest cells form. First, the neural plate thickens in the midline above the notochord. Second, the neural folds containing premigratory neural crest cells elevate and begin to close. Third, the neural tube closes containing premigratory neural crest cells in the dorsal portion. Finally, neural crest cells emigrate from the neural tube and begin their migration along characteristic pathways through the somites. NT, neural tube; No, notochord, Som, somite.

3. Induction of the neural crest

Throughout neurulation, prospective neural crest cells are located at the boundary between neural plate/neural tube and epidermis. Therefore, one possibility is that interactions between these two cell populations are responsible for the formation of neural crest. Indeed,

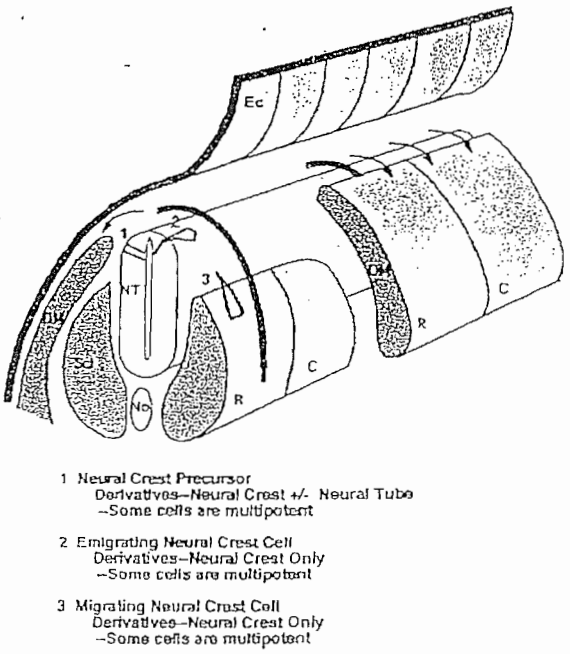

Fig. 2. Schematic diagram of the trunk region showing the results of cell lineage experiments performed on premigratory, emigrating and migrating neural crest cells. Individual cells were labeled with lineage tracers and their subsequent derivatives were examined. For cells labeled within the dorsal neural tube (1), progeny were found in multiple neural crest and neural tube lineages. For emigrating cells labeled as they were leaving the neural tube (2), progeny were found in multiple neural crest lineages. Similarly, when migrating neural crest cells were labeled (3), some progeny were found in multiple neural crest derivatives. (Data from [3,4,6]).

experiments in amphibian embryos [9] indicate that neural crest cells are generated wherever these tissues approximate. To test whether neural plate-epidermal interactions lead to the formation of neural crest cells in the avian embryo, we have performed experiments in which prospective neural plates from definitive streak stage chick embryos (stage 4) were grafted adjacent to prospective epidermis or the two tissues were juxtaposed in a collagen gel explant system grown in serum-free medium [4,5,10]. We found that neural crest cells were produced by this juxtaposition. By using cell marking techniques, we found that, in addition to the neural plate, presumptive epidermal cells generate neural crest if they are "induced" by the neural plate. This result shows that there are bidirectional signals from epidermis to neural plate and vice versa.

4. Role of BMP signaling in neural crest formation

Bone morphogenetic proteins (BMP) are members of the TGF-β family of secreted signaling molecules. BMP4 and BMP7 have been shown to be sufficient to substitute for the non-neural ectoderm in inducing neural crest cells [8], making BMPs a good candidate for the inducer within the ectoderm. An important question is whether BMPs are necessary for neural crest formation. Careful analysis of the expression patterns of BMP-4 and -7 demonstrate that they are highly dynamic. Although they are expressed initially in the ectoderm and absent from the open neural plate (Fig. 3), they subsequently are down-regulated in the ectoderm and then become highly expressed in the neural folds [11]. Interestingly, the ectoderm remains competent to

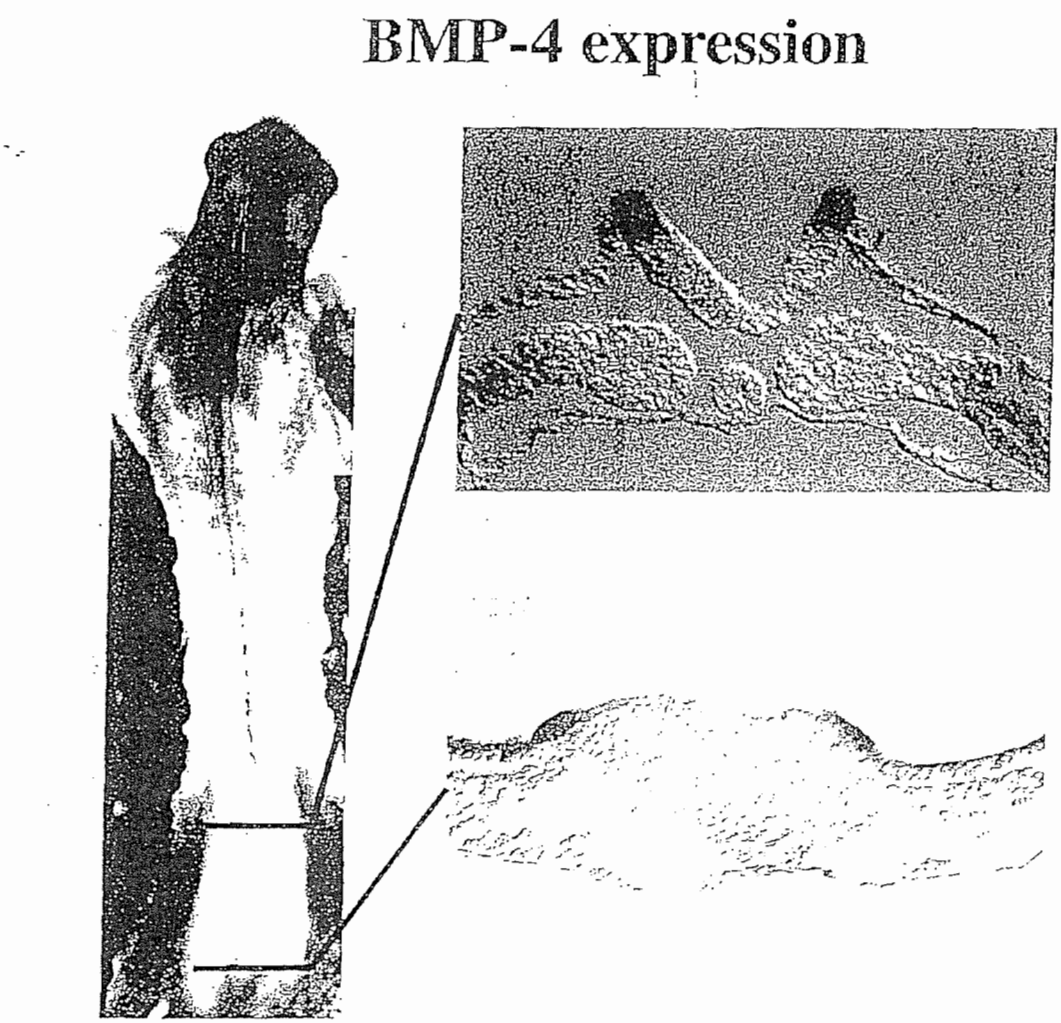

Fig. 3. BMP-4 expression in the stage 10 embryo shows that BMP is expressed in the ectoderm caudal to Hensen's node, but later is expressed in the dorsal neural folds. Whole mount in situ hybridization (left) illustrates that BMP-4 is broadly expressed in the early embryo; lines indicate positions of sections at the right.

induce neural crest cells well after BMP has been down-regulated [2]. This raises the interesting question as to the role and the timing of BMP signaling activity in neural crest formation.

To define the time of BMP function, we used the potent BMP antagonist, Noggin, as an inhibitor of BMP signaling in vivo and in vitro. Noggin-producing cells were introduced into the neural plate/ectodermal border either at open neural plate stages (Fig. 4), or into the dorsal neural tube shortly after tube closure (Fig. 5). We found that Noggin does not repress neural crest formation if injected at the open neural plate stage, but can do so if injected into the lumen of the closing neural tube. The period of Noggin-sensitivity corresponds to the time when BMPs are expressed in the dorsal neural tube but are down-regulated in the non-neural

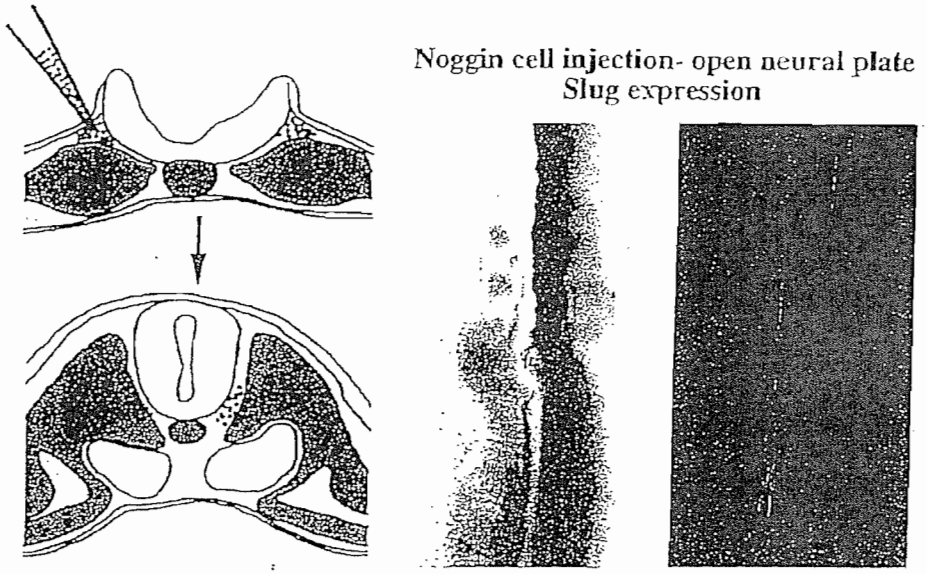

Fig. 4. The BMP inhibitor, Noggin, fails to inhibit neural crest formation when introduced into embryos prior to neural tube closure. On the left, a schematic diagram illustrates that noggin expressing cells were introduced below the closing neural folds at the time of neural crest induction and later moved lateral to the neural tube. The middle panel is a whole mount showing that Slug is expressed uniformly in the dorsal neural tube despite the presence of Noggin cells (right). (Data from [11]).

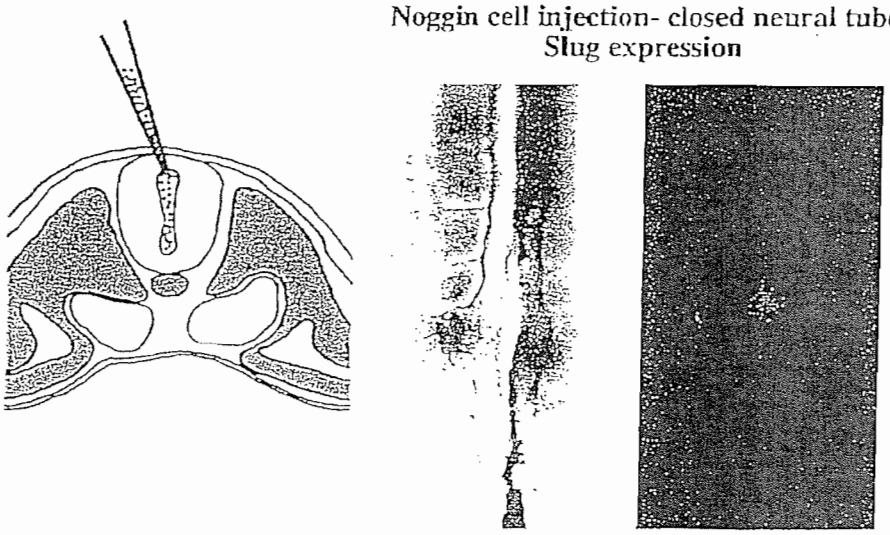

Fig. 5. Noggin inhibits Slug expression when introduced into the closed neural tube. When Noggin producing cells are microinjected into the lumen of the neural tube after neural tube closure (left), Slug expression as seen in whole mount (middle) is inhibited in the vicinity of the Noggin cells (right). (Data from [11]).

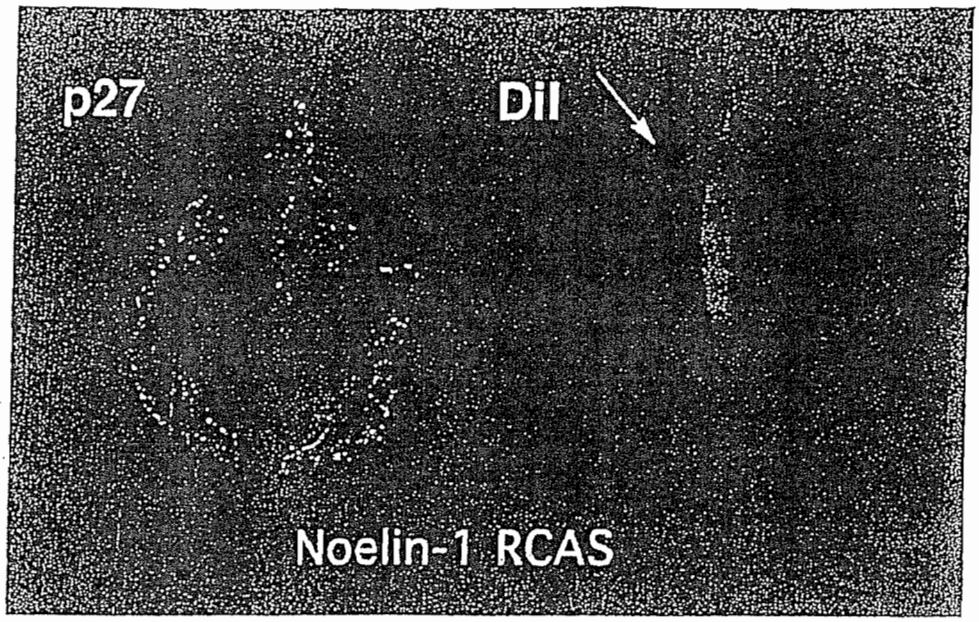

Fig. 6. Noelin-1 prolongs the time of neural crest cells emigration from the neural tube. An embryo infected with Noelin-1 RCAS retrovirus has infected cells in the neural tube and neural crest, as illustrated by the distribution of p27 stained virally infected cells (left). The neural tube of this embryo was labeled with DiI (right) well after the time that neural crest emigration has ceased in normal embryos (>20 somite stage). However, neural crest cells (arrow) continue to emigrate from the neural tube in Noelin-1 over-expressing embryos. (Data from [1]).

ectoderm. Together, our results suggest three phases of neural crest formation—an early BMP-independent phase, a later BMP-dependent phase mediated by BMPs in the dorsal neural tube and a third, BMP-insensitive, phase characterized by the presence of partially committed neural crest precursors.

These results suggest that other inducers are likely to play a role in formation of the neural crest. Experiments in Xenopus embryos suggest that neural crest induction is a two-step process involving multiple components [7]. One important molecular constituent of neural crest induction in Xenopus is a Wnt signal, which functions cooperatively with other signals (either combined with an inhibition of BMP signaling or together with Slug). Like BMPs, They activate translocation of β-catenin to the nucleus. These experiments suggest that neural crest formation is a multi-step and multifactorial process.

5. Role of Noelin-1 in rendering the neural tube competent to form neural crest

Our previous cell lineage analyses suggest that the neural crest is not a pre-segregated population of cells within the neural tube; instead, a single progenitor in the dorsal neural tube can contribute to the neural tube (presumptive central nervous system) and neural crest [4]. Furthermore, we have found that neural crest induction and formation occur in the open neural plate and are largely completed by the time of neural tube closure. Because many marker genes that are expressed in the dorsal neural tube (e.g. Dorsalin-1, Wnt-1, Pax-3) are expressed after induction of neural crest cells is well underway, it is important to identify molecules that are expressed in the neural plate at earlier times.

Induction of the neural crest requires both a source of inducer (the ectoderm) and a competent receiving tissue (the neural plate). Thus, molecules expressed in the early neural plate may be involved in rendering the tissue "competent" to respond to neural crest induction.

By screening neural crest/tube libraries, we have identified a secreted factor, Noelin-1, which is expressed in the prospective avian neural plate and may play a role in making the neural tube competent to form neural crest. Noelin-1 mRNA is expressed in a graded pattern in the closing neural tube, with highest expression in the neural folds and no detectable expression at the ventral midline. Its expression precedes that of Slug, a zinc finger transcription factor and vertebrate homologue of Drosophila Snail, that represents the earliest known neural crest marker gene. As the neural tube closes, Noelin-1 becomes restricted to the dorsal neural folds and subsequently to migrating neural crest cells and peripheral ganglia. Noelin-1 mRNA predicts a protein with a signal peptide, N-glycosylation sites and an olfactomedin-related domain; it is secreted when over-expressed in frog oocytes.

The distribution pattern of Noelin-1 is consistent with a role in bestowing competence onto the neural tube to generate neural crest cells. In the early neural plate and neural tube, its expression correlates with the time course of competence to make neural crest. Furthermore, after ablation of the dorsal cranial neural tube, Noelin-1 is re-expressed prior to regeneration of neural crest cells.

To test the functional importance of this molecule, we have conducted preliminary experiments to establish a biological function of Noelin-1 [1]. Three lines of evidence suggest that Noelin-1 has an important function in bestowing competence to form neural crest. First, over-expression in the neural tube using recombinant retroviruses causes an excess of neural crest emigration and prolongs the time that the neural tube is competent to generate neural crest cells (Fig. 6). Second, fibroblast cells over-expressing Noelin-1 into early chick embryos elicit neural crest emigration when implanted lateral to the neural tube. Third, regeneration of the neural crest by the neural tube after neural fold ablation continues well past the critical period in the presence of excess Noelin-1.

These results support an important role for Noelin-1 in regulating the production of neural crest cells by the neural tube. Thus, Noelin-1 appears to function in rendering the neural tube competent to respond to such inductive cues to generate neural crest. This promises to be a particularly exciting factor since little is known about the biological properties underlying tissue competence.

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Cells in focus

Glial cells

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Abstract

The nervous system is built from two broad categories of cells, neurones and glial cells. The glial cells outnumber the neurones and the two cell types occupy a comparable amount of space in nervous tissue. The main glial cell types are, in the central nervous system, astrocytes and oligodendrocytes and, in the peripheral nervous system, Schwann cells, enteric glial cells and satellite cells. In the embryo, glial cells form a cellular framework that permits the development of the rest of the nervous system, and regulate neuronal survival and differentiation. The best known function of glia in the adult is the formation of myelin sheaths around axons thus allowing the fast conduction of signalling essential for nervous system function. Glia also maintain appropriate concentrations of ions and neurotransmitters in the neuronal environment. Increasing body of evidence indicates that glial cells are essential regulators of the formation, maintenance and function of synapses, the key functional unit of the nervous system.

Keywords: Nervous system; Astrocytes; Oligodendrocytes; Schwann cells

Cell facts

- Throughout the brain, spinal cord and peripheral nerves, neurones are never found except in a close association with glial cells.
- The turnover rate of mature glia is normally close to zero but most of them respond to injury by rapid proliferation.
- Glial cells come in many types and have multiple functions in the developing and mature nervous system.
- Following injury, glia are major regulators of neuronal repair and they are largely responsible for the difference in regeneration capacity between the central and peripheral nervous system.

1. Introduction: the main glial types

Two main cell types build the nervous system. These are neurones, which are directly involved in electrical transmission and information processing, and glial cells. In all parts of the nervous system, glial cells outnumber neurones by some margin, and they make up a large part of nervous tissue. For instance, glial cells occupy about half the volume of the brain. These cells carry out many indispensable functions, both in development and during the normal function of the mature system (Jessen & Richardson, 2001). They are also major players in the reaction of the nervous system to disease and trauma.

The term "glial cell" denotes in fact a broad category of cells that is made up of many sub-types.

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1.1. Glia in the central nervous system

In the central nervous system (CNS), consisting of the brain and spinal cord, the major glial types are astrocytes and oligodendrocytes. The astrocytes, which are more numerous, have many radiating processes that interweave in complex and intimate ways between neuronal cell bodies and fibres. Some astrocyte processes contact blood vessels and may control the blood-brain barrier which protects the CNS from unwanted substances in the general circulation. Others form cuffs or veils around individual synapses, and synaptic transmission can be modified by signals between nerve terminals and these glial elements (Fig. 1). They also have high affinity uptake sites for major brain neurotransmitters that help to remove excess transmitter following release from nerve terminals. Together this provides compelling evidence that gial cells are directly involved in information processing in the brain. Astrocytes also help to control the levels of potassium in the extracellular space and have major roles in CNS development.

Oligodendrocytes form one of the most highly specialised cellular structures in the body, the myelin sheath, which forms electrical insulation around nerve fibres thereby making rapid transmission of electrical signals in the brain possible. The CNS also contains microglia, resident, macrophage-like cells that originate from blood monocytes rather than the neurectoderm.

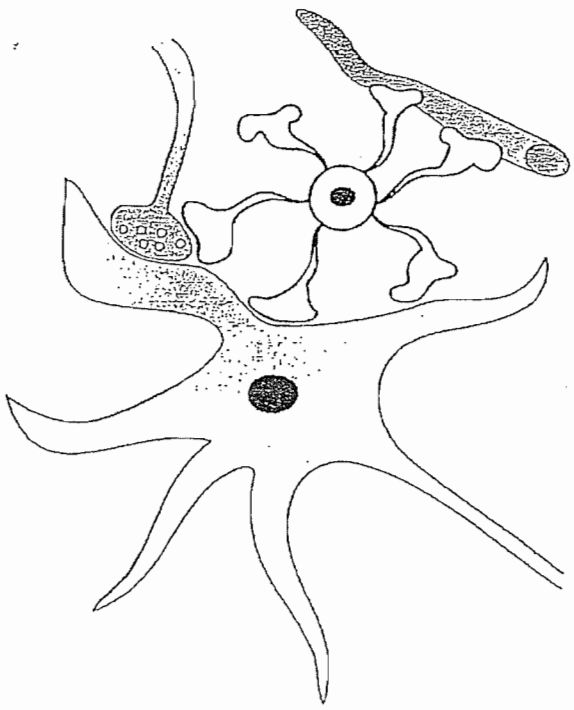

Fig. 1. The general arrangement between astrocytes, capillaries and neurones. Astrocytes (white) typically carry numerous processes many of which spread along capillaries (brown), abut on to neurones (green) or associate intimately with axon terminals at synaptic junctions (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

1.2. Glia in the peripheral nervous system

In the peripheral nervous system (PNS), the major glial cells are Schwann cells. They ensheath all axons in peripheral nerves and are found in two types, myelinating and non-myelinating. The myelinating Schwann cells form insulating sheaths around axons that are comparable in structure and function to those made by oligodendrocytes in the CNS. The non-myelinating cells show similarities with astrocytes and are likely to have metabolic and mechanical support functions. There is evidence that Schwann cells are indispensable for neuronal survival during development, and in damaged nerves Schwann cells control successful regeneration and restoration of function.

Olfactory ensheathing cells represent a special category of glia that resembles non-myelinating Schwann cells and associate with both the CNS and PNS part of the primary olfactory axons. Another important category of PNS glia is the enteric glia. They are found in the autonomic ganglia of the gut (the enteric nervous system). Unlike other parts of the PNS, the enteric system has complex synaptic interactions and high integrative capacity, and the enteric glia are remarkably like astrocytes in structure and biochemistry. The cell bodies of other autonomic ganglia and sensory ganglia are enveloped by simpler satellite glial cells, while the synapses between nerve terminals and skeletal muscle are covered by terminal glia, also called teloglia or perisynaptic glia. They help to maintain a stability of the neuromuscular junction and regulate synaptic transmission.

2. Development

The glial cells and neurones of the CNS develop from neural precursor cells of a germinal layer called the ventricular zone, that lines the lumen of the developing spinal cord and the ventricles of the brain. Oligodendrocyte development is better understood in the spinal cord than in the brain. In the cord, oligodendrocytes appear to originate from a tightly restricted area of the ventricular zone in a process that depends on the transcription factors Olig 1 and 2 and the signalling molecule sonic hedgehog (Nave & Trapp, 2000). From this location, oligodendrocyte progenitor cells migrate to reach all parts of the cord while progressing through defined differentiation stages and remaining in the cell cycle. They fall out

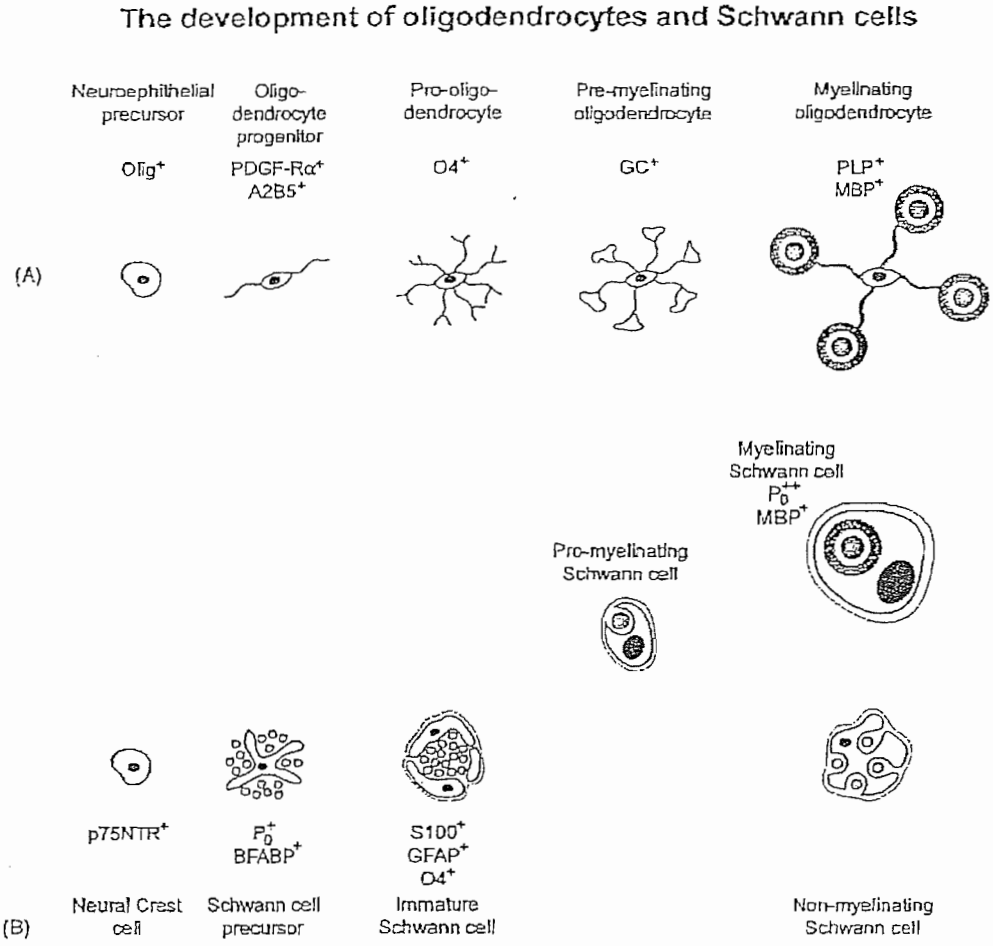

Fig. 2. The diagram shows the main stages in (A) oligodendrocyte and (B) Schwann cell developement, and some of the molecular markers that can be used to differentiate each stage from the preceding one. The lineages arise from multipotent cells in steps that are broadly comparable. Note that only the Schwann cell lineage generates two distinct cell types and includes a fate decision point, the immature Schwann cell stage. Also, fully differentiated Schwann cells retain an unusual plasticity throughout life and can readily de-differentiate to form cells similar to immature Schwann cells (open arrows). Basal lamina (gray line) is associated with Schwann cells but not with Schwann cell precursors or cells in the oligodendrocyte lineage. Blue: axons. Red: myelin. Olig: Olig1/2 transcription factors. PDGF-R alpha: platelet derived growth factor receptor alpha. GC: galactocerebroside. PLP: proteolipid protein. MBP: myelin basic protein. p75NTR: low affinity p75 neurotrophin receptor. P0: protein zero. BFABP: brain fatty acid binding protein. GFAP: glial fibrillary acidic protein.

of division after they have associated with their target axons. At this point, they start to express high levels of myelin gene products and form myelin sheaths around axons (Fig. 2A). In more anterior regions of the CNS, the origin of oligodendrocytes is less clear, although also here, ventral structures are likely to be a major source of these cells, in analogy with the cord. The adult CNS retains a population of cells, the adult oligodendrocyte precursor, that is in many ways similar to early cells in the lineage. It is likely that these cells take part in myelin repair under certain circumstances, such as in early multiple sclerosis lessons (see below).

Astrocytes are generated from two sources (Goldman, 2001). Early in development, they form from elongated precursors that have their cell body in or near the ventricular zone and a process that stretches radically to terminate at the surface of the neural tube or developing brain. These cells show many molecular and morphological features that characterise astrocytes later in development and are generally known as radial glia. Later in development, astrocytes also originate from a distinct set of germinal areas, the subventricular zone.

Surprisingly, recent work has shown that radial glia and another astrocyte-like cell, the subventricular zone astrocyte, are also active generators of neurones during development and even, in some cases, in the adult brain. These findings challenge traditional notions about the function of glia, since these astrocytes are acting like multipotent neural stem cells. This has caused much head-scratching, with some workers arguing that these neurogenic cells should no longer be classified as glia, but regarded as neural precursors that happen to have some glial-like features, while others are happy to embrace the new concept that some glia have the novel and exciting role of generating neurones in the developing and adult brain.

Schwann cells develop from the neural crest, which is a transient population of cells that migrates away from the dorsal aspect of the neural tube as it closes (Jessen & Mirsky, 2002; Mirsky & Jessen, 2001). Crest cells are multipotent cells that give rise not only to the neurones and glia of the PNS but also to pigment cells in the skin and to some smooth muscle and connective tissue cells. The generation of Schwann cells from crest cells requires the transcription factor Sox-10 and involves, first, the formation of Schwann cell precursors that, in turn, form immature Schwann cells. This population diverges, about half the cells forming myelin around larger diameter axons, while the others associate with smaller diameter axons and become non-myelinating cells (Fig. 2B). This last step of Schwann cell development remains reversible throughout life. The lineage is strikingly dependent on signals from axons. In early development, the most important of these signals is neuregulin-1. Myelination also depends on signals from axons but the identity of this key signal is not known. The transcription factor Krox-20 is indispensable for Schwann cell myelinaton, although it is not required for oligodendrocyte myelination.

3. Functions

3.1. Role in development

3.1.1. Guidance

In the developing brain, neurones are often formed at what in cellular terms is a very long way from their final site of residence. Development therefore involves a remarkable amount of neuronal migration, a process in which glial cells play a major role. This has probably been studied most thoroughly in the cerebral cortex and the cerebellum. Here, the radial glial cells mentioned before act as indispensable scaffolds for extensive neuronal migration, involving astrotactin and neuregulin-1 signalling, that establishes the layered architecture of these structures (Rakic, 2003). Glia are also implicated in directing axonal growth during development, although it can be disputed whether some of the cells that perform such early guiding functions should be classified as glial cells.

3.1.2. Survival

Some of the best evidence for the common notion that glia support neuronal survival comes from the PNS. In mice in which the transcription factor Sox-10, or neuregulin-1 signalling, have been inactivated, Schwann cell precursors and later, Schwann cells are missing. This is accompanied by the death of large numbers of motor neurones and dorsal root sensory (DRG) neurones, both of which send their axons into peripheral nerves, suggesting that these neurones depend on survival signals from developing glia, a function that is perhaps carried out via

neuregulin-1 back-signalling (Bao, Wolpowitz, Role, & Talmage, 2003; Jessen & Mirsky, 2002). Another example comes from the experimentally induced loss of enteric glia that leads to death of enteric neurones (Bush et al., 1998). A number of mutations in glial genes that initially cause glial malfunction eventually also lead to axonal abnormalities and neuronal death, again pointing to the trophic dependence of neurones on glia (see below; Berger, Young, & Suter, 2002).

3.1.3. Synapse formation

The synapse is the key functional unit in the nervous system and synapses are found in astonishing numbers between neurones and between neurones and other cells. Provocative evidence now indicates that not only the formation, but also the efficiency and maintenance, of synapses depends on signals from astrocytes (Barres & Smith, 2001). These observations, that come from cell culture studies, have important implications, not least for the mechanisms underlying the deterioration of synaptic transmission implicated in age-related memory loss and cognitive dysfunction.

3.2. Myelination

Myelin is made by oligodendrocytes in the CNS and myelinating Schwann cells in the PNS (Fig. 2). While each Schwann cell forms myelin around a single axon, an oligodendrocyte can myelinate up to 30–40 axons by carrying processes each of which ends in a myelin sheath. The sheath forms by spiralling movements of a flattened cellular process around the axon and involves several thousand fold increase in membrane area. Extrusion of the cytoplasm and compaction of the stacked membrane bilayers leads to the formation of a myelin segment which provides electrical insulation around the axon. Sodium and potassium channels are concentrated in the axonal membrane at the meeting points between adjacent myelin segments. This alternating arrangement of electrically excitable and insulated areas along the axon leads to a saltatory conduction of electrical signals that is about ten times faster than impulse conduction along an unmyelinated axon of a similar diameter. This difference has undoubtedly provided the evolutionary pressure for the emergence of myelinating cells (Colman, Pedraza, & Yoshida, 2001).

3.3. Control of synaptic function

Glial cells have long been known to possess neurotransmitter receptors (Porter & McCarthy, 1997). The important new idea now being established is that these receptors are activated during synaptic activity, leading to elevation of Ca^{2+} and release of glial glutamate that acts back to modulate synaptic transmission and neuronal excitability (Haydon, 2001; Verkhratsky, Orkand, & Kettenmann, 1998). Because Ca^{2+} elevation can be propagated between astrocytes as a Ca^{2+} wave over long distances, activation of astrocytes at one site could modulate neuronal activity at a distant location. Comparable events take place at PNS synapses (Rochon, Rousse, & Robitaille, 2001). These exciting findings suggest that the synapse is best viewed as a tripartite entity, consisting of three functional parts, the pre- and post-synaptic element and the surrounding glia.

3.4. Homeostatic regulation of neurotransmitter and potassium ion concentrations

Glial cells have molecular pumping mechanisms and intracellular enzymes that enable them to take part in removing major neurotransmitters, including glutamate and GABA, from synaptic sites and metabolise them, thereby helping to terminate postsynaptic action following transmitter secretion. During electrical activity of neurones, K^+ is transferred from neurones to the extracellular space where, if it accumulated, it could disastrously alter the electrical excitability of neurones. Reuptake into neurones and diffusion in the extracellular space are not enough to prevent K^+ buildup, and it is now clear that removal of K^+ via carrier and channel molecules in glial membranes is an essential function of astrocytes (Walz, 2000).

4. Pathology

4.1. Multiple sclerosis (MS)

This is perhaps the most widely recognised disease associated with glial cells (Lucchinetti & Lassmann, 2001). Multiple sclerosis (MS) is a progressive disease with a significant immune involvement that primarily affects oligodendrocytes. It is characterised by

the formation of multiple lesions in the CNS in which myelin is destroyed and oligodendrocytes die. Axons are also adversely affected. The causes of MS are not well understood and effective treatment remains to be developed.

4.2. Type 1 Charcot–Marie–Tooth disease (CMT)

CMT is a collection of inherited diseases all of which cause malfunction of peripheral nerves (Berger et al., 2002). The majority of these are due to mutations in some five Schwann cell genes coding for structural proteins of myelin or proteins related to cell-cell communication, the cytoskeleton or control of myelin gene transcription. While the direct effects of most of these mutations is instability and breakdown of Schwann cell myelin, clinical disability is thought to relate better to axonal damage resulting from this disturbance of normal Schwann cell function.

4.3. Tumours

The majority of malignant brain tumours is derived from glial cells or their progenitors (Nistér, Uhrbom, Hesselager, & Westermark, 2001). Most of these tumours have an astrocytic component, but a high degree of heterogeneity often makes it difficult to determine the cell of origin. It is not clear to what extent the frequency of glial tumours relates to the ongoing proliferative potential of adult glial cells, or whether the multipotent neural progenitors now known to persist in the adult CNS (above) are significant targets of malignant mutations.

In the PNS, Schwann cell tumours arise in the context of two diseases, neurofibromatosis (NF) type 1 and NF type2, caused by mutations in two genes important for Schwann cell function, neurofibromin and merlin (Schwannomin), respectively (Ratner & Daston, 2001). Neurofibromin is a GTP activating protein (GAP) for Ras proteins, while the tumour suppressor merlin links the actin cytoskeleton to transmembrane proteins.

4.4. Signalling and homeostatic functions of astrocytes

Astrocytes have the potential to secrete a variety of signalling molecules including a large number of immune modulators, metalloproteases and nitric oxide. In this way, and by their ability to remove potentially cytotoxic amino acids such as glutamate (above), astrocytes are likely to be important regulators of many pathological processes, including stroke and inflammatory conditions such as Alzheimer's disease and MS.

4.5. Injury to the nervous system

In the event of mechanical damage, such as spinal or peripheral nerve injury, glial cells act as major determinants of repair by expressing molecules that block or promote axon regrowth (Fawcett & Asher, 1999; Fu & Gordon, 1997; Houle & Tessler, 2003). In the PNS, axons have a good chance of regrowing following nerve cut or crush and many of them may reach correct targets leading to restoration of function. This is largely due to the remarkable response of Schwann cells in the distal part of injured nerves. They re-enter the cell cycle, lose their myelin sheaths and de-differentiate to adopt the phenotype of immature Schwann cells, which, due to expression of trophic factors and adhesion molecules, provide a particularly favourable environment for axonal re-growth. The situation is quite different in the CNS. There, injury prompts astrocytes to hypertrophy and reorganise to form the glial scar, that forms a barrier to regeneration, and both astrocytes and oligodendrocytes express factors that potentially block the re-growth of axons. These molecules include Nogo-A, myelin associated glycoprotein, oligodendrocyte-myelin glycoprotein, tenascin and factors associated with chodroitin heparan sulphate. As a result, the prognosis for spinal cord injury, for instance, is poor, although novel treatments, including the neutralisation of inhibitory molecules and insertion of PNS glia or other agents that promote regeneration, are starting to yield promising results.

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CHAPTER

11

Oligodendroglial Lineage

Robert Miller and Richard Reynolds

CHARACTERIZATION OF OLIGODENDROCYTE DEVELOPMENT *IN VITRO*

Introduction

The oligodendrocyte lineage represents one of the most intensely studied and best understood cell lineages in the vertebrate CNS. In part, this reflects the fact that the cells of the oligodendrocyte lineage can be grown extensively in culture, and their biochemical, morphological and molecular characteristics have been more accessible to identification. As this information has been forthcoming it has been possible to go back into the more complex cellular environment of the intact CNS and validate the data obtained in simpler systems. Remarkably, the vast majority of major advances in understanding revealed by culture studies have proven to illuminate the development process or injury responses in the intact CNS.

As with the other major classes of CNS neural cells the oligodendrocyte lineage is derived from cells of the neural tube. Presumably early in development, all neural tube cells have the capacity to generate oligodendrocytes, although during the course of normal embryogenesis only some cells manifest that potential. It seems likely these early neural tube cells have many characteristics of stem cells. That is, they undergo asymmetric divisions generating an identical cell and a sibling that is more restricted in either differentiative or proliferative potential. Currently there are no prospective isolation approaches for such cells in the CNS, only retrospective identification of the progeny, although recent studies have begun to facilitate enrichment of putative neural stem cells in the adult CNS. As a result, this discussion is restricted only to those cells where the steps to a differentiated oligodendrocyte are reasonably well understood.

During normal development there is a unidirectional progression of cells from immature to more mature states, and in the oligodendrocyte lineage this occurs in a series of overlapping phases that are demarcated by distinct morphological, biochemical, and behavioral characteristics. In general, younger animals contain more immature oligodendrocyte lineage cells than do older animals, although, as in most other lineages, individual cells within an organism, and even within a particular region of the CNS, mature and differentiate at different rates. Indeed, the genesis of differentiated oligodendrocytes is extremely protracted and can be ongoing in the adult CNS. It is therefore critical to define maturational characteristics of individual cells rather than the overall maturation characteristics of the source animal.

Biochemical and Morphological Characteristics of Oligodendrocyte Lineage Cells *in Vitro*

The capacity to identify biochemical characteristics on the surface or in the cytoplasm of individual cells has proven enormously important in allowing rational cellular classification. Pioneered in the hematopoetic system, the use of cell markers continues to be a cornerstone of cellular neuroscience. The usefulness of any marker depends on it being used in the appropriate context. In general, the rat CNS has the most verified markers, although this is likely to change with our increasing understanding and identification of transcription factors and their targets. Much of the earliest work on the development of the oligodendrocyte lineage focused on the optic nerve, a region selected due to its relatively simple cytoarchitecture and lack of neuronal cell bodies (Raff *et al.*, 1987). For example, cell cultures derived from rodent optic nerve contain exclusively glial cells with small contaminants from the meningies and blood vessels (Raff *et al.*, 1987). The earliest well characterized cells of the oligodendrocyte lineage in the rodent optic nerve express cell surface antigens recognized by the monoclonal antibody A2B5 (Raff *et al.*, 1984). These cells have a characteristic morphology in that they are either bipolar or unipolar with a large cell body and one or two major processes (Fig. 11.1). Several other antigenic characteristics have been ascribed to early oligodendrocyte precursors, including the expression of the NG2 antigen (Nishiyama *et al.*, 1996), the embryonic form of polysialic acid containing neural cell adhesion molecule (E-NCAM or PSA-NCAM), and antigens recognized by a number of different monoclonal antibodies including GD3. A major growth factor for oligodendrocyte precursors is platelet-derived growth factor (PDGF) (Noble *et al.*, 1988; Richardson *et al.*, 1988), and expression of the alpha receptor for PDGF (PDGFαR) is a further characteristic of oligodendrocyte precursors (Pringle *et al.*, 1992). Cells with similar antigenic phenotypes have been identified in other regions of the CNS, including spinal cord, hindbrain, and forebrain, and precursors of the oligodendrocyte lineage clearly reside among this population. Unlike the optic nerve, however, in these more complex regions of the CNS, no single reagent allows for the unambiguous identification of early oligodendrocyte precursors.

Oligodendrocyte precursors undergo a number of structural and biochemical changes as they mature (Pfeiffer *et al.*, 1993). Structurally, the cells begin to develop a more complex morphology and frequently develop multiple processes (Fig. 11.1B), although they retain a relatively large cell body. Biochemically, maturing oligodendrocyte precursors begin to express antigens on their surface that bind the monoclonal antibody O4 (Bansal and Pfeiffer, 1992; Bansal *et al.*, 1992; Sommer and Schachner, 1981;). Among these antigens is the POA antigen (Bansal and Pfeiffer, 1992) and galactosulfatide that subsequently becomes a structural component of myelin (Sommer and Schachner, 1981). Cells at this particular stage of development have been termed pro-oligodendrocytes denoting their progression toward differentiated oligodendrocytes (Pfeiffer *et al.*, 1993). Pro-oligodendrocytes also express a number of other characteristic antigens, including early myelin proteins (Fig. 11.2). The differentiation of oligodendrocyte precursors into oligodendrocytes is associated with the loss of expression of precursor antigens such as those recognized by mAbA2B5 (Raff *et al.*, 1983, 1984) and the gain of expression of oligodendrocyte antigens such as galactocerebroside (Raff *et al.*, 1978), a major glycolipid of myelin. Structurally, the morphology of oligodendrocytes becomes far more complex, with multiple cellular processes and a smaller phase dark cell body (Fig. 11.1). Under certain conditions, cultured oligodendrocytes will begin to express broad membranous sheets that have been suggested to represent the early stages of myelin formation. Continued maturation of oligodendrocytes results in elevated expression of the major myelin proteins such as myelin basic protein (MBP) and proteolipid protein (PLP) (Campagnoni, 1995; Lemke 1993).

Potential, Proliferation, and Migration of Oligodendrocyte Lineage Cells

A remarkable feature of immature A2B5+ oligodendrocyte precursors is that they have the potential to generate more than one type of cell (Kondo and Raff, 2000; Raff *et al.*, 1983).

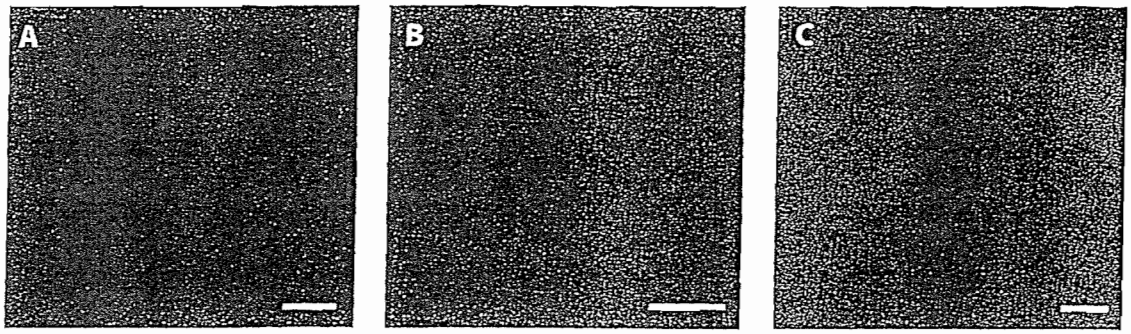

FIGURE 11.1
Examples of rodent spinal cord cells at different stages of development of the oligodendrocyte lineage *in vitro*. (A) Immature oligodendrocyte precursors labeled with mAb A2B5. These immature cells with a bipolar morphology are highly migratory and proliferate in response to their major mitogen PDGF. During development, A2B5+ cells differentiate constitutively into oligodendrocytes and can be induced to differentiate into type 2 astrocytes under the influence of serum or CNTF stimulation, and even into neurons in response to the correct stimulation. (B) A pro-olgiodendrocyte labeled with mAb O4. Compared to immature oligodendrocyte precursors, O4+ cells have a larger cell body and a more process-bearing morphology. The O4+ cells are less proliferative, responding primarily to FGF rather than PDGF. Furthermore, 04+ cells are considerably less migratory than A2B5+ cells, although they actively extend processes *in vitro*. (C) A newly differentiated oligodendrocyte labeled with mAb 01, which recognizes galactocerebroside as well as other myelin. Compared to oligodendrocyte precursors, differentiated oligodendrocytes have a more complex morphology with multiple cellular processes. Differentiated oligodendrocytes seldom divide *in vitro*, or under normal conditions *in vivo*, and are not migratory. As these cells mature *in vitro*, they extend wide processes or sheets of membrane that may correspond to uncompacted myelin sheaths *in vivo*. Bar = XXmm?? in all figures.

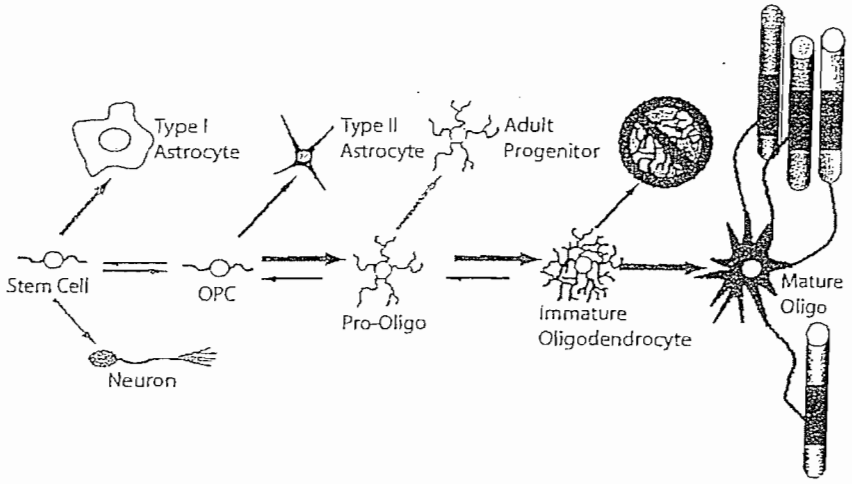

FIGURE 11.2
Schematic representation of the major cellular stages in the development of myelinating oligodendrocytes *in vitro*. Like other lineages of the CNS, oligodendrocyte precursors (OPC) arise from stem cells that can develop into either neurons or type –1 astrocytes, depending on environmental signals. Oligodendrocyte precursors are bipotential *in vitro*, giving rise to type 11 astrocytes or pro-oligodendrocytes. *In vivo*, these cells mature to give rise to pro-oligodendrocytes or adult progenitor cells. During development, pro-oligodendrocytes differentiate into immature oligodendrocytes that either undergo cell death due to a lack of survival factor or mature into myelinating cells that myelinate several axonal segments depending on axonal diameter. The major molecule regulators of each stage in the development of oligodendrocytes is discussed in detail in the text.

While they constitutively differentiate into oligodendrocytes, under the influence of different environmental signals these cells can give rise to the major classes of neural cells (Kondo and Raff, 2000). The first evidence of the multipotential nature of the A2B5+ cells from the developing optic nerve was the finding that under the influence of serum these cells gave rise to a distinct population of astrocytes termed type 2 astrocytes (Raff *et al.*, 1984). This bipotential nature led to these cells being termed oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells (Raff *et al.*, 1984). Type 2 astrocytes can be distinguished from other type 1 astrocytes, which express intermediate filaments composed of glial

fibrillary acidic protein (GFAP) based on the process-bearing morphology and different antigenic characteristics, including the short-term expression of mAb A2B5 binding (Raff *et al.*, 1983). Subsequent studies have identified some environmental factors that can induce O2A progenitor cells to differentiate into type 2 astrocytes. Such factors include serum and more specifically ciliary neurotrophic factor (CNTF) (Hughes *et al.*, 1988; Lillien *et al.*, 1990). More recently it has become clear that O-2A progenitors can be induced to give rise not only to type 2 astrocytes but also to type 1 astrocytes and neurons, suggesting these cells have stem cell like properties (Kondo and Raff, 2000). Indeed, in regions of the CNS such as the spinal cord, mAb A2B5 labeling can be used to isolate cells with stem cell like properties (Rao *et al.*, 1998; Rao and Mayer-Proschel, 1997). Since, however, it is unclear if A2B5+ cells manifest any potential differentiative diversity apart from the generation of oligodendrocyte *in vivo* (discussed later), they will be termed oligodendrocyte precursors in this chapter. Other markers identify bipotential oligodendrocyte precursors, including expression of NG2 (Levine and Card, 1998; Nishiyama *et al.*, 1996). It is currently unclear whether A2B5+ and NG2 cells represent the same stage in the oligodendrocyte lineage or whether one is an earlier cell in the lineage. Circumstantial evidence suggests that NG2+ cells may generate A2B5+ cells and thus represent an earlier stage in the oligodendrocyte lineage.

During development, the capacity of oligodendrocyte precursors to generate type 2 astrocytes is transient and is lost as the cells begin to express POA and other antigens recognized by mAbO4. While O4+ cells appear to be able to generate type 2 astrocytes under certain conditions, they may have to revert to a more immature cell phenotype to do so. The capacity to revert to more immature cells as well as the capacity to generate type 2 astrocytes is lost when oligodendrocyte precursors differentiate into oligodendrocytes.

The growth factors that promote oligodendrocyte precursor proliferation are different depending on the developmental stage of the cells (Fok-Seang and Miller, 1994; Pfeiffer *et al.*, 1993). In general, more immature oligodendrocyte precursors have a greater proliferative capacity than do more mature oligodendrocyte precursors. The major mitogen for immature A2B5+ oligodendrocyte precursors is platelet-derived growth factor (PDGF) (Noble *et al.*, 1988; Richardson *et al.*, 1988). This mitogenic response is mediated through the PDGF alpha receptor (PDGFαR) (Pringle *et al.*, 1992), the expression of which overlaps with that of A2B5 immunoreactivity and therefore characterizes oligodendrocyte precursors in specific regions of the developing CNS. Immature oligodendrocyte precursors also respond to fibroblast growth factor (FGF), although to a somewhat lesser extent (Fok-Seang and Miller, 1994), while a combination of PDGF and FGF promotes extended proliferation and inhibits differentiation (Bogler *et al.*, 1990; Gard and Pfeiffer, 1993; Mayer *et al.*, 1993; McKinnon *et al.*, 1990). Several other cytokines and growth factors influence the proliferation of immature oligodendrocyte precursors. These include the neurotrophin NT3 (Barres *et al.*, 1994), insulin-like growth factor and the chemokine CXCL1 (Robinson *et al.*, 1998). While not a mitogen in its own right, CXCL1 enhances the proliferative response of immature oligodendrocyte precursors to PDGF (Robinson *et al.*, 1998; Wu *et al.*, 2000), and this synergy is mediated through the chemokine receptor CXCR2 (Robinson *et al.*, 1998; Tsai *et al.*, 2000). In contrast to A2B5+ cells, O4+ prooligodendroblasts are largely refractory to PDGF but proliferate largely in response to bFGF (Fok-Seang and Miller, 1994). The response to bFGF is mediated through different receptors depending on the maturity of the precursor cells (Bansal *et al.*, 1996). Exposure of O4+ cells to FGF not only promotes proliferation but also modifies cell fate. Initial studies suggested that treatment with FGF induced a reversion of the O4+ cells to a more immature phenotype (Grinspan *et al.*, 1993); however, subsequent analyses indicates that rather than a reversion, FGF exposure induces a novel cell phenotype (Bansal and Pfeiffer, 1997), whereby cells may become more susceptible to differentiation inducing signals. Upon differentiation into GC+ oligodendrocytes, cells largely drop out of the cell cycle and in the majority of cases become post-mitotic (Pfeiffer *et al.*, 1993).

Not only do growth factors promote the proliferation of oligodendrocyte precursors, but inhibitory signals suppress the proliferation of oligodendrocyte precursors. For example, soluble signals such as TGFβ (Louis *et al.*, 1992; McKinnon *et al.*, 1993) tend

inhibit the proliferation of oligodendrocyte precursors and can counteract the prolifera- e stimulation of growth factors. More localized signals can also inhibit the proliferation oligodendrocyte precursors in response to growth factors (Nakatsujji and Miller, 2001; .ang and Miller, 1996). Like many other cell types, oligodendrocyte precursors demon- ate contact dependent inhibition of proliferation (Wieser *et al.*, 1990). This phenomenon ell type specific and can significantly truncate the number of cell divisions a precursor l undergoes (Nakatsujji and Miller 2001). The ligand/receptor complexes mediating ntact inhibition of proliferation are as yet uncharacterized; however, the cell cycle achinery utilized to inhibit cell proliferation is better understood and involves coordinate wn-regulation of specific cyclins and an up-regulation of the cell cycle inhibitor p27 lengst and Reed 1996; Kato *et al.*, 1997; Nakatsujji and Miller 2001). Similar molecular echanisms are utilized during the cessation of cell proliferation that accompanies the nsition from precursor cells to differentiated oligodendrocytes (discussed later).

Oligodendrocyte precursors are highly migratory. In cultures of developing optic nerve, e most immature cells are the most migratory (Noble *et al.*, 1988). For example, bipolar 2B5+ cells are extremely motile and are some of the most motile cells in the body (Noble *al.*, 1988). By contrast, O4+ cells are far less migratory and differentiated oligodendro- tes are largely non-motile (Warrington *et al.*, 1993). The migration of immature oligo- ndrocyte precursors is influenced by a variety of environmental cues. Type 1 astrocytes imulate both the proliferation and migration of A2B5+ cells (Noble *et al.*, 1988). This tivity probably reflects the synthesis and secretion of PDGF by these cells (Noble *et al.*, 988). Consistent with this hypothesis, PDGF promotes both proliferation and migration f oligodendrocyte precursors (Noble *et al.*, 1988). Not only does PDGF promote oligo- endrocyte precursor migration, but it may also act as a chemoattraction factor (Arm- rong *et al.*, 1990, 1991; Frost *et al.*, 2000). For example, in Boyden chamber studies ligodendrocyte precursors migrated toward higher concentrations of PDGF (Armstrong *t al.*, 1990). Likewise, bFGF may also act as a chemoattractant for oligodendrocyte recursors (Armstrong *et al.*, 1990). Other guidance molecules such as the semaphorins nd netrins (Sugimoto *et al.*, 2001) also influence oligodendrocyte migration while the hemokine CXCL1 acts as a stop signal for migratory oligodendrocyte precursors (Tsai *et l.*, 2000). The issues of migrational control of oligodendrocyte precursors are discussed in nore detail in a later chapter.

Differentiation and Survival of Oligodendrocyte Precursors: Control of Cell Number

Oligodendrocyte precursors differentiate into immature oligodendrocytes before they gen- erate myelin (Figs. 11.1 and 11.2). This is a critical transition in the lineage and has many important cellular consequences. For example, as cells exit the cell cycle they become acutely dependent on survival factors (Barres *et al.*, 1992, 1993). The mechanisms mediat- ing the transition from precursor to differentiated cell are still not clearly understood. Both cell intrinsic and extrinsic signals appear to regulate this critical transition. *In vitro*, clonally related oligodendrocyte precursors derived from the rat optic nerve differentiate at ap- proximately the same time and after similar numbers of divisions (Temple and Raff, 1985, 1986), suggesting differentiation is in part regulated through an intrinsic clock that meas- ures the number of cell divisions or elapsed time (Raff *et al.*, 1985; Raff and Lillien, 1988). The nature of the clock is unclear but appears to be comprised of several components including the cell cycle regulator p27 (Casaccia-Bonnefil *et al.*, 1997). Extrinsic signals also influence oligodendrocyte differentiation (Bogler *et al.*, 1990; McKinnon *et al.*, 1990). Axonal signals promote the differentiation of oligodendrocyte precursors (Payne and Lemmon, 1993), and considerable circumstantial evidence suggests that axons provide a signal that initiates the process of myelination (Trapp 1990). One potential candidate for an oligodendrocyte differentiation signal is Neuregulin 1 (Marchionni *et al.*, 1993). Neuregulin is expressed by many myelinating axons and induces process outgrowth in oligodendrocytes (Vartanian *et al.*, 1994). Neuregulins signal by binding to the ErbB receptors (Lemke, 1996). In the absence of ErbB2 signaling *in vitro* oligodendrocyte precursors fail to differentiate and

remain as precursors (Park *et al.*, 2001). In the intact CNS, treatment with high doses of Neuregulin induces a demyelination, possibly as a result of driving a de-differentiation of precursors. Axonal signals may not only promote oligodendrocyte differentiation, they may also inhibit differentiation. For example, many axons express delta and other notch ligands on their surface. Stimulation of the notch signaling pathway by these ligands appears to inhibit oligodendrocyte precursor differentiation (Wang *et al.*, 1998). Soluble signals also regulate differentiation of oligodendrocytes. For example, exposure to thyroid hormone elevates the number of mature oligodendrocytes as well as directly enhancing the expression of myelin-specific genes (Barres *et al.*, 1994). The influence of other signals such as retinoic acid varies depending on either the developmental stage of the cells or their origin. For example, retinoic acid promotes the differentiation of postnatal optic nerve oligodendrocyte precursors (Barres *et al.*, 1994) while it inhibits the differentiation of embryonic rat spinal cord oligodendrocyte precursors (Noll and Miller, 1994).

Although oligodendrocyte precursors are highly proliferative, mature differentiated oligodendrocytes do not proliferate extensively; for this reason, the regulation of oligodendrocyte precursor number is crucial for generating sufficient oligodendrocytes in the adult. The final number of oligodendrocytes that develop in a specific region of the CNS is closely correlated with the number of axons that require myelination (Barres and Raff, 1994; Burne *et al.*, 1996). This matching of cell number is accomplished both by regulation of cell proliferation and cell survival (Barres *et al.*, 1992; Barres and Raff, 1994) (Fig. 11.1). As with neuronal lineages, oligodendrocyte precursors are produced in excess and apoptosis and programmed cell death remove extraneous cells (Barres *et al.*, 1992). Newly formed oligodendrocytes depend on PDGF for survival both *in vitro* and *in vivo*, while more mature oligodendrocytes depend on insulin growth factor–1 (IGF-1) and the neurotrophin NT3 for survival (Barres *et al.*, 1992, 1994).

Increased understanding of the biology of oligodendrocyte development has revealed that, at least *in vitro*, cells of this lineage respond to an extremely broad range of different molecular cues. Why such diverse signaling exists is unclear but may be a reflection of the multiple cellular interactions that impinge on the cells destined for an oligodendrocyte fate. The challenge for current and future studies is to discern which cues predominate in the developing intact CNS.

CHARACTERIZATION OF OLIGODENDROCYTE DEVELOPMENT *IN VIVO*

Overview

Much of the basic developmental biology of the oligodendrocyte lineage first revealed in a variety of *in vitro* systems has proven to be directly applicable to understanding oligodendrocyte development in the intact CNS. Regional differences in the cytoarchitecture of the developing CNS as well as the detailed molecular control of oligodendrocyte development do, however, add considerable complexity to the system as well as providing critical insights into the fundamental pathways. For example, recent advances on understanding the transcriptional control of oligodendrocyte development stem directly from the localization of the earliest cells of the lineage in different regions of the CNS. In many cases, the underlying molecular mechanisms controlling specific aspects of oligodendrocyte development are less clearly understood in the intact CNS due presumably to the existence of multiple regulators at each stage.

Oligodendrocyte Precursors Arise in Restricted Regions of the CNS during Development

Although the majority of mature oligodendrocytes are located in white matter, the founder cells of the oligodendrocyte lineage arise early in development in restricted regions of the neural tube (Warf *et al.*, 1991). Early oligodendrocyte development has been most exten-

FIGURE 11.3
Developing oligodendrocytes *in vivo*. Transverse section of the ventral spinal cord labeled with antibodies to myelin basic protein. Individual differentiating oligodendrocytes are seen throughout the developing white matter surrounded by 1-6 myelin profiles. Bar = XXmm.

sively studied in selected regions of the CNS such as the spinal cord and the optic nerve. (Fig. 11.3) In the rat optic nerve, tissue culture studies suggested the founder cells of the oligodendrocyte lineage originated in the brain or optic chiasm and migrated along the nerve during subsequent development (Small *et al.*, 1987). For example, isolated cultures of the retinal end of embryonic and early postnatal rat optic nerve do not develop oligodendrocytes, while parallel cultures of the regions of the nerve closer to the optic chiasm do (Small *et al.*, 1987). A similar migration of oligodendrocyte precursors occurs during development of the chick optic nerve where a source of at least a subset of optic nerve oligodendrocytes has been defined in the floor of the third ventricle (Ono *et al.*, 1997). In rodents, the migration of oligodendrocyte precursors is inhibited at junction between the nerve and the retina (ffrench-Constant *et al.*, 1988), while in chick the cells continue into the retina where they myelinate retinal ganglion cell axons (Ono *et al.*, 1998, 2001).

In the spinal cord, the earliest oligodendrocyte precursors arise in the ventral ventricular zone after the majority of neurogenesis is complete (Ono *et al.*, 1995; Pringle and Richardson 1993). Several independent approaches have localized the source of spinal cord oligodendrocytes to a domain of the neural tube dorsal to the floor plate of the neural tube including their localized proliferation (Noll and Miller, 1993), expression of growth factor receptors (Pringle and Richardson, 1993), and biochemical profile (Ono *et al.*, 1995; Timsit *et al.*, 1995). Oligodendrocyte precursors can be identified by expression of the PDGFα-Receptor (PDGFα-R) (Pringle *et al.*, 1992) and *in situ* hybridization indicated PDGFα-R+ cells are initially seen in the same region of developing spinal cord as cells expressing mRNA for the myelin genes CNP (2',3'-cyclic-nucleotide 3'-phosphodiesterase) (Yu *et al.*, 1994) and DM20, an isoform of the major myelin proteolipid protein (PLP) (Timsit *et al.*, 1995) gene as well as in chick antigens recognized by the O4 monoclonal antibody (mAb) (Ono *et al.*, 1995). These cells arise in the same ventricular domain that generates motor neurons and appear around the time the generation of motor neurons is complete (Ono *et al.*, 1995; Pringle *et al.*, 1992; Richardson *et al.*, 1997).

The localized origin of oligodendrocyte precursors is not restricted to the spinal cord. In more rostral regions of the CNS, oligodendrocyte precursors arise in specific regions of the ventricular and subventricular zone at particular stages of development (Ono *et al.*, 1997). For example, cells in the ventricular mantle zone of the ventral diencephalon of the E13 rat express mRNA for the PDGFα-R (Pringle and Richardson 1993). During subsequent

development, these cells appear to migrate into the developing thalamus and hypothalamus as well as to more dorsal regions including the developing cerebellum (Pringle and Richardson, 1993). While most regions of the CNS seem to be populated by precursor cells derived from a single region, in the telecephalon multiple oligodendrocyte precursor domains have been described (Spassky *et al.*, 1998, 2001). These include the anterior penducle area and the olfactory bulb (Olivier *et al.*, 2001; Spassky *et al.*, 2001). It is currently unclear if the progeny of each of these domains are identical and whether they contribute oligodendrocytes to overlapping or nonoverlapping axon tracks.

Molecular Control of Oligodendrocyte Precursor Specification

The mechanisms by which neuroepithelial cells become specified to the oligodendrocyte lineage are best understood in the developing spinal cord where two general mechanisms may account for their ventral origin. It could be that cells in dorsal regions lack the intrinsic potential to generate oligodendrocytes regardless of extrinsic cues. Alternatively, both dorsal and ventral cells have the capacity to generate oligodendrocytes, but only ventral cells receive the appropriate cues. Several lines of evidence demonstrate that the ventral origin of spinal cord oligodendrocyte precursors is a reflection of local signaling. For example, local influences from the notochord, a ventrally located mesodermally derived structure, are required for the appearance of oligodendrocytes in adjacent spinal cord in both mouse (Pringle *et al.*, 1996) and Xenopus embryos (Maier and Miller, 1997). Signals from the notochord are involved in formation of the dorsal/ventral axis in the developing CNS, which results in the subsequent specification of distinct populations of spinal cord neurons (Jessell and Dodd, 1990; van Straaten *et al.*, 1988, 1989). Transplantation of an additional notochord adjacent to the dorsal spinal cord at the appropriate stage in development resulted in the local induction of oligodendrocyte precursors in chick and Xenopus embryos (Maier and Miller, 1997; Orentas and Miller, 1996;), while co-culture of notochord and dorsal spinal cord results in oligodendrocyte induction in the spinal cord tissue (Orentas and Miller, 1996; Poncet *et al.*, 1996; Pringle *et al.*, 1996; Trousse *et al.*, 1995).

Many of the inductive properties of the notochord appear to be due to its production of the signaling molecule sonic hedgehog (Echelard *et al.*, 1993; Roelink *et al.*, 1994). Sonic hedgehog, the vertebrate homologue of the Drosophila pattern forming gene *hedgehog*, is localized to the notochord and adjacent floor plate (Roelink *et al.*, 1994). *In vitro*, sonic hedgehog induces the development of floor plate and motor neurons in a concentration dependent manner (Roelink *et al.*, 1994). *In vitro*, oligodendrocytes are induced at similar concentrations of Shh required for the induction of motor neurons (Pringle *et al.*, 1996), suggesting that the development of these two cell types is closely linked (Richardson *et al.*, 1997; Rowitch *et al.*, 2002).

In more rostral regions of the CNS, the expression of Shh and the appearance of oligodendrocytes is spatially and temporally closely linked (Davies and Miller, 2001; Nery *et al.*, 2001; Spassky *et al.*, 2001; Tekki-Kessaris *et al.*, 2001). Furthermore, ectopic expression of Shh leads to concomitant local development of oligodendrocytes (Nery *et al.*, 2001). Whether Shh is essential for the development of all rostral populations of oligodendrocytes is less clear. In cell cultures derived from Shh knockout animals, considerable numbers of oligodendrocytes develop, indicating that oligodendrocytes can arise in the absence of Shh signaling (Nery *et al.*, 2001). It seems likely, however, that other members of the hedgehog family can substitute for Shh in its absence and blocking all hedgehog family member signaling with cyclopamine (Incardona *et al.*, 1998) appears to block all oligodendrocyte development (Tekki-Kessaris *et al.*, 2001).

In vitro, the development of oligodendrocyte precursors is inhibited by exposure to members of the TGF□ family (Mabie *et al.*, 1997). Specifically, bone morphogenetic proteins 2 and 4 inhibit the development of oligodendrocytes (Mabie *et al.*, 1997, 2000). This appears to be in part a reflection of the commitment of cells to astrocyte lineages at the expense of the oligodendrocyte lineage (Mabie *et al.*, 1997, 2000). Whether BMP signaling contributes to the spatial patterning of oligodendrocyte precursor induction in

e developing intact CNS is currently unclear although recent studies suggest that BMPs nibit spinal cord oligodendrocyte development (Mekki-Dauriac *et al.*, 2002) and it seems ely that the lack of dorsally derived oligodendrocytes in the spinal cord reflect active hibition by BMPs. Indeed, if the BMP source was adjacent to the spinal cord, this pothesis would explain why oligodendrocyte develop in isolated explants of dorsal inal cord over time (Sussman *et al.*, 2000). Additional, as yet uncharacterized, inhibitors oligodendrocyte precursor development may also exist, since dorsal spinal cord contains inhibitor of early oligodendrocyte development (Wada *et al.*, 2000) functionally distinct om any known BMP (Wada *et al.*, 2000).

The generation of motor neurons and oligodendrocytes is closely linked (Richardson *et* ., 1997; Rowitch *et al.*, 2002). Not only do they arise from the same region of the spinal ord and require the same concentration of Shh (Pringle *et al.*, 1996), neurons and igodendrocytes also arise from clonally related cells *in vitro* and *in vivo* (He *et al.*, 2001; eber *et al.*, 1990; Leber and Sanes, 1991; Williams *et al.*, 1991). The differentiation of europepithelial cells into a neuronal or glial fate is regulated by expression of distinct ombinations of transcription factors (Kessaris *et al.*, 2001). For example, the ventricular one of the ventral spinal cord contains several specific cellular domains identified by ifferent transcription factor expression and generating distinct cell populations (Briscoe *et l.*, 2001; Jessell, 2000). Oligodendrocyte precursors arise during later development from ie motor neuron pool that is characterized by expression of the transcription factor Olig2 Zhou *et al.*, 2001; Zhou and Anderson 2002). Olig2 is thought to combine with the Basic Helix-Loop-Helix transcription factors neurogenin 1 and 2 to generate motor neurons. As evelopment proceeds, however, the expression of neurogenins is down-regulated (Zhou *et l.*, 2001) and this allows for an alteration in the distribution pattern of a more ventrally xpressed transcription factor, Nkx2.2, into the Olig2 domains such that they overlap. Cells that express both transcription factors subsequently develop into spinal cord oligo-lendrocytes rather than motor neurons (Kessaris *et al.*, 2001; Zhou *et al.*, 2001). Several ines of evidence support such a model. For example, oligodendrocytes can be ectopically enerated in other regions of the CNS by expression of both Olig2 and either Nkx2.2 or omponents of the Notch signaling pathway (Zhou *et al.*, 2001). Confirmation of the equirement and roles of the Olig transcription factors in the genesis of oligodendrocytes as come from targeted disruption of these genes. Olig 2 is required for the specification of oth motor neurons and oligodendrocytes, while Olig1 is required for the later develop-nent of oligodendrocytes, particularly in rostral regions of the CNS (Lu *et al.*, 2002). In he absence of both Olig1 and 2, the cells that would normally give rise to motor neurons nd oligodendrocyte generate a specific class of interneurons and surprisingly astrocytes (Zhou and Anderson, 2002). The simple model of a restricted motor neuron oligodendro-yte precursor will, however, require further refinement since in the mouse CNS some early oligodendrocyte precursor cells arise outside the Nkx2.2+ domains of the CNS (Lu *et al.*, 2000; Sun *et al.*, 1998). It may be that there is more than one population of oligodendrocyte precursors, which differ in the mechanism by which they become specified (Spassky *et al.*, 1998, 2000) or some cells previously characterized as oligodendrocyte precursors are in fact astrocyte precursors or glial restricted precursors (Rao and Mayer-Proschel 1997).

Control of Oligodendrocyte Number *in Vivo*

The functioning of the adult CNS depends critically on matching the correct number of oligodendrocytes to axons. In the developing nervous system, oligodendrocyte cell number is regulated by several independent mechanisms. The proliferation of oligodendrocyte precursors is mediated in large part by the availability of mitogens such as PDGF (Richardson *et al.*, 1988). Thus, increasing levels of PDGF result in increasing numbers of oligodendrocyte precursors, at least in the spinal cord (Calver *et al.*, 1998). This increase in precursor cells may also reflect increased cell survival, since PDGF is a strong survival factor cells of the oligodendrocyte lineage (Barres *et al.*, 1992; Barres and Raff, 1994; Calver *et al.*, 1998). The final number of differentiated oligodendrocytes in any particular region of the CNS is a combination of precursor proliferative control, cell differentiation,

and regulation of cell survival (Barres and Raff, 1994). The differentiation of oligodendr cytes is regulated by thyroid hormone (Barres *et al.*, 1994) and the intrinsic redox state individual cells (Smith *et al.*, 2000), which also alters the potential for cell proliferatio Likewise, modulation of cell cycle regulators such as $p27^{kip-1}$ and $p21^{cip-1}$ influence olig dendrocyte development (Casaccia-Bonnefil *et al.*, 1997; Casaccia-Bonnefil, 2000; Duran *et al.*, 1997). In the absence of p27Kip-1 the number of oligodendrocytes is significantl altered, although they differentiate on time (Casaccia-Bonnefil *et al.*, 1997). By contrast, i the absence of p21cip-1 the differentiation of oligodendrocytes is disrupted (Zezula *et al* 2001), suggesting this molecule is required for their timely differentiation.

The control of cell survival is a major component in determining oligodendrocyt number in the developing CNS. In the developing optic nerve as well as other regions the CNS, the final number of oligodendrocytes that ultimately differentiate appears to b regulated by competition for local survival factors (Barres and Raff, 1994) includir PDGF and possibly axonally derived neuregulin (Park *et al.*, 2001). A significant numb of newly generated oligodendrocytes die during normal development, indeed in the opt nerve this has been estimated to be as high as 50% (Barres *et al.*, 1992). This extent of c death can be reduced dramatically by increased expression of PDGF or insulin-like grow factor (Barres *et al.*, 1992). Since the ultimate goal of oligodendrocytes is to myelinate available axons during development, it seems likely that expression of oligodendrocy survival signals will be either directly or indirectly controlled by the number of availal axons (Barres and Raff, 1994; Burne *et al.*, 1996). Evidence to support this hypothe comes from the increased oligodendrocyte number in animals in which the number retinal axons is increased as a result of inhibition of cell death while removal of axo results in decreases in the number of optic nerve oligodendrocytes (Burne *et al.*, 1996).

Not all oligodendrocyte precursors in the developing nervous system either differenti or die. A significant population of oligodendrocyte precursors persist as potent progenitor cells in the adult CNS and these enigmatic cells are discussed further in following sections.

THE OLIGODENDROCYTE LINEAGE IN THE ADULT CNS

The Existence of a Population of Glial Progenitors in the Adult CNS

By late embryonic stages in the rodent, oligodendrocyte progenitors (OPCs), identified their expression of either $PDGF\alpha R^+$, NG2, or O4, have become evenly distributed throu out the presumptive gray and white matter of the developing brain and spinal c (Nishiyama *et al.*, 1996; Pringle *et al.*, 1992; Reynolds and Hardy, 1997). (Fig. 4) described earlier in this chapter, a combination of the action of soluble and cont dependent growth factor signals, together with an intrinsic timing mechanism, leads to differentiation of the progenitors into myelin producing oligodendrocytes. It is clear t unlike progenitors, the distribution of myelinating oligodendrocytes is not even through the CNS but reflects the different tract specific requirements for myelin. There is evide that newly formed oligodendrocytes are dependent on axonal signals for their survival undergo apoptosis if they do not contact competent axons that require myelination. In way oligodendrocyte number is matched to the requirement for myelin. Thus, the two m fates of oligodendrocyte progenitors are to differentiate into mature myelinating ol dendrocytes or to die by apoptosis. There is, however, considerable evidence to sug that not all perinatal oligodendrocyte progenitors undergo these two fates. Here we rev the evidence that a proportion of progenitors persist in the adult CNS in a phenotypic immature form and discuss the origins and functions of these cells.

In Vitro Studies

A number of studies have demonstrated that cells with the phenotype and expected gro characteristics of oligodendroglial progenitors can be isolated from the optic n

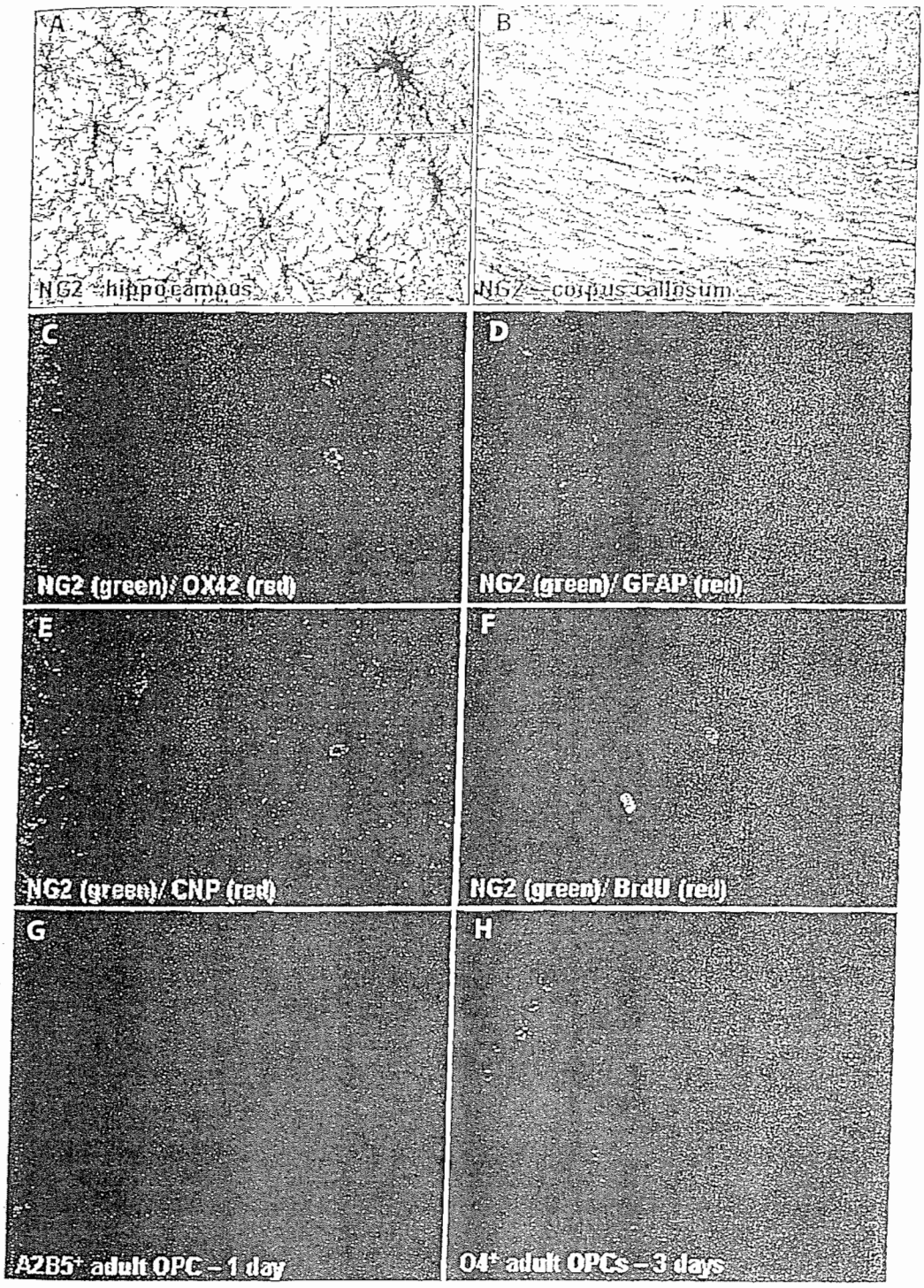

FIGURE 11.4

Appearance of oligodendrocyte lineage cells in the adult CNS. NG2 expressing cells seen in the rat hippocampal gray matter (A) and in the corpus callosum (B). The multiple processed nature of these cells is clearly seen in the hippocampus (A, inset). NG2-expressing cells are seen to be intermingled with, but distinct from, OX-42+ microglia (C, cerebral cortex), GFAP+ astrocytes (D, corpus callosum), and CNP+ oligodendrocytes (E, cerebral cortex). A pair of NG2+BrdU+ cells are seen in the rat cerebral cortex 6 days after a 6-hour pulse of BrdU (F). OPCs can be isolated from the adult forebrain using O4 and A2B5 double immunopanning. (G) A bipolar A2B5+ cell 1 day after isolation. (H) A field of galatocerebroside expressing cells 3 days after isolation.

(ffrench-Constant and Raff, 1986; Shi *et al.*, 1998; Wolswijk and Noble, 1989) and cerebellum (Levine *et al.*, 1993) of adult rats. These cells express the A2B5, O4, PDGF αR, and NG2 antigens and therefore have a more mature phenotype than the early perinatal progenitors that do not yet express O4 (Dawson *et al.*, 2000). When grown in serum-free chemically defined medium they differentiate rapidly into oligodendrocytes, and when grown in 10% fetal calf serum containing medium they take on an astrocyte phenotype, indicated by the expression of glial fibrillary acidic protein (GFAP). Because of the similarity of these cells to the O-2A progenitor cells isolated from neonatal optic nerves (Raff *et al.*, 1983), they were termed O-2A^{adult} progenitors (Wolswijk and Noble, 1989). A number of fundamental differences between perinatal and adult progenitors have been identified. Progenitor cells derived from perinatal optic nerves are highly motile (Small *et al.*, 1987) with a cell cycle of about 18 hours (Noble *et al.*, 1988; Temple and Raff, 1986) and can be induced to differentiate into either oligodendrocytes or astrocytes in less than 3 days (Raff *et al.*, 1983). In contrast, adult OPCs migrate at a slower rate, have an average cell cycle of 65 hours, and also differentiate at a slower rate (Shi *et al.*, 1998; Wolswijk and Noble, 1989). Reports that the morphology of progenitors isolated from the adult optic nerve differed from those isolated from the developing optic nerve (Wolswijk and Noble, 1989) are not substantiated in other studies (ffrench-Constant and Raff, 1986; Shi *et al.*, 1998). Later studies have identified two populations of OPCs in the adult rat brain: an A2B5$^+$ O4$^-$ population that is bipotential and an O4$^+$ population that appears committed to the oligodendrocyte lineage (Gensert and Goldman, 2001). These two populations differ in their responsiveness to growth factors *in vitro* (Mason and Goldman, 2002). Although the precise relationship of these cells to one another and to those identified *in vivo* is not clear, it is clear that the mature CNS contains a population of cells with the potential to give rise to oligodendrocytes when isolated in culture (Fig. 11.4G,H).

In Vivo Morphological Studies

The glial component of the adult CNS is generally thought to consist of astrocytes, oligodendrocytes, and microglia. However, there have been many reports over the past 40 years that a proportion of cells in the CNS do not fall easily into these categories. Although these cells have been largely ignored for a long time, there is increasing interest in these populations mainly because they express characteristics of immature cells and, therefore, may be important for regenerative purposes. Smart and Leblond (1961) identified a population of cells in the adult mouse corpus callosum that had the morphological characteristics of immature glia and were mitotically active. They were shown to have some ultrastructural features in common with oligodendrocytes and were classified as light and medium oligodendrocytes, precursors to the post-mitotic dark oligodendrocytes (Mori and Leblond, 1970). Vaughn and Peters (1968) identified cells in the developing and adult rat optic nerve lacking intermediate filaments and microtubules. They showed these cells to comprise 5% of all glia in the optic nerve and suggested that they might represent a multipotential stem cell. A population of cells with similar characteristics were found in the adult rat cortical gray matter (Reyners *et al.*, 1982, 1986) and were called beta-astrocytes. They were found to be mitotically active and were suggested to be glial precursor cells. However, until very recently it was not possible to unequivocally identify these cells as part of the oligodendrocyte lineage.

Characterization and Distribution of Oligodendrocyte Progenitors in the Adult CNS

Antibodies to both the NG2 chondroitin sulphate proteoglycan and the PDGF α-receptor have been used extensively to identify OPCs in the developing CNS (reviewed in Levine *et al.*, 2001) and more recently have been applied to studies of the mature CNS. In the adult rodent, these antibodies identify a widespread and numerous population of cells that have the phenotype of late oligodendroglial progenitors (Dawson *et al.*, 2000; Fig. 11.4A,B). Although NG2-immunopositive, process-bearing cells present in the mature rat cerebellum were initially identified as smooth protoplasmic astrocytes on the

basis of their light microscope and ultrastructural characteristics (Levine and Card, 1987), later studies suggested that they corresponded to the *in vivo* counterparts of the optic nerve O-2A^{adult} progenitors described *in vitro* (Levine *et al.*, 1993; Wolswijk and Noble, 1989). In all regions of the adult CNS, both gray and white matter, NG2 expression completely overlaps with the expression of the PDGF α-receptor (Dawson *et al.*, 2000; Nishiyama *et al.*, 1996; Reynolds *et al.*, 2001). More compelling evidence that these cells represent OPCs comes from studies demonstrating that they also express the O4 antigen *in vivo* (Dawson *et al.*, 2002; Reynolds and Hardy, 1997), but not antigens characteristic of more mature cells of the oligodendrocyte lineage, for example, CNP, galactocerebroside and myelin basic protein (Dawson *et al.*, 2002; Levine *et al.*, 1993; Reynolds and Hardy, 1997; Reynolds *et al.*, 2001). However, there have been a number of recent reports that OPCs in the adult CNS may express low levels of galactocerebroside and myelin/oligodendrocyte glycoprotein (MOG) (Li *et al.*, 2002; Shi *et al.*, 1998). All this evidence taken together suggests that these cells are phenotypically part of the oligodendrocyte lineage and are closely related to the late NG2$^+$/PDGF-αR$^+$/O4$^+$ progenitor seen during development. They appear to stop differentiating at the point in the lineage before they differentiate into post-mitotic young oligodendrocytes. However, this does not mean that their only function is that of becoming oligodendrocytes following demyelination or that they are all capable of such differentiation.

A number of studies have looked at the relationship between NG2$^+$/PDGF-αR$^+$ cells and the other major glial cell types of the mature CNS. Although when isolated into culture OPCs from the adult CNS appear to be bipotential, there is no evidence of expression of markers known to be specific for astrocytes, such as GFAP and S100a, (Dawson *et al.*, 2000; Nishiyama *et al.*, 1996) *in vivo* (Fig. 11.4D). In addition, in the normal resting CNS, NG2$^+$/PDGF-αR$^+$ cells do not express microglial markers such as OX-42 (Fig. 11.4C) (Levine *et al.*, 1993; Reynolds and Hardy, 1997) or GSA I-B$_4$ lectin (Nishiyama *et al.*, 1997). NG2$^+$ cells are found in close association with MAP2$^+$ neurons in the cerebral cortex but do not themselves express MAP2 (Reynolds *et al.*, 2001). It remains to be seen whether they are capable of a fate other than oligodendrocyte following appropriate stimulation *in vivo*, but data available at present clearly demonstrate that in the resting situation they are phenotypically part of the oligodendrocyte lineage.

Adult OPCs display a highly branched morphology that is very different when compared to developing OPCs and one that does not fit easily into our concept of immature progenitor cells (Dawson *et al.*, 2000; Levine *et al.*, 2001), although it may be closely related to their function. In gray matter areas, the process network is extensive and radial and reflects the particular spatial arrangement of groups or layers of neuronal cell bodies (Dawson *et al.*, 2002). In white matter tracts, the cell bodies of OPCs are found among the rows of interfascicular oligodendrocytes in a regular pattern and extend processes both perpendicular and parallel to the bundles of axons (Berry *et al.*, 2002; Butt *et al.*, 1999). Within any one gray or white matter area there does not appear to be any morphological heterogeneity in the NG2$^+$ OPC population. Any heterogeneity between CNS regions is likely to be a consequence of differences in the cytoarchitecture that determines the number and course of the processes.

NG2$^+$ and PDGF-αR$^+$ OPCs are found in abundance in both gray and white matter regions throughout the entire CNS comprising between 3 to 9% of all cells depending on the region (Dawson *et al.*, 2002). The gray:white matter ratio in OPC cell number of approximately 1:1.5 is surprising and demonstrates that the numerical density of these cells is not simply a reflection of the abundance of myelin. These results also reinforce the idea that they are likely to play other roles in the CNS, in addition to providing a reserve cell population for both physiological and pathological requirements for new myelin synthesis. When considering the role of these cells in remyelination, of particular importance is the consistent ratio of OPCs to oligodendrocytes of approximately 1:4 across the spinal cord in both gray and white matter, but only 1:1 in the cerebral cortex and hippocampus (Dawson *et al.*, 2002), which has significance for the efficiency and speed of remyelination in these different areas.

The Origin of Oligodendrocyte Progenitors in the Adult CNS

It is a matter of much debate why there should be such a large population of cells in the adult gray matter with the potential to become oligodendrocytes. The answer to this conundrum may lie in the developmental origin of these cells. During embryonic development in the rodent, PDGF-αR^+NG2^+ OPCs migrate out from the germinal zones and become evenly distributed in both prospective white and gray matter areas prior to any myelination (Nishiyama *et al.*, 1996; Pringle *et al.*, 1992). As myelination begins in the cerebral cortex, a wave of differentiation of OPCs can be observed spreading from the corpus callosum out toward the pial surface (Reynolds and Hardy, 1997). However, as this wave of differentiation occurs a proportion of the $O4^+NG2^+$ cells do not begin to express GalC and myelin proteins and remain among the immature oligodendrocytes (Reynolds and Hardy, 1997). The same pattern of differentiation can be seen in the optic nerve. Therefore, a population of immature $O4^+NG2^+$ cells would be expected to remain in all areas of the CNS irrespective of the density of myelinated fibers. This may in fact explain the 1:1 ratio of OPCs to oligodendrocytes in most gray matter areas, because a smaller proportion of cells would be induced to differentiate into oligodendrocytes compared with the white matter. The lower degree of expansion of OPCs in the gray matter in response to growth factors would account for the overall lower numbers of oligodendroglial lineage cells. It is a common misconception that because PDGF-αR^+NG2^+ cells have the capability of becoming oligodendrocytes, then there should be more of them in the white matter than in the gray matter.

Wren *et al.* (1992) used time-lapse microscopy to demonstrate that perinatal OPCs isolated from the rat optic nerve can give rise to adult progenitors over two or more cell divisions. The results suggest that both perinatal and adult type OPCs would be expected to coexist during the later stages of development and that the proportion of adult OPCs would increase as myelinogenesis proceeds. This is in agreement with studies on the optic nerve and cerebral cortex *in vivo* (Fulton *et al.*, 1992; Reynolds and Hardy, 1997). More recent investigations, using transgenic mice in which EGFP expression is driven by the PLP gene promoter, have demonstrated two populations of $NG2^+$ OPCs in the cerebral cortex from as early as postnatal day 1 (Mallon *et al.*, 2002). One population expresses both NG2 and PLP gene promotor activity and is suggested to give rise to myelinating oligodendrocytes while the other population does not express PLP gene promoter activity and is suggested to persist into adulthood as a population of progenitor cells. The question of whether these two $NG2^+$ populations represent two distinct lineages that diverge during early development of the oligodendrocyte lineage or whether the two populations represent two different stages of the differentiation schedule of a single lineage remains to be answered. It is possible that the $NG2^+EGFP^+$ cells give rise to the $NG2^+EGFP^-$ cells during postnatal development as suggested by the *in vitro* experiments of Wren *et al.* (1992). The two populations of cells described in the transgenic mice have similar morphology, distribution, and proliferative characteristics, and therefore it is likely that they are closely related.

The preceding experiments raise the question of whether two populations of $NG2^+$ OPCs continue to be present in the adult CNS, and therefore adult OPCs are a heterogeneous population. Evidence to date from studies conducted *in vivo* suggest that the $NG2^+PDGF\text{-}\alpha R^+$ population of cells is homogeneous with respect to immunological phenotype and radiation sensitivity (Dawson *et al.*, 2000; Li *et al.*, 2002), and it remains to be seen whether this extends to the expression of PLP gene promoter activity (Mallon *et al.*, 2002). Although *in vitro* experiments suggest that there are several populations of OPCs in the adult brain (Gensert and Goldman, 2001; Mason and Goldman, 2002), it is difficult to reconcile these data with the *in vivo* results. The majority of *in vitro* experiments have been conducted under conditions in which the immunological phenotype of adult OPCs *in vivo* is unlikely to have been preserved following isolation, and therefore the results are difficult to interpret.

The Maintenance of Adult OPCs in an Undifferentiated State

Adult OPCs have been suggested to represent a slowly dividing stem cell population in the rodent CNS (Wren *et al.*, 1992), capable of generating oligodendroglial lineage cells

broughout life. $NG2^+$ OPCs have been demonstrated to be a cycling population in all reas of the CNS (Dawson *et al.*, 2000; Horner *et al.*, 2000), although their ability to divide symmetrically *in vivo* has received little attention and is still a matter of debate *in vitro* (Shi *et al.*, 1998). It is suggested that OPCs may progressively change their properties during development, lengthening their cell cycle time, possibly as a result of p27 accumulation (Durand *et al.*, 1997; Shi *et al.*, 1998), and eventually attaining the properties of adult OPCs. The availability of neuronally derived growth factors, such as the neuregulins, would be expected to decline as myelination proceeds and might also contribute to slowing the rate of OPC proliferation. Continued production of PDGF by astrocytes might be expected to provide a continuous survival signal and also allow a slow rate of proliferation. The further differentiation of adult OPCs following myelination is suggested to be prevented by activation of the Notch-Jagged signaling pathway (Wang *et al.*, 1998), thereby maintaining a pool of undifferentiated progenitor cells. Notch receptors on OPCs would be activated by the Jagged ligand on nearby oligodendrocytes or at nodes of Ranvier. In addition, myelin has been demonstrated to inhibit the further differentiation of OPCs *in vitro* (Robinson and Miller, 1999). However, experiments on cells isolated from the fully mature CNS have yet to be performed (Wang *et al.*, 1998). It remains to be demonstrated whether Notch becomes asymmetrically distributed following OPC division in the adult CNS, thus allowing the daughter cell to differentiate into an oligodendrocyte, while also maintaining OPC number.

A Mitotically Active Population of Cells in the Adult CNS

Although adult OPCs divide slowly compared to perinatal cells, there are a significant number of studies demonstrating that they represent the major cycling cell population in the adult CNS. In the rat spinal cord, up to 70% of cells incorporating BrdU after a single injection were found to express NG2 (Horner *et al.*, 2000), whereas in both white and gray matter areas of the forebrain, 70 to 75% of BrdU incorporating cells were $NG2^+$ after a 2-hour pulse, representing 1 to 4% of the OPC population (Fig. 11.4F) (Dawson *et al.*, 2002). Although a number of studies of the characteristics of cycling cells in the adult rat CNS *ex vivo* have suggested that they do not express NG2, but rather are either $O4^+$ or $A2B5^+$ (Gensert and Goldman, 2001; Mason and Goldman, 2002), these conflicting results are most likely the result of technical difficulties associated with NG2 antigenicity. Six days after a BrdU pulse, labeled CNP^+ oligodendrocytes are observed *in vivo* in both gray and white matter (Dawson *et al.*, 2002; Wu *et al.*, 2001), indicating that cycling progenitors give rise to small numbers of oligodendrocytes throughout adult life, although direct evidence that $NG2^+PDGF\text{-}\alpha R^+$ OPCs give rise to oligodendrocytes in the normal adult CNS in response to physiological demands for new myelin is still lacking. Retroviral lineage studies also suggest that oligodendrocytes are generated from $NG2^+$ cycling cells in the adult CNS (Levison *et al.*, 1999). It remains possible that new OPCs and oligodendrocytes generated in the adult CNS undergo apoptosis, although TUNEL labeled OPCs or oligodendrocytes are only very rarely seen (Dawson *et al.*, 2002). The recent report of no change in the number of PDGFαR-expressing OPCs in the rat cerebellar peduncles during aging (Sim *et al.*, 2002) also suggests that the newly generated $NG2^+$ cells do not remain as OPCs. Thus, it is likely that a small but consistent number of myelinating oligodendrocytes differentiate from non-myelinating $NG2^+$ cycling cells during the course of adult life. However, the possibility that BrdU-labeled oligodendrocytes arise from $BrdU^+$ $NG2^-$ cells cannot be ruled out at this stage.

The Function of Oligodendrocyte Progenitors in the Normal Adult CNS

The very abundance in the adult white and gray matter of cells with the phenotype of OPCs, and their extensive process network, has been taken as evidence for a role in the adult CNS other than as oligodendrocyte progenitors. The presence of processes of $NG2^+$ cells at the node of Ranvier (Butt *et al.*, 1999) and at synapses (Bergles *et al.*, 2000; Ong and Levine, 1999) is suggestive of a role in the modulation of neuronal activity. The presence of

AMPA receptors on OPCs, the surprise observation of glutaminergic synaptic input onto these cells in the hippocampus (Bergles *et al.*, 2000), and their ability to transport glutamate (Domercq *et al.*, 1999; Reynolds and Herschkowitz, 1987), also indicates a role in glutamate neurotransmission and homeostasis. However, it is not known whether this occurs throughout the CNS. The location of OPC processes also places the cells in an ideal position to monitor and respond to electrical activity in both white and gray matter, although little is known concerning the cell-cell contacts that OPCs make in the gray matter, other than at synapses. Thus, it is possible that electrical activity may play an indirect role in cell cycle regulation in OPCs. Therefore, there is accumulating evidence for multiple roles for $NG2^+$ cells in the adult CNS, although this is not incompatible with their role as oligodendrocyte progenitors. There is little doubt that these cells have the capability of differentiating into oligodendrocytes in certain circumstances, both physiological and pathological, in response to a demand for new myelin.

The Response of Adult OPCs to Demyelination and Other Pathologies

Thus, a population of slowly dividing OPCs is retained in the adult CNS, as a reservoir of cells that have the potential to generate new oligodendrocytes. Changes in the CNS environment following injury or myelin loss appear to enable adult OPCs to reacquire the rapidly dividing phenotype of neonatal OPCs so large numbers of oligodendrocytes can be replaced. Adult OPCs react to a variety of pathological insults to the CNS (for review, see Levine *et al.*, 2001) by up-regulating NG2 expression and a thickening of their primary processes. Processes are then retracted and cell bodies become swollen. In the presence of demyelination (Cenci di Bello *et al.*, 1999; Levine and Reynolds, 1999) or severe tissue damage (Levine, 1994), this is followed by proliferation. A consistent feature of the adult OPC response to demyelination is that it is highly restricted in space and is extremely rapid. Only OPCs in the area of demyelination and immediate border proliferate (Cenci di Bello, 1999; Reynolds *et al.*, 2002; Watanabe *et al.*, 2002). In response to inflammation, induced by passive transfer of activated MBP-specific T cells (Cenci di Bello *et al.*, 1999) or herpes virus infection (Levine *et al.*, 1998), OPCs show reactive changes but do not proliferate. These data suggest that OPC proliferation in demyelinated lesions in which OPCs are preserved is brought about in part by signals from the now bare axons, in combination with synergistic signaling from astrocytes and microglia/macrophages (Franklin, 2002). This reactive process appears to generate more than enough cells to carry out remyelination, which is rapid in nearly all animal models of demyelinating disease. The increase in the number of OPCs within demyelinated areas and subsequent decrease on remyelination, and the close physical association between the processes of reactive OPCs and demyelinated axons, strongly suggests adult OPCs are able to revert to a neonatal phenotype in order to undergo rapid proliferation and migration before differentiating into myelin forming cells. However, a direct demonstration that a reactive adult OPC can mature into a myelin-forming cell *in vivo* is still lacking. Destruction of both oligodendrocytes and myelin would be likely to both release OPCs from the block on differentiation mediated by Notch-Jagged signaling and also increase the availability of the axonally derived GGF. *In vitro*, adult OPCs can be stimulated to divide rapidly by a combination of PDGF and GGF (Shi *et al.*, 1998). Therefore, rapid repopulation of demyelinated lesions by OPCs would be expected to occur in the presence of bare axons and reactive astrocytes.

Following remyelination, numbers of $NG2^+$ or PDGF-aR^+ OPCs in the immediate area of demyelination return to approximately pre-lesion levels (Cenci di Bello *et al.*, 1999) or remained elevated (Levine and Reynolds, 1999). This would be expected if OPCs were acting as a stem cell population, dividing asymmetrically in order to maintain a population of cells for future repair. This is in agreement with *in vitro* studies showing asymmetrical division of OPCs isolated from adult rat optic nerve (Wren *et al.*, 1992). A small decrease in $NG2^+$ OPC number has been noted at the lesion border following remyelination of lesions induced by the injection of GalC antibodies in the rat spinal cord (Keirstead *et al.*, 1998), although ethidium bromide induced lesions in the same location did not result in depletion of OPCs from the areas surrounding the lesion (Chari and Blakemore, 2002).

Conclusions

In conclusion, the adult CNS contains a substantial population of slowly dividing cells with the phenotype of oligodendroglial progenitors. These cells are perfectly situated to be able to rapidly respond to a variety of insults, and all the experimental evidence produced using animal models of demyelinating disease is highly suggestive of $NG2^+PDGF\text{-}\alpha R^+$ OPCs as the source of new oligodendrocytes. Their number and location in the resting CNS is also suggestive of a number of additional roles that have yet to be explored in detail.

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THE ORIGIN AND DEVELOPMENT OF GLIAL CELLS IN PERIPHERAL NERVES

Kristjan R. Jessen and Rhona Mirsky

Abstract | During the development of peripheral nerves, neural crest cells generate myelinating and non-myelinating glial cells in a process that parallels gliogenesis from the germinal layers of the CNS. Unlike central gliogenesis, neural crest development involves a protracted embryonic phase devoted to the generation of, first, the Schwann cell precursor and then the immature Schwann cell, a cell whose fate as a myelinating or non-myelinating cell has yet to be determined. Embryonic nerves therefore offer a particular opportunity to analyse the early steps of gliogenesis from transient multipotent stem cells, and to understand how this process is integrated with organogenesis of peripheral nerves.

Before the onset of gliogenesis in the spinal cord, neural crest cells (BOX 1) have already given rise to the early glial cells that are found among the axons of nascent nerves as they work their way through body tissues to reach distal targets and establish functional links between the CNS and the rest of the body[1,2]. These early embryonic nerves are compact columns built exclusively from axons and tightly associated Schwann cell precursors (SCPs). Notably, they have no reinforcing connective tissue or protective covering, and do not even have their own blood supply. These features arise later, at about the time that nerves reach their targets (REFS 3,4; A. Kumar, R.M. and K.R.J., unpublished observations).

A surprising finding is that although SCPs are intimately associated with the axon bundles of these nerves, they are not actually required for the nerve to grow and reach its final target fields[5–7]. Rather, SCPs have four main functions. Their most obvious role is, of course, as an intermediary precursor stage between neural crest stem cells and Schwann cells and, therefore, as the immediate source of the Schwann cells present in perinatal nerves[8,9]. Another major function of these cells is likely to be the provision of essential trophic support for sensory and motor neurons — at limb levels of the spinal cord most of these neurons die in mouse mutants in which SCPs are absent[10]. In addition, SCPs are essential for normal nerve fasciculation[10]. Finally, SCPs might be the source of not only Schwann cells, but also the relatively small population of fibroblasts that is found in peripheral nerves[11].

This last finding accords with previous work showing that SCPs can generate non-glial lineages, including neurons, *in vitro* (see below). In principle, these observations on SCPs parallel the recent observation that radial glia give rise to CNS neurons during development[12,13]. In both cases, cells that are unambiguously glial-like, both in molecular phenotype and also, in the case of SCPs, in showing intimate association with axons, seem to generate unexpected progenies that were previously thought to arise from different lineages. These findings are a useful reminder of the tentative nature of many of the dogmas that shape the field.

Notwithstanding these uncertainties, recent progress in understanding neural crest and Schwann cell development has allowed us to generate a coherent baseline picture of glial development in early peripheral nerves, which we describe in this review. We also discuss the molecules that control glial differentiation and highlight new data that point to unexpected functions, developmental potential and the fate of early glial cells in the PNS.

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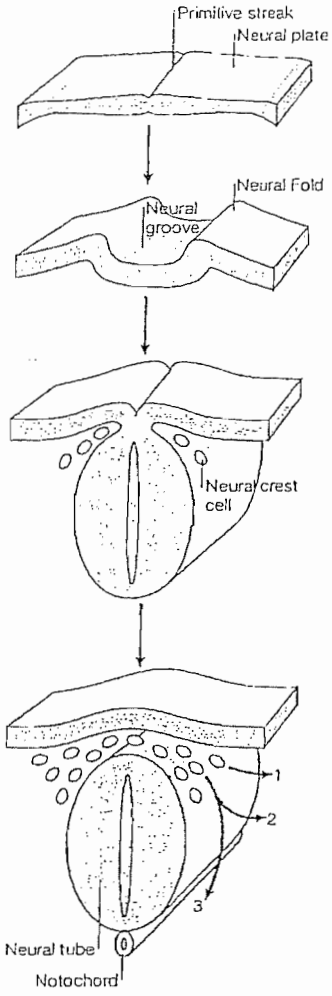
Box 1 | The neural crest

In a process known as neurulation, the neural plate, which is found along the dorsal surface of an embryo, gradually folds in on itself to generate the neural groove. As the neural folds fuse to form the neural tube, neural crest cells segregate from the tips of the folds. After taking up an initial position at the dorsal surface of the tube, the crest cells in the trunk region soon migrate along one of two major streams: in a lateral direction (1) to give rise to melanocytes in the skin, and in a ventral direction (2,3) to give rise to neurons in dorsal root sensory ganglia and glia (2), or glia, autonomic neurons and chromaffin cells (3). Neural crest cells in the most anterior part of the trunk, the cardiac crest, also generate fibroblasts and smooth muscle cells, and the cephalic crest in the head region forms the cells of cartilage and bone.

The mechanisms that allow the apparently homogenous population of crest cells to generate such diversity have been intensively studied. It is now considered likely that some neural crest cells are already committed to certain fates, whereas others are multipotent. Although some of these cells may enter lineages in a stochastic and undirected manner, a combination of positive and negative instructive signals probably play an important part in directing neural crest cell differentiation.

How migrating neural crest cells, which initially move through immature connective tissue on each side of the nascent neural tube, end up as Schwann cell precursors in tight association with axons in early embryonic nerves is not clear, either in terms of their detailed migratory route or the signals that cause these cells to adopt an early glial phenotype.



Outline of the Schwann cell lineage

Schwann cells in spinal nerves originate from the neural crest, although the origin of cells in spinal roots is more complex (see below). The end point of Schwann cell development is the formation of myelinating and non-myelinating cells that ensheath large and small diameter axons, respectively, throughout the PNS[4,11–20] (FIG. 1). The other main glial cell types in the PNS are described in BOX 2.

Schwann cell formation is preceded by the generation of two other cell types: SCPs, which are the glial cells of embryonic day (E) 14–15 rat nerves (mouse E12–13), and immature Schwann cells, which are generated from the SCPs from E15 to E17 (mouse E13–15). The latter are the glial cells found in rat nerves from E17–18 to about the time of birth[21] (FIG. 2). The postnatal fate of immature Schwann cells is determined by which axons they randomly associate with, with myelination being selectively activated in those cells that happen to envelop single large diameter axons.

These events can be viewed as three main transitions, that is, the transition from migrating neural crest stem cells to SCPs, from SCPs to immature Schwann cells and, lastly, the divergence of this population to form the two mature Schwann cell types. These events are strikingly dependent on survival factors, mitogens and differentiation signals from the axons with which SCPs and Schwann cells continuously associate[1,15]. Another notable feature is plasticity, as much of this developmental sequence is readily reversible: mature myelinating and non-myelinating cells respond to nerve injury by reverting to a phenotype similar to that of immature Schwann cells, and SCPs can be diverted, at least *in vitro*, to other neural crest derivatives[21–25]. Only the middle transition — from SCPs to immature Schwann cells — seems to involve irreversible commitment.

Neural crest cells, SCPs and immature Schwann cells all proliferate rapidly *in vivo*, and the onset of myelination is the only step of differentiation in the entire lineage that is clearly linked to cell cycle exit. Even in this case, cessation of proliferation is reversible, because myelinating cells re-enter the cell cycle as they start to de-differentiate in response to nerve injury[24–26].

Numerous molecules have now been implicated in the regulation of Schwann cell development. It is notable that our knowledge of perinatal events greatly exceeds what we know about the control of early steps in the differentiation of the Schwann cell lineage (FIG. 3).

Markers of lineage progression

A partially overlapping set of molecular differentiation markers can be used to characterize each stage of the Schwann cell lineage. However, each stage can also be identified by additional criteria that relate to signalling responses and changing relationships to other cells and tissues (FIG. 4). Until a few years ago, analysis of lineage progression in this system was limited by a paucity of markers — only S100 calcium-binding protein (S100) and glial fibrillary acidic protein (GFAP) were used, both of which appear late in embryonic nerve development. Now, a number of markers are emerging that offer insights into the intervening developmental steps, allowing new questions to be asked about the mechanisms that control lineage progression.

The markers shown in FIG. 4 fall into 5 main groups: (1) those that are present at all developmental stages, for example, SRY (sex determining region Y) box 10 (SOX10); (2) those expressed by neural crest cells and SCPs, but downregulated by immature Schwann cells, such as activator protein 2α (AP2α); (3) a gene that is expressed only by SCPs — at present cadherin 19 (Cad 19) is the only marker in this category; (4) those present on SCPs and immature Schwann cells but not on migrating neural crest cells, such as brain fatty acid-binding protein (BFABP); and (5) those present on immature Schwann cells but absent or at much lower levels in SCPs, for example, GFAP and S100.

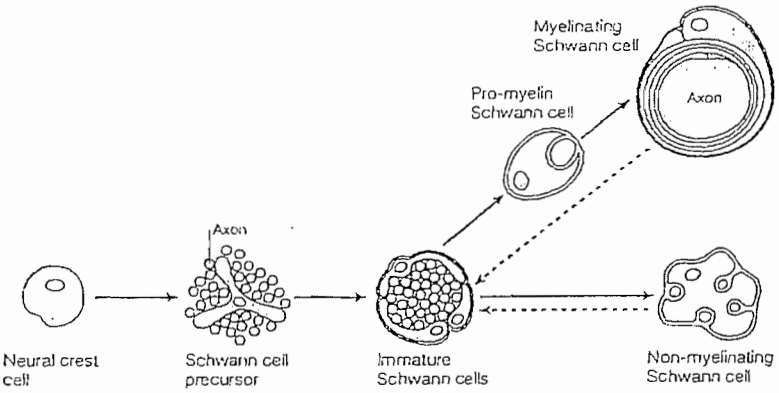


Figure 1 | **The Schwann cell lineage.** Schematic illustration of the main cell types and developmental transitions involved in Schwann cell development. Dashed arrows indicate the reversibility of the final, largely postnatal transition during which mature myelinating and non-myelinating cells are generated. The embryonic phase of Schwann cell development involves three transient cell populations. First, migrating neural crest cells, which are discussed further in BOX 1. Second, Schwann cell precursors (SCPs). These cells express various differentiation markers that are not found in migrating neural crest cells, including brain fatty acid-binding protein (BFABP), protein zero (P0) and desert hedgehog (DHH) (FIG. 4). At any one time, a rapidly developing population of cells — such as the glia of embryonic nerves — will contain some cells that are rather more advanced than others. However, the cells[24] isolated from embryonic day (E) 14 rat nerves by the P07 monoclonal P0 antibody[97] (referred to as 'neural crest stem cells'[23]) are unlikely to be significantly different from the bulk of cells in the nerve, which are referred to here as SCPs (for a detailed discussion, see REFS 98,99). Third, immature Schwann cells. All immature Schwann cells are considered to have the same developmental potential, and their fate is determined by the axons with which they associate. Myelination occurs only in Schwann cells that by chance envelop large diameter axons — Schwann cells that ensheath small diameter axons progress to become mature non-myelinating cells.

Other differences between the three main stages of embryonic Schwann cell development, also shown in FIG. 4, include the following. First, SCPs and immature Schwann cells, but not migrating neural crest cells, are intimately associated with neurons (axons), a characteristic attribute of glial cells (FIGS 2,5). Second, SCPs and migrating neural crest cells show numerous differences in their responses to survival factors[4,15,27]. Third, when compared with migrating neural crest cells, SCPs are also relatively insensitive to the neurogenic action of bone morphogenetic protein 2 (BMP2), and are strongly biased towards Schwann cell generation[28,29].

Additional differences between immature Schwann cells and SCPs include the presence of a basal lamina, which starts to form soon after Schwann cells are generated (A. Kumar, R.M. and K.R.J., unpublished observations). But perhaps the most striking difference between these cells is the ability of Schwann cells to ensure their own survival through the help of autocrine survival circuits[30]. These are missing in SCPs, leaving these cells wholly dependent on survival signals from axons, which are probably mediated to a large extent by β-neuregulin 1 (NRG1; see BOX 3 and below).

Box 2 | Glial cells of the peripheral nervous system

The PNS contains a number of distinct glial cells, each of which is intimately associated with different parts of the neuron or with specific neuronal cell types[94]. Neuronal cell bodies in dorsal root sensory ganglia and in sympathetic and parasympathetic ganglia are covered by flattened sheet-like cells known as satellite cells, whereas axons in nerve trunks are ensheathed by non-myelinating or myelinating Schwann cells, the best known of all peripheral glia. Unlike oligodendrocytes, the myelinating cells of the CNS, each Schwann cell forms myelin around one axon only. For reasons that are not understood, only the larger diameter (>~1 μm) axons of peripheral nerves are myelinated — smaller axons lie in troughs in the surface of non-myelinating Schwann cells. Distinct cells known as olfactory ensheathing cells envelop the axons of the olfactory nerve. Axon terminals at the skeletal neuromuscular junction are tightly covered by terminal glia (teloglia), whereas the terminals of autonomic neurons show only irregular associations with processes of non-myelinating Schwann cells. Many sensory nerve endings in the skin associate with glial cells that form the innermost part of larger structures, for example, the Pacinian corpuscle. Lastly, the complex ganglia of the enteric nervous system harbour glial cells, the enteric glia, that are remarkably similar to astrocytes.

The control of gliogenesis from the neural crest

It has been suggested that in the CNS, glial differentiation represents a 'default pathway' of neural stem cell differentiation[12,13]. We do not yet know whether this is a useful way of thinking about glial cell development in the PNS. However, the functions of the major signalling pathways that have been implicated in the control of gliogenesis from the neural crest — those that involve NRG1, BMP2 and 4, and Notch — are consistent with a default mode. This is because the most obvious functions of these signals in the neural crest are to suppress glial development, or to suppress or activate neuronal development[31–33]. It has been hard to prove that these factors positively initiate glial differentiation from neural crest cells. Similarly, although the transcription factor SOX10 is required for glial development, it is expressed by all neural crest cells and therefore does not seem to be part of a classical inductive signalling cascade for the activation of glial development[34].

A default model would also provide a relatively simple hypothesis explaining how, during gangliogenesis, neurons and glia could form in the same location. It would be sufficient to envisage neurogenic signals acting on neural crest cells, tempered by signals from early neurons (for example, NRG1 or Delta–Notch signalling; see below) that suppress excessive neurogenesis in neighbouring cells.

SOX10. Before investigating the often uncertain function of cell–cell signals in the initiation of glial development, we discuss the transcription factor SOX10, because *Sox10* is the only gene known to be essential for the generation of the glial lineage from trunk crest cells. SOX10 appears to be present in all migrating neural crest cells[34,35]. Expression persists in developing satellite glia in dorsal root sensory ganglia (DRG) and in SCPs in spinal nerves, but is downregulated in early neurons. Matching this pattern, satellite glia and SCPs are missing in mice in which *Sox10* is inactivated, whereas neurons are initially generated in normal numbers[34]. In these mutants, early DRGs contain neural crest-like cells instead of BFABP$^+$ satellite cells (FIG. 4), and nerve trunks also contain a few neural crest-like cells that lack BFABP$^+$. This indicates that in the absence of SOX10 glial specification is blocked, whereas neural crest cells thrive and are able to generate neurons. *In vitro* experiments also support a role for SOX10 in establishing

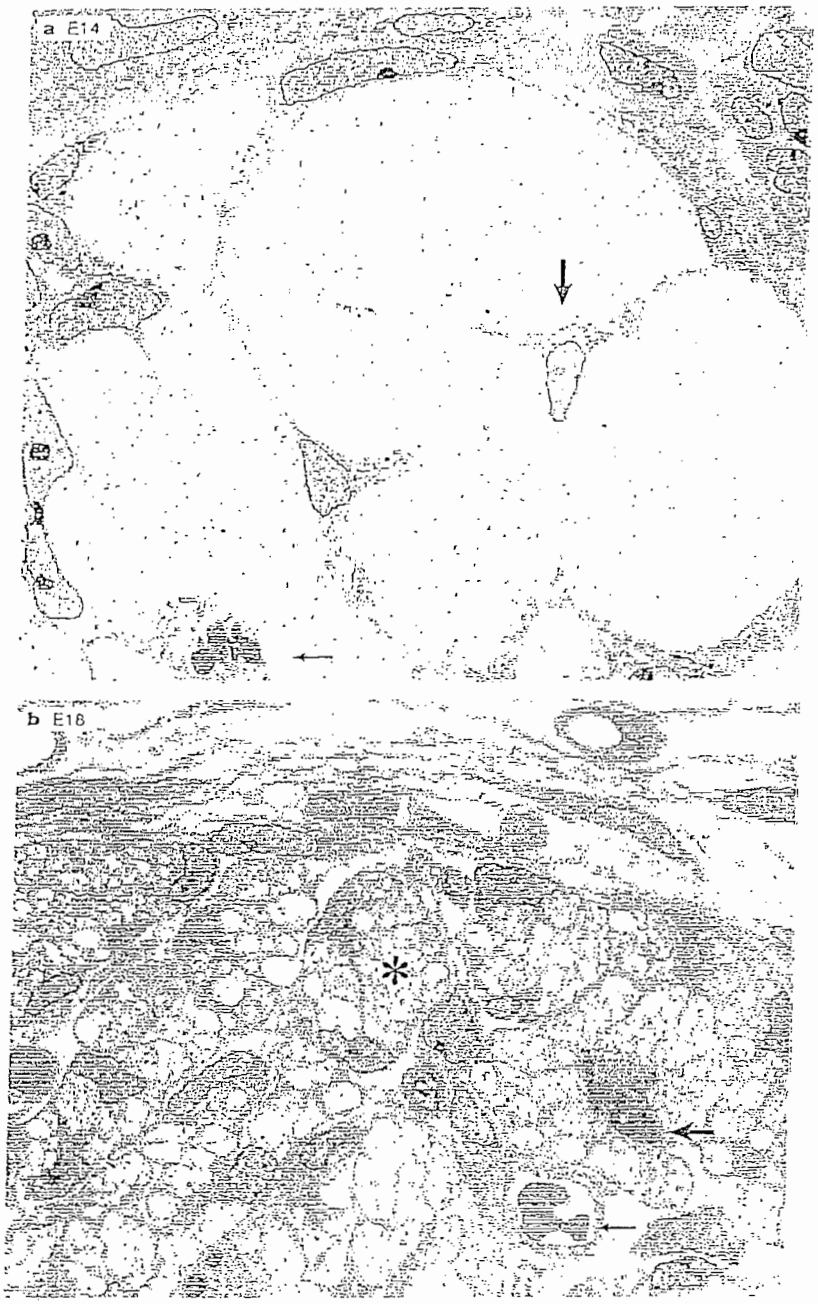


Figure 2 | **The appearance of early cells in the Schwann cell lineage.** a | An electron microscopic image of a transverse section of a nerve in the hindlimb of a rat embryo at embryonic day (E) 14. Schwann cell precursors (SCPs) branch among the axons inside the nerve (big arrow) and are also found in close apposition to axons at the nerve surface. One precursor cell is undergoing mitosis (small arrow). Extracellular connective tissue space (turquoise), which contains mesenchymal cells, surrounds the nerve but is essentially absent from the nerve itself. These nerves are also free of blood vessels and the axons are of smaller and more uniform diameter than those seen in mature nerves. Magnification, x2000. b | Schwann cells in a transverse section of the sciatic nerve of a rat embryo at E18, shown at the same magnification. In marked contrast to the nerve at E14, connective tissue spaces now branch throughout the nerve among compact bundles of immature Schwann cells and their associated axons ('Schwann cell families'[35], for example see asterisk). Blood vessels (small arrow) and fibroblasts (for example, directly above the vessel) have also appeared inside the nerve. One Schwann cell is undergoing mitosis (big arrow). Outside the nerve (in the uppermost part of the picture) connective tissue, which contains flattened fibroblasts of the early developing perineurium and two blood vessels, can be seen.

and maintaining a glial phenotype[35]. One function of SOX10 might be to maintain the ability of early glia to respond to NRG1, as the neuregulin receptor ErbB3 is downregulated in *Sox10* mutants[34].

Neuregulin 1. In neural crest cell cultures, NRG1 inhibits the development of neurons[31], a function that might lead, indirectly, to increased gliogenesis. It is not clear whether NRG1 also restrains neurogenesis *in vivo*, as overproduction of neurons has not been noted in NRG mutants. In similar cultures, GFAP$^-$ Schwann cells appear readily even without the addition of NRG1 (REF. 31), and the same is true if the activation of protein zero (P0) expression, an earlier SCP marker, is used (N. Kazakova, R.M. and K.R.J., unpublished observations). A major population of neural crest-derived glia, the satellite cells of DRGs, is also apparently unaffected in mouse mutants in which NRG1 or the NRG1 receptors ErbB2 or 3 are missing (although SCPs are lost; see below)[16]. Together, these findings indicate that NRG1 signalling is not required for glial differentiation from the neural crest.

The NRG mutants point to two important alternative functions for NRG1 in the development of neural crest derivatives. First, NRG1 is required for the migration of neural crest cells past the location of the DRGs to reach the ventral sites of sympathetic gangliogenesis, as shown by the finding that sympathetic ganglia are hypoplastic in neuregulin mutants[36]. Second, SCPs and, later, Schwann cells are absent or seriously depleted in spinal nerve trunks of the mutant animals[6,7,35]. This probably reflects the role of NRG1 as an axon-derived survival factor and mitogen for SCPs, and failure of migration of precursors from DRGs into spinal nerves might also play a part[9,35]. NRG1 is an essential survival factor for SCPs *in vitro*, and stimulates SCP proliferation. It is also the signal by which DRG neurons rescue SCPs from death *in vitro*[9]. *In vivo*, NRG1 is present at the right time and place to control SCP survival, because it is found in DRG and motor neurons at the stage at which SCPs populate spinal nerves, and it accumulates along axon tracts[39–42]. SCPs depend on axons for survival *in vivo*, and the SCP death that follows nerve injury can be prevented by the application of NRG1 (REF. 43). Comparable results have been obtained with neonatal Schwann cells and terminal glia (known as teloglia)[41,45].

All of these observations indicate that NRG1 is an essential survival factor for SCPs in embryonic nerves. It is likely that the main NRG1 variant responsible for this function is the transmembrane type III isoform, which is the main neuregulin in DRG and motor neurons. In mice that selectively lack this isoform, SCPs initially populate spinal nerves, but their number is severely depleted by E14, which shows that the type III isoform is necessary for the survival of SCPs *in vivo*[35].

NRG1 also promotes Schwann cell survival and proliferation and is likely to be an important component of the axon-associated signal that drives Schwann cell division prior to myelination[16]. There is also evidence that NRG1 accelerates the production of Schwann cells from SCPs (see below).

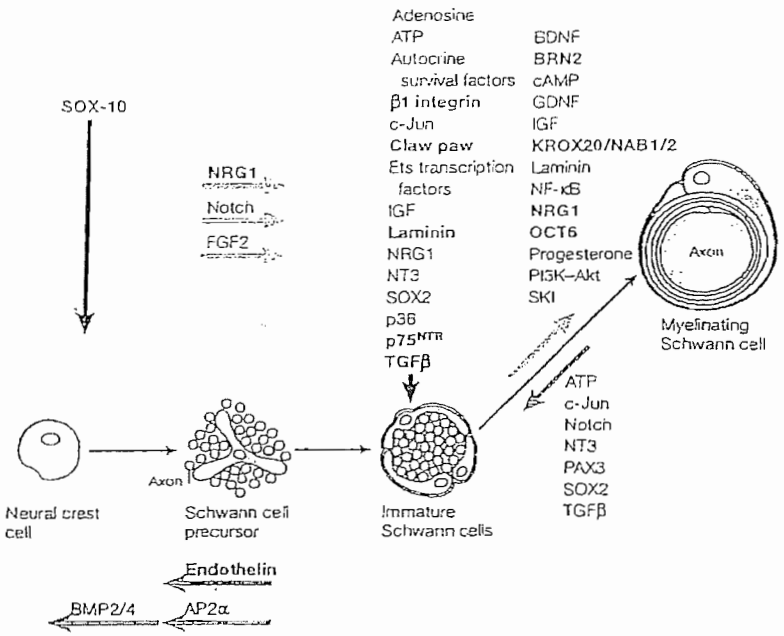


Figure 3 | Some of the factors that have been implicated in the control of early Schwann cell development and myelination. Evidence for molecules shown in bold is based on *in vivo* observations in mutant animals. The other molecules have been implicated *in vitro*. In some cases the *in vitro* evidence is substantially more complete than in others. SRY (sex determining region Y) box 10 (SOX10) is required for the generation of all peripheral glia from the neural crest[34], whereas bone morphogenetic proteins (BMPs) inhibit glial differentiation[32]. Axon-derived neuregulin 1 (NRG1), in particular the type III isoform, is necessary for the survival of Schwann cell precursors (SCPs) in embryonic nerves both *in vitro* and *in vivo*[9,10,35]. NRG1, fibroblast growth factor 2 (FGF2) and Notch accelerate the SCP–Schwann cell transition (REFS 33,55,100,101; A. Woodhoo, R.M. and K.R.J., unpublished observations), whereas the transcription factor activator protein 2α (AP2α) and endothelins delay it[55]. In immature Schwann cells, survival is supported by autocrine survival factors, NRG1, Ets transcription factors and laminin[30,44,45,74,76,102], whereas transforming growth factor-β (TGFβ) and the p75 neurotrophin receptor (p75^{NTR}) induce Schwann cell death (REFS 81,82; M. D'Antonio, A. Droggiti, R.M. and K.R.J., unpublished observations). *In vitro* experiments indicate that NRG1 is an axon-associated Schwann cell mitogen, but proliferation is also supported by TGFβ, laminin and various other factors (REFS 46,73,74,104; M. D'Antonio, A. Droggiti, R.M. and K.R.J., unpublished observations). The transcription factors SOX2 and c-Jun support proliferation[83,84], although c-Jun is also required for cell death[83]. ATP and adenosine, however, inhibit Schwann cell division[87]. NRG1, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), insulin-like growth factors (IGFs) and the p38 pathway function in Schwann cell migration and/or association with axons prior to myelination[75–78]. Radial sorting is impaired in claw paw, laminin and β1 integrin mutants[70–72,77]. Myelination is promoted by the transcription factors early growth response 2 (EGR2, also known as KROX20) with NGFI-A-binding (NAB) proteins, octamer-binding transcription factor 6 (OCT6) and brain 2 class III POU-domain protein (BRN2)[89–91], and inhibited by c-Jun, paired box gene 3 (PAX3) and SOX2 (REFS 83,85,86; D. B. Parkinson, R.M. and K.R.J., unpublished observations). Cell-extrinsic signals that promote myelination include glial cell line-derived neurotrophic factor (GDNF), NRG1, IGFs, BDNF, progesterone and laminin[1,42,105–108]. Intracellular phosphatidylinositol 3-kinase (PI3K)–Akt (v-akt murine thymoma viral oncogene homologue) and cyclic AMP (cAMP) activated pathways also promote myelination, whereas it is blocked by Notch activation. NT3 and ATP (REFS 4,87,88,107,109; A. Woodhoo, R.M. and K.R.J., unpublished observations). TGFβ also inhibits myelination, whereas v-ski sarcoma viral oncogene homologue (SKI), which suppresses TGFβ, stimulates it[93,110,111]. NF-κB, nuclear factor-κB.

Therefore, NRG1 acts in a number of different ways to promote the generation and expansion of immature Schwann cells. Furthermore, the inhibitory effect of NRG1 on neurogenesis[31] might act indirectly to increase the production of glial cells, by prolonging the time available for uncommitted neural crest cells to adopt a glial fate. It is likely that the enhancement of Schwann cell generation by NRG1 that has been seen in various *in vitro* situations occurs through a combination of these mechanisms.

Studies on zebrafish indicate that although ErbB2/3 signalling is not needed for Schwann cell generation, it is required for proliferation and migration of Schwann cells along the posterior lateral line[37].

Notch. In the CNS, enforced Notch activation *in vivo* directly or indirectly promotes the generation of glial cells[48]. In the Schwann cell lineage there are intriguing similarities between the actions of Notch and NRG1. Notch activation inhibits the generation of neurons in neural crest cell cultures and increases the number of GFAP$^+$ Schwann cells[29,33,49]. There is also evidence that Notch activation, like NRG1 activation, stimulates the formation of Schwann cells from SCPs (see below) and stimulates Schwann cell proliferation (A. Woodhoo, R.M. and K.R.J., unpublished observations). Cooperative interactions between Notch and NRG1 signalling have been noted previously in the development of astrocytes[50]. It is not yet clear whether Notch acts instructively on neural crest cells to promote gliogenesis. As with NRG1, alternative explanations for the Notch-mediated increase in GFAP$^+$ Schwann cells in neural crest cultures include indirect effects due to inhibition of neurogenesis, stimulation of the SCP–Schwann cell transition, and stimulation of Schwann cell proliferation.

BMP2 and 4. BMPs are important for the generation of sympathetic neurons *in vivo* and stimulate the formation of neurons in neural crest cultures[32,51]. *In vitro*, BMPs are also powerful blockers of glial differentiation from the neural crest[32]. Whether this has a role in normal neural crest development *in vivo* remains to be determined.

The formation of immature Schwann cells

In rats, SCPs convert to immature Schwann cells between E15 and E17 (E13–E15 in mice). This entails a coordinated change in molecular expression, and response to survival signals and mitogens (FIG. 4). At this time connective tissue spaces also open up within the nerves, which become vascularized, and a distinct layer of developing perineurium appears at the nerve surface. The SCP–Schwann cell transition therefore correlates with an important step in the organogenesis of peripheral nerves.

Control of cell survival also changes at this transition, because Schwann cells (but not SCPs) can support their own survival in an autocrine way by secreting a cocktail of survival factors, which, *in vitro*, has been shown to include insulin-like growth factor 2 (IGF2), neurotrophin 3 (NT3), platelet-derived growth factor-β (PDGFB), leukaemia inhibitory factor (LIF) and lysophosphatidic acid (LPA)[30,52–53]. These autocrine survival circuits are probably important in maintaining the survival of Schwann cells in injured nerves even after axons have degenerated. The switch from paracrine dependence on axonal signals to autocrine support of

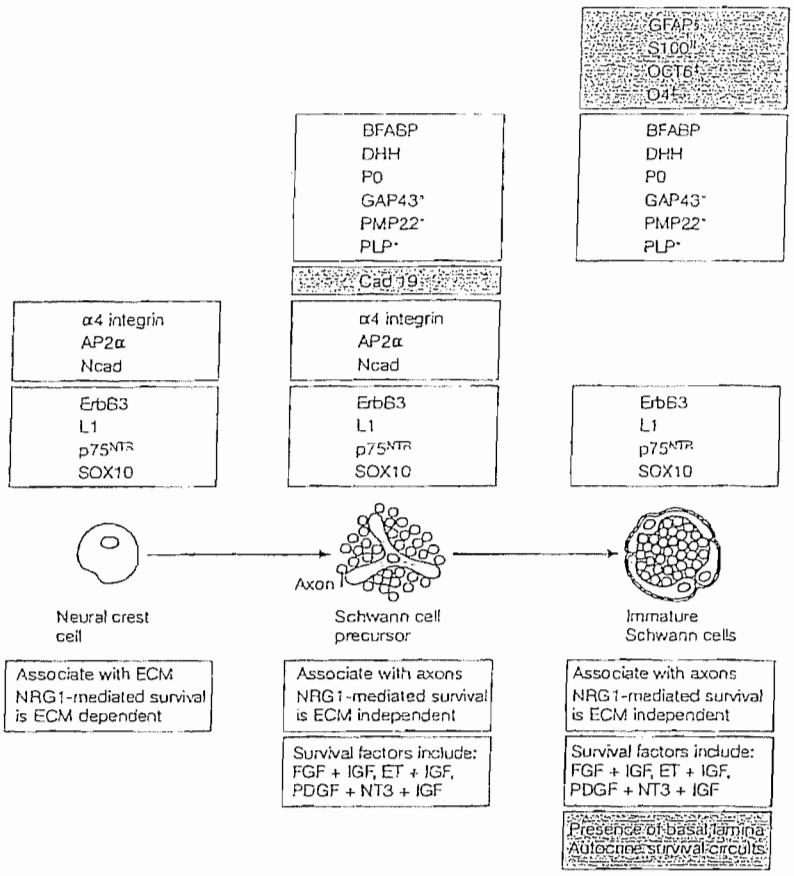


Figure 4 | **Changes in phenotypic profile as cells progress through the embryonic Schwann cell lineage.** Shared profiles are indicated by distinct colours. The boxes above the lineage drawing indicate the changes in gene expression that take place during embryonic Schwann cell development. The gene expression shown here is based on observations of endogenous genes rather than on observations of reporter genes in transgenic animals. Note that Cadherin 19 (Cad 19) is exclusively expressed in Schwann cell precursors (SCPs)[113]. Each developmental stage also involves characteristic relationships with surrounding tissues, and distinctive cell signalling properties (boxes below lineage drawing). For instance, neural crest cells migrate through extracellular matrix. By contrast, SCPs and Schwann cells are embedded among neurons (axons) with minimal extracellular spaces separating them from nerve cell membranes, a characteristic feature of glial cells in the CNS and PNS. Basal lamina is absent from migrating crest cells and SCPs, but appears on Schwann cells. *In vitro*, β-neuregulin 1 (NRG1) only supports neural crest survival in the presence of extracellular matrix (ECM), although this is not required for the NRG1-mediated survival of SCPs and Schwann cells[27]. Migrating neural crest cells also fail to survive in the presence of several factors that support the survival of SCPs and Schwann cells[27], including combinations such as fibroblast growth factor (FGF) plus insulin-like growth factor (IGF), endothelin (ET) plus IGF, and platelet-derved growth factor (PDGF) plus neurotrophin 3 (NT3) and IGF. Schwann cells also have autocrine survival circuits that are absent from SCPs[30]. *Proteins that also appear on neuroblasts/early neurones. ‡Markers that are acutely dependent on axons for expression. §Glial fibrillary acidic protein (GFAP) is a late marker of Schwann cell generation, as significant expression is not seen until about the time of birth. GFAP is reversibly suppressed in myelinating cells. The early expression of GFAP has not yet been carefully examined in mice. ||SCPs have been shown to be S100 calcium-binding protein (S100)-negative and Schwann cells S100-positive using routine immunohistochemical methods — however, low levels of S100 are detectable in many mouse SCPs when the sensitivity of the assay is significantly increased. α4 integrin[11] (V. Sahni and K.R.J. unpublished observations); AP2-α, activator protein 2α[54]; BFABP, brain fatty acid-binding protein[31]; DHH, desert hedgehog[114,115]; ErbB3, neuregulin receptor[10]; GAP43, growth associated protein 43 (REF. 8); L1, L1 adhesion molecule[4]; N-cad, N-cadherin (I. Wanner and K.R.J., unpublished observations); OCT6, octamer-binding transcription factor 6 (REF. 116); O4, lipid antigen[116]; PLP, proteolipid protein[117]; PMP22, peripheral myelin protein, 22-kDa[22]; P0, protein zero[118]; p75^{NTR}, p75 neurotrophin receptor[4]; SOX10, SRY (sex determining region Y) box 10 (REF. 34).

survival makes biological sense. The former provides a mechanism for matching the numbers of axons and SCPs, whereas the axon-independence of Schwann cell survival ensures that if postnatal nerves are injured, Schwann cells survive to provide essential support for axon regrowth.

Little is known about the transcription factors that control the SCP–Schwann cell transition. It has been suggested that AP2α provides negative regulation, because this factor is strongly downregulated as Schwann cells are generated *in vivo*, and enforced expression in SCPs delays Schwann cell generation *in vitro*[54]. Another negative regulator of Schwann cell generation is endothelin. Endothelins and their receptors are present in embryonic nerves, and in rats with defective endothelin B receptors Schwann cells form prematurely[55]. Evidence for positive regulation of the SCP–Schwann cell transition comes from *in vitro* experiments indicating that NRG1 accelerates the conversion of SCPs to Schwann cells, as well as promoting SCP survival and proliferation[55]. Notch activation also promotes the generation of Schwann cells from SCPs *in vitro* (REF. 33; A. Woodhoo, R.M. and K.R.J., unpublished observations).

Novel origin of Schwann cells in spinal roots

Most of our detailed knowledge about the development of SCPs and immature Schwann cells comes from studies on the spinal nerve trunks that innervate the limbs. How much of it holds true for Schwann cell development in the dorsal and ventral roots of the spinal cord? The observation that S100 appears relatively early in the roots had already indicated that the developmental schedule, at least, differs between root and limb nerves[56]. Now, a study of the fate of boundary cap cells of the spinal cord has revealed a more fundamental difference between these Schwann cell populations[57]. Boundary cap cells originate in the neural crest and take up positions in clusters where dorsal and ventral roots enter and exit the spinal cord. They can be identified by the expression of early growth response 2 (*Egr2*, also known as *Krox20*) long before this gene appears in myelinating Schwann cells. When the fate of these cells is traced *in vivo* using *Krox20*-driven Cre recombinase, it transpires that these cells not only give rise to a small subset of nociceptive neurons and some satellite cells within the DRG, but also give rise to all of the Schwann cells in dorsal roots and many of the Schwann cells in ventral roots. Therefore, boundary cap cells constitute a relatively late reservoir of neural precursors that contribute to the generation of neurons and glia in the PNS. In spinal nerves, few or no boundary cap cell-derived glia were detected. Therefore these findings do not affect the classical notion that the Schwann cells of limb nerves originate in migrating neural crest cells.

The function of Schwann cell precursors

It is generally believed that glial cells provide trophic support for developing neurons. Persuasive *in vivo* evidence for this mechanism comes from investigations

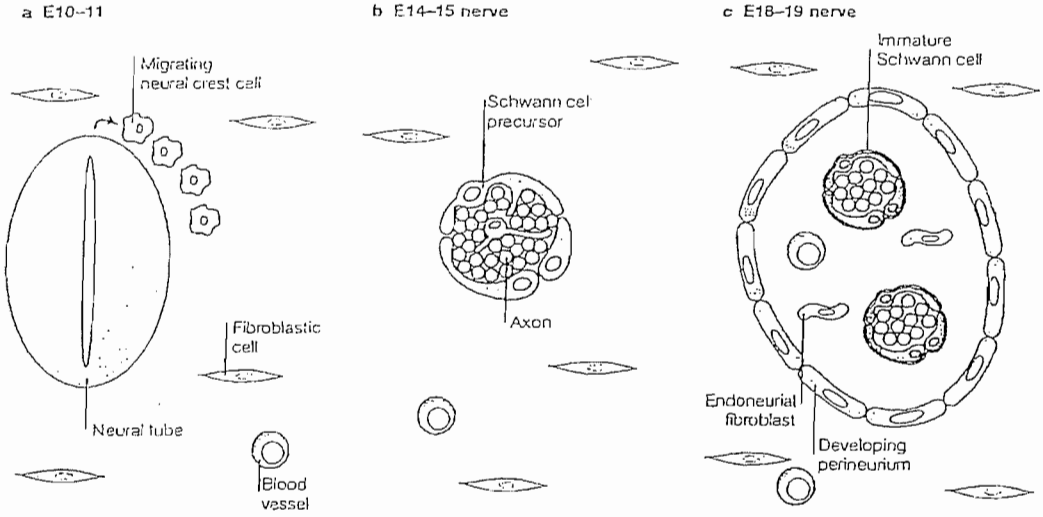


Figure 5 | **Cell and tissue relationships at key stages of Schwann cell development in rodents.** There is a simple relationship between the main stages of embryonic Schwann cell development and organogenesis of spinal nerves. **a** | Migrating crest cells move through immature connective tissue before the time of nerve formation. **b** | Schwann cell precursors (SCPs) are tightly associated with axons and are found in early nerves that are still compact and do not yet contain blood vessels or connective tissue. **c** | Immature Schwann cells are found in nerves that have acquired the basic tissue relationships of adult nerves. By this time, the developing perineurium defines the endoneurial space that now contains, in addition to axon–Schwann cell units, blood vessels, endoneurial fibroblasts and extracellular matrix.

of embryonic nerve development, in which it has been possible to examine what happens when glial cells are deleted from a significant part of the nervous system. This occurs in the major limb nerves of mouse embryos that lack SOX10, isoform III of NRG1, or the NRG1 receptors ErbB2 or ErbB3, owing to the importance of these molecules in gliogenesis and glial survival, as discussed above. Strikingly, in these mutants most of the DRG neurons and motor neurons that project into limb nerves die by E14 and E18 respectively, although these cells are initially generated in normal numbers. This suggests that one of the functions of SCPs and immature Schwann cells is to provide essential survival signals for developing neurons[6,10,34]. Impaired axon–target contacts probably contribute to sensory and motor neuron death in the isoform III neuregulin mutants[38].

Taken together with the finding that axons control the survival of SCPs (see above), these studies identify a discrete phase in early nerve development when neurons and glia depend on each other for survival. An intriguing possibility is the control of neuronal survival by SCPs through back-signalling by the intracellular domain of NRG1 (REF. 58). If this is the case, mutual survival of neurons and glia in embryonic nerves would be ensured by a bidirectional effect of the same molecular interaction, namely the binding of NRG1 to ErbB2 and ErbB3 receptors.

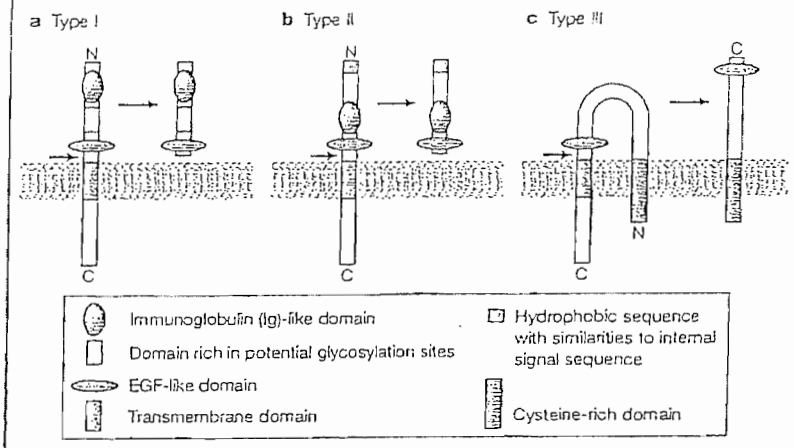
An unexpected morphogenetic role has also been identified for early glia of ventral roots[59]. This function has been seen in a number of mouse lines in which these cells are missing. Such lines include *Sox10* mutants, which fail to generate all glial neural crest derivatives (of which boundary cap cells are a subset), mice in which the boundary cap cells have been selectively killed by diphtheria toxin inserted into the *Krox20* locus, and paired box gene 6 (*Pax6*) mutants, in which failure of neural crest migration leads to the absence of glia in caudal peripheral nerves. In all of these animals the cell bodies of motor neurons are displaced into ventral roots[59]. It seems, therefore, that one of the functions of boundary cap cells, or the SCPs that are derived from them, is to maintain the position of motor neurons in the spinal cord.

Another morphogenetic function of neural crest-derived glia in peripheral nerves is seen in the lateral line of the zebrafish, in which developing glial cells control the formation of secondary neuromasts, organs that are specialized for the detection of water movement[60,61]. SCPs and immature Schwann cells are also important for normal fasciculation of peripheral nerves[7,37].

Recently, a number of classical ideas about developmental potential and lineage restrictions have been challenged by unexpected observations. Probably the most notable example from the nervous system is the finding that neurons can arise from cells that show obvious similarities to astrocytic glial cells, both during development and in the adult[12,62,63].

Another example of a surprising lineage relationship involving glial cells seems to occur in embryonic nerves[11]. In this case, genetic lineage tracing indicates that the relatively small population of fibroblasts found inside nerves (5–10% of the number of Schwann cells at birth) originates from cells in the nerve that are neural crest derived and express desert hedgehog (DHH), and are, therefore, presumably SCPs (FIG. 4). The idea

Box 3 | **Neuregulin 1**



Neuregulin 1 (NRG1) seems to have exceptionally numerous and varied functions in Schwann cell biology. It is involved in neural crest migration, and has been implicated in the lineage specification of neural crest cells and shown to be essential for the survival of Schwann cell precursors (SCPs). It is also involved in Schwann cell generation, proliferation and survival. In postnatal nerves, NRG1 is a positive regulator of myelin sheath thickness, but, paradoxically, also appears to drive the de-differentiation of myelinating cells in injured nerves. No other signalling molecule has been proposed to be so comprehensively involved in the control of Schwann cell development.

There are a surprisingly large number (>15) of NRG1 protein isoforms. The schematic structures of the main isoforms found in the nervous system are shown in the panel. Although splice variants without the transmembrane domain exist for all of these isoforms, transmembrane isoforms (as shown here together with the products of a proteolytic cleavage in the juxta-membrane area) predominate in the nervous system. The epidermal growth factor (EGF) domain is found in all bioactive forms of NRG1 and is sufficient for activation of ErbB receptor-kinase activation. The type III isoform is expressed in axons and is the main regulator of survival of SCPs and myelin sheath thickness. It is thought to have two membrane-spanning domains and to undergo proteolytic cleavage that generates a membrane attached protein carrying the EGF domain[95,96].

NRG1 shows high affinity binding to two receptors, ErbB3 and ErbB4, whereas a related protein, ErbB2, acts as a co-receptor in ErbB3–ErbB2 and ErbB4–ErbB2 complexes. The former is the main NRG1 receptor in peripheral glial cells. The action of axonal NRG1 type III on ErbB3–ErbB2 in developing Schwann cells is probably the best established molecular signalling pathway between neurons and glia in the PNS.

that SCPs generate some fibroblasts in addition to Schwann cells fits well with the observation that both cell types appear in the nerve at the SCP–Schwann cell transition (A. Kumar, R.M. and K.R.J., unpublished observations; also, see above). It is also consistent with the principle, established in previous experiments, that early PNS glia from rodents and birds can generate cells other than Schwann cells. This was first shown in the quail, when P0$^-$ cells from embryonic nerves were found to generate melanocytes *in vitro*[21,61]. More recently, chick SCPs expressing the glial-specific protein SMP (Schwann cell myelin protein) were induced to generate melanocytes by exposure to endothelin, a signal that also seems to act more broadly to promote plasticity of neural crest derivatives[25,65]. The appearance of melanocytes has even been documented in injured nerves of adult mice, particularly in lines that are heterozygous for the neurofibromin 1 gene[66]. In addition, in the rat, P0$^-$ cells from early nerves or DRGs have been shown to have the potential to generate neurons and fibroblasts[22,23].

The generation of fibroblasts from SCPs *in vivo*, if confirmed, would bring the trunk crest lineage in line with the cardiac and cephalic crest lineages, which have long been known to generate connective tissue[67]. But these observations on the developmental potential of SCPs are also in agreement with the emerging concept that early glia can act as multipotent progenitors in the developing nervous system[12,13].

Direct experimental evidence for the more intuitive, if prosaic, idea that SCPs generate Schwann cells includes the following. It is the simplest explanation for the observation that, in spinal nerves of the limbs, SCPs disappear as Schwann cells appear at E15–E17, with substantial numbers of both cell types being present only at E16. Also, in SCP cultures prepared from E14 nerves, the gradual appearance of cells with the phenotype of Schwann cells can be observed directly as SCPs disappear. *In vitro*, the conversion of most of the SCPs to Schwann cells is completed in 3–4 days, which is close to the time course of the SCP–Schwann cell transition *in vivo*[8,9]. Lastly, in mouse mutants that lack SCPs, Schwann cells are not generated[10,31].

A significant narrowing of developmental options clearly takes place at the SCP–Schwann cell transition. Immature Schwann cells have not yet been seen to de-differentiate to SCPs and, *in vitro*, immature Schwann cells are resistant to signals, including BMP2 and fibroblast growth factor 2 (FGF2), that are able to induce the generation of other neural crest derivatives from SCPs[21,23,33]. Their only option seems to be the reversible generation of myelinating or non-myelinating cells, fates that are determined by axon associated signals (see above).

Preparing for myelination

The transition of SCPs to Schwann cells coincides with a major change in the cellular architecture of peripheral nerves. At E14 in rats, limb nerves consist of axons and SCPs. The SCPs are found at the outer margin of the nerves, and inside them as well, connecting to each other through sheet-like processes that communally envelop large numbers of axons. These nerves are compact structures — there are no significant connective tissue spaces, the cellular elements are separated by only minimal gutters and blood vessels are absent (FIG. 2). By E18, the nerves consist of irregular axon–Schwann cell bundles (similar to the 'families' described in newborn nerves[68]) that are surrounded by connective tissue spaces containing endoneurial fibroblasts and blood vessels (A. Kumar, R.M. and K.R.J., unpublished observations). This is the basic relationship between nervous tissue, connective tissue and blood vessels that is seen in adult nerves. We still know little about the signals that control these complex changes and govern the organogenesis of peripheral nerves (FIG. 5).

Around E18 in rats, immature Schwann cells are found communally ensheathing large groups of axons, whereas myelination starts some 3 days later, at birth. This requires radial sorting — a process of radical change in cellular relationships that allows individual Schwann cells to start myelinating single large diameter axons. At the same time, Schwann cell numbers are adjusted by controlling survival and proliferation. While these events take place, premature myelination seems to be prevented by the activity of a number of signalling systems that function as 'myelination brakes'.

Radial sorting. In a process that continues postnatally, the Schwann cell families of late embryonic nerves gradually erode as individual Schwann cells, each associated with a single large diameter axon, segregate from them to form the pro-myelin stage, which is characterized by a 1:1 relationship between axons and Schwann cells[68]. The molecular control of this crucial step is poorly understood. It is impaired in laminin-defective mutants and in the absence of β1 integrin, which is a component of Schwann cell laminin receptors, and also in the claw paw mutant, which has a defect in myelination[69–71].

Various factors affect Schwann cell migration in cell culture and it is possible that these signals govern Schwann cell movements during radial sorting *in vivo*. They include NRG1, IGFs, NT3 and brain-derived neurotrophic factor (BDNF)[72–77]. There is also evidence that activity in the p38 mitogen-activated protein kinase (MAPK) pathway is required prior to myelination, perhaps to attain the correct alignment between axons and Schwann cells[78].

The control of Schwann cell numbers. Another challenge during the late postnatal period is to match the numbers of Schwann cells and axons. Because the period of neuronal death is largely over, this comes down to controlling the rates of Schwann cell survival and proliferation.

Evidence that the axons themselves are major stimulators of Schwann cell proliferation comes from *in vitro* experiments[79], and the idea is supported by the observation that, *in vivo*, Schwann cell proliferation decreases as Schwann cells lose contact with axons in transected nerves in newborn animals[80]. Co-culture studies using Schwann cells and neurons indicate that NRG1 is a major axonal mitogen, but this has not yet been confirmed in mammals *in vivo*[16,17].

Another potential Schwann cell mitogen is transforming growth factor-β (TGFβ). This is found in embryonic nerves, and excision of the type II TGFβ receptor reduces Schwann cell proliferation in E19 mouse sciatic nerves, which shows that TGFβ is directly or indirectly involved in controlling Schwann cell division *in vivo* (M. D'antonio, J. Roes, R.M. and K.R.J., unpublished observations). Interactions with laminin also promote Schwann cell proliferation in developing nerves[73,74].

The survival of immature Schwann cells in late embryonic and perinatal nerves is probably controlled by a balance between factors that support survival and factors that cause death. Survival support comes from axon associated NRG1 and autocrine circuits, as discussed above, and from laminin associated with the basal lamina[30,71]. Two death signals have been identified *in vivo*. One of these acts through the p75 neurotrophin receptor ($p75^{NTR}$), perhaps following activation by binding to nerve growth factor (NGF), and is required for the elevated Schwann cell death that is seen in newborn nerves following injury[81]. The other is likely to be TGFβ, as deletion of type II TGFβ receptors suppresses the normal developmental death in E18 to newborn nerves. The elevated death that follows injury in neonates is also suppressed by injection of TGFβ-blocking antibodies (REF. 82; M. D'antonio, J. Roes, R.M. and K.R.J., unpublished observations) and by deletion of the TGFβ receptor in Schwann cells.

Differentiation brakes. There is now evidence that various signalling pathways that inhibit myelin differentiation are active in immature Schwann cells, and that these pathways are suppressed at the onset of myelination. For instance, the c-Jun-amino (N)-terminal kinase (JNK) pathway is active in Schwann cells of E18 to newborn nerves, where it is required for NRG1 and TGFβ signalling[83]. This pathway is inactivated in individual cells as they start to myelinate by a mechanism that depends on the myelin-associated transcription factor KROX20. If this is prevented, and the JNK pathway remains active, myelination in neuron–Schwann cell co-cultures is blocked and myelin gene expression that would normally result from pro-myelin signals, such as KROX20 or the elevation of cyclic AMP, is inhibited (REF. 84; D. B. Parkinson, A. Bhaskaran, R.M. and K.R.J., unpublished observations). Similarly, Notch signalling promotes proliferation of immature Schwann cells, but is suppressed as cells start to myelinate, and if this is prevented, myelination is blocked (A. Woodhoo, R.M. and K.R.J., unpublished observations). An analogous pattern of action is seen in the transcription factors PAX3 and SOX2: these are expressed before myelination and are involved in proliferation. They are downregulated in myelinating cells and exert a negative effect on myelin differentiation[85,86]. In neuron–Schwann cell co-cultures, axon-derived ATP also delays myelination[87].

These studies are starting to define the signals that determine the immature Schwann cell state. Myelination is activated by inhibition of these pathways together with activation of pro-myelin pathways, which involve the transcription factors KROX20, octamer-binding transcription factor 6 (OCT6) and brain 2 class III POU-domain protein (BRN2), NGFI-A-binding proteins 1 and 2 (NAB1/2), phosphatidylinositol 3-kinase (PI3K) signalling and v-ski sarcoma viral oncogene homologue (SKI)[86,88–93]. The mechanism that initiates these switches remains unclear.

Summary and perspectives

Some 10 years ago, the first potent molecular regulators of glial development from the neural crest were identified, and it was realised that embryonic nerves contain a distinct cell type that is positioned between

neural crest cells and Schwann cells. The first transcription factors that control Schwann cell myelination were also discovered.

Our knowledge of PNS glial development has since been transformed. The main transitions from migrating neural crest cells to myelinating and non-myelinating Schwann cells have been established and each developmental stage can now be unambiguously defined by molecular profile, signalling responses and tissue relationships. Although many important cell-intrinsic, paracrine and autocrine signals have been identified, NRG1, in particular the axon-associated type III isoform, has emerged as a signalling molecule of fundamental importance and considerable versatility, as it is likely to carry out different functions at different stages of the lineage. Understanding how the glial lineage is established from the neural crest, and the role that positive and/or negative inductive signals or default mechanisms have in this key event remain challenging areas. Another important step will be the clarification of the signals that induce myelination.

In many ways our ideas about CNS and PNS glial cells have changed along a similar trajectory in recent years. In both cases, new and unexpected glial functions have been determined and glial cells have been shown to have surprisingly broad developmental potential. It is even possible that they are interchangeable in development, although this has not been tested. Both classes of glia are also increasingly recognized as sources of signals that are essential for the survival and function of neurons and other cells. This key function of glial cells is likely to be more tractable in peripheral nerves because of their relative simplicity. A related issue is the question of to what extent glial cells determine and maintain the higher order organization of the tissue in a specific environment. In peripheral nerves, this issue can be addressed by testing the role of glial-derived signals not only in organogenesis — namely the processes that establish correct tissue relationships during development — but also in the maintenance of tissue function and integrity throughout life.

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Signals to promote myelin formation and repair

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Abstract

The myelin sheath wraps large axons in both the CNS and the PNS, and is a key determinant of efficient axonal function and health. Myelin is targeted in a series of diseases, notably multiple sclerosis (MS). In MS, demyelination is associated with progressive axonal damage, which determines the level of patient disability. Few treatments are available for combating myelin damage in MS and related disorders. These treatments, which largely comprise anti-inflammatory drugs, only show limited efficacy in subsets of patients. More-effective treatment of myelin disorders will probably result from early intervention with combinatorial therapies that target inflammation and other processes—for example, signaling pathways that promote remyelination. Indeed, evidence suggests that such pathways might be impaired in pathology and, hence, contribute to the failure of remyelination in such diseases. In this article, we review the molecular basis of signaling pathways that regulate myelination in the CNS and PNS with a focus on differentiation of myelinating glia. We also discuss factors such as extracellular molecules that act as modulators of these pathways. Finally, we consider the few preclinical and clinical trials of agents that augment this signaling.

Introduction

Most large axons in the mammalian nervous system are surrounded by a lipid-rich membrane known as the myelin sheath, which promotes rapid conduction of nerve impulses and protects against axonal damage. Myelin sheaths form during development and consist of compacted spiral wraps of membrane that are supplied by oligodendrocytes in the CNS and Schwann cells in the PNS (Box 1 and Figure 1). These myelinating glia and their target axons form intimate units, with the glia and axons regulating each other's phenotype through the reciprocal exchange of signals (Boxes 2–4).

During development, glia provide survival signals to neurons, define the molecular domains of the axolemma and determine the diameter of axons.[1,2] In turn, axons provide signals that regulate the proliferation, survival and differentiation of glia, as well as myelin formation.[3–5] In adulthood, myelinating glia maintain axolemmal organization, axonal diameter and neuronal health, while axons maintain glial differentiation and myelin integrity.[2] At least in

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Competing interests
The authors declare no competing interests.

Review criteria
We searched PubMed for articles published up until January 2010 using the terms "axon signaling and glia", "myelin", or "remyelination". We also visited the Inherited Peripheral Neuropathies Mutation Database (http://www.molgen.ua.ac.be/CMTMutations/default.cfm), and consulted our personal collections of literature. In addition, we searched the clinical trial registries http://clinicaltrials.gov/ and http://www.controlled-trials.com/mrct/ with the search terms myelin or remyelination.

the PNS, axonal damage alters these maintenance signals and initiates an active program of Schwann cell dedifferentiation, which probably facilitates myelin reabsorption.[6]

Multiple diseases target myelin, including multiple sclerosis (MS) and hereditary leukodystrophies in the CNS, and Guillain–Barré Syndrome and hereditary demyelinating neuropathies in the PNS.7 In general, the degree of disability observed in myelin disorders correlates best with the level of associated axonal damage.8, 9 In most disorders, axonal damage is recognized after myelin damage, suggesting that signals from glia or myelin to axons might be altered by disease. New evidence from studies of MS and of globoid (Krabbe) leukodystrophy indicates that axonal damage might occur contemporaneously with myelin damage in some conditions.8, 10 Thus, loss of non-myelin-related glial signals or, even, addition of toxic signals from glia might damage axons. Finally, non-myelin forming Schwann cells are not innocent bystanders. They respond to nearby demyelination by proliferating, thereby impairing their relationship with small caliber axons subserving pain sensation.11 Taken together, these observations call into question which cell type (that is, myelinating or non-myelinating glial cells or neurons) is the most appropriate target for therapy in diseases of myelin.

Treatment of the most common myelin disorders is limited to anti-inflammatory therapies, which can have notable adverse effects.12 Where can we find other therapeutic targets beyond inflammation? Remyelination occurs in both the CNS and PNS after damage and might provide adjunctive targets for therapy that is aimed at limiting destruction of myelin and axons.13 In MS, remyelination occurs in the CNS after initial myelin damage, but fails after multiple episodes of demyelination. This failure of remyelination subsequently leads to augmented axonal degeneration and progressive disability.[13] Thus, early intervention with treatments that promote remyelination might slow the progression of MS. Proliferation and migration of oligodendroglial precursor cells (OPCs) near MS plaques has been the focus of much work,[13] but failure of differentiation in OPCs has also been recognized. This inability to differentiate might reflect inhibitory influences, or destruction or dysfunction of axons—an important source of differentiation signals for OPCs.13

A starting point for consideration of how differentiation of OPCs might be promoted during repair is the assumption that remyelination depends on signals that are similar to those that occur in developmental myelination. After all, oligodendrocyte precursors or immature Schwann cells very likely recapitulate differentiation as they remyelinate axons.[13] Here, we review the various signals that are crucial to the axon–glia interaction (Figure 1). As the molecular basis of glial support of axons is poorly understood,[4] the main focus of this Review is on the axon to glia signaling pathways that might promote differentiation and, hence, the formation or maintenance of myelin. We also discuss the extent to which developmental studies can guide remyelination therapies, and provide an overview of the limited number of preclinical and clinical trials that have tested such treatments.

Axolemma-based signaling pathways

Glial myelination can be influenced by axonal contact,[14–17] diameter[18–20] or electrical activity.[21–25] These findings suggest that signals originating from the axonal membrane regulate myelination (Box 2; Figure 2, Figure 3).

Neuregulins and ErbB receptors

In the PNS, the neuregulin (NRG) family of proteins and their receptors, which belong to the ErbB family of tyrosine kinase receptors (heterodimeric ErbB2–ErbB3 in Schwann cells), have emerged as important regulators of most aspects of Schwann cell development (Figure 2).[26] The NRGs are encoded by at least four genes, of which *Nrg1* is the best characterized.

[27, 28] *Nrg1* encodes multiple isoforms, all containing an epidermal growth factor-like 1 domain. Most isoforms are shed, whereas others remain anchored in the membrane [28]. In the PNS, NRG1 type III, which is expressed on the axonal membrane, is a key determinant of whether a Schwann cell will form myelin and, if so, how thick the myelin sheath becomes. [29, 30] The potency of NRG1 type III for inducing myelination was demonstrated in cultured superior cervical ganglion neurons, which normally exhibit small-diameter unmyelinated axons. When this NRG was ectopically expressed in such neurons, the associated Schwann cells responded by changing fate and myelinating axons, even if the axons retained their small diameter.[30]

In the CNS, the role of NRGs in myelination remains controversial. Several studies have reported that rodents deficient in either NRG1 or ErbB receptors had impaired oligodendrocyte differentiation, proliferation, survival and myelination.[31–35] These findings suggested that NRGs might be important in CNS myelination. Brinkmann *et al.*, however, reported that mice lacking all NRG1 isoforms or both ErbB3 and ErbB4 receptors showed normal CNS myelination during development. Interestingly, these researchers also showed that NRG1 overexpression in the CNS of transgenic mice induced precocious formation and increased thickness of myelin.[36] Taken together, these data suggest that multiple parallel signals probably control myelination in the CNS, and that the regulation of myelination in CNS and PNS differs.[35, 36] In support of these assertions, animals with mutations in genes encoding neuronal growth factors,[35, 37] extracellular matrix components[38] or intracellular mediators[39, 40] displayed region-specific myelination in the CNS.

NRG-induced signals are clearly important for myelination during development; however, the involvement of these signals in myelin maintenance, damage or remyelination is unclear. The partial reduction of ErbB2 function in Schwann cells of adult transgenic mice had no effect on myelin, suggesting that neuregulin signals might be dispensible for maintenance of the myelin sheath in the PNS.41 Activation of the ErbB receptors in either myelinating neuron-Schwann cell co-cultures, or upon nerve injury, induced demyelination,[42,43] although activation might involve shed NRG1 ligands (not membrane-anchored NRG1 type III). Interestingly, and in agreement with a possible role for ErbB receptors in CNS remyelination, overexpression of ErbB1 in a mouse model of demyelination promoted oligodendrocyte differentiation and remyelination.[44]

The secretases

In general, the number of growth factors is much lower than the number of biological functions that these molecules regulate. Diversification of function is achieved by altering ligands or downstream signals. For example, a growth factor presented in a soluble or membrane-bound form can activate different signaling pathways. Secretases, like the alpha disintegrin and metalloproteases (ADAM), β-secretase (β-site amyloid precursor protein cleaving enzyme 1; BACE1), or the γ-secretase complex all change the presentation of membrane-associated growth factors.

Many myelin-related growth factors (for example, NRG1) and growth factor receptors (for example, p75 neurotrophin receptor ($p75^{NTR}$) and Notch-1, undergo proteolytic cleavage at the axonal membrane, suggesting that this process is a common way of regulating these myelin-associated signaling molecules. Identification of the secretases involved in such cleavage and determination of their mechanisms of action could reveal important therapeutic targets for promoting remyelination.

Several ADAMs have been implicated in myelination.[45] One study showed that *Adam22*-null mice exhibited hypomyelinated nerves,[46] while data from another study suggested that ADAM22 might bind leucine-rich glioma-inactivated proteins,[47] which had been previously

implicated in PNS myelination.[48] In addition, ADAM19 was upregulated following axonal injury and *Adam19*-null mice had delayed remyelination.[49]

Research has revealed that NRG1 type III can be cleaved by BACE1, and that this process is probably regulated by the zinc metallopeptidase nardilysin.[50] In agreement with these findings, either BACE1 or nardilysin-null mice showed marked hypomyelination in the PNS[50, 51] and in the CNS[52, 50]. The level of hypomyelination was similar to that observed in mice with a 50% reduction in NRG1 type III expression.[29, 30] Taken together, the results from these studies indicate that BACE1 promotes myelination. The data from one study have also revealed that loss of BACE1 impairs remyelination during regeneration after a crush injury to sciatic nerve in mice.[53]

Inhibition of γ-secretase, enhances the onset and the amount of myelin formed by cultured rat oligodendrocytes.[54] Moreover, data from various studies have suggested that γ-secretase might cleave NRG1,[55, 56] $p75^{NTR}$[57] or Notch-1[58], potentially activating multiple intracellular signaling pathways in neurons. Regulated intramembrane proteolysis, a highly conserved method for intracellular communication, appears to be an important control of downstream signals in both axons and glial cells.

The secretases have generated strong interest from pharmaceutical companies as potential therapuetic targets, because these proteases are accessible and multiple secretase inhibitors are already available. Therapeutic strategies that target these proteases must consider the problem of specificity and, hence, collateral effects, as single secretases target multiple molecules. For example, BACE1, a potentiator of myelination, cleaves not only neuregulins, but also amyloid precursor protein, generating the amyloid beta42 peptide, that is probably pathogenetic in Alzheimer disease.

The Akt-1 signaling pathway

Akt-1 is emerging as an integrator of various signals that increase myelination in both the CNS and PNS (Box 3). Studies have shown that, Akt-1 is phosphorylated by phosphatidylinositol 3-kinase (PI3K) in response to growth factors that promote myelination, in particular NRG1 type III,[30] insulin growth factor 1 (IGF-1)[59, 60] and steroids.[61] In addition, the expression of constitutively active Akt-1 in mouse oligodendrocytes led to an increase in myelination.[62] PI3K–Akt-1 augments myelination through activation of the kinase mammalian target of rapamycin (mTOR), as inhibition of mTOR *in vivo* was shown to limit myelination in the developing mouse brain.[63] mTOR activation induces formation of both mTORC1 and mTORC2 complexes, which in turn regulates the terminal differentiation of oligodendrocytes, and myelin protein and lipid expression.[64] The transcription factors targeted by the Akt-1/mTOR signals have yet to be fully elucidated in oligodendrocytes,[65] although studies have identified two possible candidates, namely myelin regulatory factor (MRF)[66] and zinc finger protein 191 (ZFP191).[67]

In Schwann cells, most signals that promote myelination act through the transcription factors POU3F1 (POU domain class 3 transcription factor 1; Oct6), EGR2 (early growth response protein 2; Krox20) and SOX-10.[68] For example, in cultured mouse Schwann cells, Akt-1 upregulated EGR2-activated myelin protein zero (P0) expression after IGF-1 stimulation.[49] Conversely, EGR2 repressed, and was repressed by, dedifferentiation factors, including SOX2 and Notch.[69–71] Such reciprocal inhibition permits active, rapid dedifferentiation of Schwann cells after nerve injury, which is necessary for facilitating myelin regeneration.[70, 71] Furthermore, these data suggest that the NRG1 type III–Akt-1 intracellular signaling pathway could be a therapeutic target in demyelinating neuropathies where dedifferentiation genes are inappropriately expressed.[6, 72].

The calcineurin–NFAT signaling pathway

A study in mice lacking expression of the calcineurin B1 subunit showed that, in Schwann cells, the calcineurin–NFAT (nuclear factor of activated T cells) pathway is involved in activating myelination [73]. This signaling pathway is downstream of NRG1 and independent of PI3K. NFAT signal transduction pathways have important roles in multiple tissues, including the developing nervous system, where these pathways promote axonal growth and guidance.74 Nonetheless, the conditional deletion of the gene encoding calcineurin B1 only in Schwann cells in motor roots or only in sensory neurons but not Schwann cells each suggest that NFAT promotes myelination autonomously in Schwann cells 73. These experiments suggest a model in which activation of ErbB2–ErbB3 heterodimers increases intracellular Ca^{2+} levels via phospholipase Cγ, and, thus, activates calcineurin. In turn, calcineurin activation promotes nuclear translocation of NFATc4, where it complexes with SOX-10 to upregulate EGR2 transcription and myelin gene transcription.[73]

Nectin-like proteins

Nectin-like proteins (NECLs), now known as cell adhesion molecules (CADMs), comprise five members and have been implicated in axon–glia interactions and myelination. The roles of CADMs 1–4 have been investigated in the rodent nervous system. Studies have reported that heterophilic interactions between NECL1 (CADM3) on the axolemma and NECL4 (CADM4) on Schwann cells might participate in myelination in the PNS.[75, 76] Schwann cells must polarize with axonal and basal lamina surfaces to form myelin, and NECLs have been previously implicated to have a role in this process.77 Thus, NECL4 might cooperate with the PAR (partitioning defective) polarity complexes, previously implicated in PNS myelination.78 Surprisingly, transgenic mice lacking expression of NECL1 displayed a mild phenotype, with no effect on PNS myelination and a developmental delay in CNS myelination.79 Functional compensation by other NECLs,[76, 80] or unrelated adhesion proteins, might have accounted for this lack of phenotype.

Notch

Notch signaling is fundamental for glial cell development and myelination in both the CNS and PNS. Notch receptors comprise four members, all of which are type I transmembrane proteins. Upon ligand binding, notch receptors are cleaved intracellularly by secretases. The γ-secretase complex generates an intracellular fragment, the notch intracellular domain (NICD), which translocates to the nucleus to activate gene transcription. The ligand engaged on the Notch receptor determines whether the canonical (mediated by the CBF1/Su(H)/Lag-1 proteins, also known as RBPJ) or non-canonical (mediated by Deltex) signaling pathway is activated [81].

In the CNS, canonical Notch1 ligands, which comprise members of the Delta or Serrate/Jagged family, are expressed by neurons at early developmental stages.[82] Notch1 is only expressed by oligodendrocytes[83]. *In vitro* and *in vivo* studies have shown that binding of Jagged-1 to Notch1 inhibits OPC differentiation and myelination,[83–86] and that such inhibition is mediated via activation of the transcription factor HES-5 (hairy and enhancer of split 5)[82]. These findings suggest that Notch1 is important for correct spatial and temporal differentiation of OPCs.

Jagged-1 expression is downregulated in retinal ganglion cells after birth; however, in MS, this protein has been reported to be re-expressed in reactive astrocytes surrounding plaques [82]. This finding suggested that the failure of OPCs to mature near such lesions could be the result of reactivation of the Notch inhibitory pathway. In support of this assertion, HES-5 expression has been detected in oligodendrocytes near to MS plaques.[82] How much of this protein is found in the nuclei of these cells, however, remains controversial [87].

Surprisingly, in adult mice that were exposed to chemicals that induce demyelination, conditional ablation of *Notch1* in oligodendrocytes did not produce a marked effect on remyelination.[88] Nevertheless, one study has shown that if *Notch1* is inactivated in mice during development, oligodendroglial differentiation is accelerated.[86] Ultimately, adult myelin was normal in these animals. In this case, remyelination after a chemical lesion was accelerated. Taken together, these results suggest that the timing of Notch 1 inhibition might be critical for achieving remyelination, and that Notch could be a therapeutic target in myelin disorders.

Contactin-1 (also know as neural cell surface protein F3) is a putative non-canonical ligand of Notch that is expressed on axons later than the canonical ligands in development. This Notch ligand has been suggested to promote rather than inhibit OPC differentiation and the expression of myelin genes in the CNS.[89] However, the observation that myelination and remyelination are accelerated and are eventually normal in mice lacking Notch in oligodendrocytes argues against this idea [86, 88].

In the rodent PNS, Notch 1 is expressed exclusively by Schwann cells, whereas Jagged-1 is present on both Schwann cells and axons.[71] Notch has been shown to promote gliogenesis over neurogenesis in neural crest stem cells *in vitro*.[90] Furthermore, *in vivo* rodent studies have demonstrated that both canonical and non-canonical Notch1 pathways determine the complex activity of Notch in PNS development and myelination.[71] Notch1, via canonical signaling pathways, promotes the transition from precursor to immature Schwann cells[71].

By contrast, Notch1 inhibits the onset of PNS myelination. Notch1 expression is tightly downregulated by EGR2—a transcription factor that is necessary for inducing the activation of the myelinating program.[91] Moreover, the overexpression of NICD delays myelination and causes hypomyelination. The inhibitory effect of Notch1 on myelination is mediated through non-canonical pathways, as such inhibition seems to be independent to the effects of RBPJ. Interestingly, myelination is also inhibited immediately following nerve injury, to allow proper Schwann cell dedifferentiation and proliferation. Reactivation of Notch1 expression in this context after injury is associated with the RBPJ canonical signal. Thus, the Notch1 pathway is differentially mediated in development and after injury. Thus, regarding the role of Notch1 in the PNS, the molecular events regulating myelination in development differ in part from those in remyelination.[71]

HES-5 and histone deacetylases

HES-5 can also be activated by Notch-independent signals in OPCs. Activation of this transcription factor can impede the differentiation of these cells during remyelination. For example, epigenetic modifications of chromatin, such as histone deacetylation, blocked the expression of inhibitory transcription factors, including HES-5, SOX-2, ID-2 (inhibitor of DNA binding 2), and ID-4 (inhibitor of DNA binding 4), and promoted myelination in the mouse [92]. Inadequate recruitment of histone deacetylases (HDAC) to the transcriptional promoters of such inhibitory factors results in their sustained expression, mirroring what happens in aged rodents, where remyelination is less efficient than in young rodents.92 This finding suggests that activation of HDACs might represent a promising strategy for promoting remyelination. Paradoxically, use of HDAC inhibitors in animal models of inflammatory demyelination have reduced demyelination and limited disability.[92, 93] One possible reason for this discrepancy is that HDAC inhibitors may act not only on oligodendrocytes, but also on cells of the immune system or axons. For example, recent *in vitro* studies have shown that HDAC1 might induce axonal damage in inflammatory demyelinating diseases when the protein is exported from the nucleus to the cytoplasm of neurons.[94] This effect could be a potential confounding factor in studies of remyelination using HDAC inhibitors.

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Signals to promote myelin formation and repair

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Abstract

The myelin sheath wraps large axons in both the CNS and the PNS, and is a key determinant of efficient axonal function and health. Myelin is targeted in a series of diseases, notably multiple sclerosis (MS). In MS, demyelination is associated with progressive axonal damage, which determines the level of patient disability. Few treatments are available for combating myelin damage in MS and related disorders. These treatments, which largely comprise anti-inflammatory drugs, only show limited efficacy in subsets of patients. More-effective treatment of myelin disorders will probably result from early intervention with combinatorial therapies that target inflammation and other processes—for example, signaling pathways that promote remyelination. Indeed, evidence suggests that such pathways might be impaired in pathology and, hence, contribute to the failure of remyelination in such diseases. In this article, we review the molecular basis of signaling pathways that regulate myelination in the CNS and PNS with a focus on differentiation of myelinating glia. We also discuss factors such as extracellular molecules that act as modulators of these pathways. Finally, we consider the few preclinical and clinical trials of agents that augment this signaling.

Introduction

Most large axons in the mammalian nervous system are surrounded by a lipid-rich membrane known as the myelin sheath, which promotes rapid conduction of nerve impulses and protects against axonal damage. Myelin sheaths form during development and consist of compacted spiral wraps of membrane that are supplied by oligodendrocytes in the CNS and Schwann cells in the PNS (Box 1 and Figure 1). These myelinating glia and their target axons form intimate units, with the glia and axons regulating each other's phenotype through the reciprocal exchange of signals (Boxes 2–4).

During development, glia provide survival signals to neurons, define the molecular domains of the axolemma and determine the diameter of axons.[1,2] In turn, axons provide signals that regulate the proliferation, survival and differentiation of glia, as well as myelin formation.[3-5] In adulthood, myelinating glia maintain axolemmal organization, axonal diameter and neuronal health, while axons maintain glial differentiation and myelin integrity.[2] At least in

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Competing interests
The authors declare no competing interests.

Review criteria
We searched PubMed for articles published up until January 2010 using the terms "axon signaling and glia", "myelin", or "remyelination". We also visited the Inherited Peripheral Neuropathies Mutation Database (http://www.molgen.ua.ac.be/CMTMutations/default.cfm), and consulted our personal collections of literature. In addition, we searched the clinical trial registries http://clinicaltrials.gov/ and http://www.controlled-trials.com/mrct/ with the search terms myelin or remyelination.

the PNS, axonal damage alters these maintenance signals and initiates an active program of Schwann cell dedifferentiation, which probably facilitates myelin reabsorption.[6]

Multiple diseases target myelin, including multiple sclerosis (MS) and hereditary leukodystrophies in the CNS, and Guillain–Barré Syndrome and hereditary demyelinating neuropathies in the PNS.7 In general, the degree of disability observed in myelin disorders correlates best with the level of associated axonal damage.8› 9 In most disorders, axonal damage is recognized after myelin damage, suggesting that signals from glia or myelin to axons might be altered by disease. New evidence from studies of MS and of globoid (Krabbe) leukodystrophy indicates that axonal damage might occur contemporaneously with myelin damage in some conditions.8› 10 Thus, loss of non-myelin-related glial signals or, even, addition of toxic signals from glia might damage axons. Finally, non-myelin forming Schwann cells are not innocent bystanders. They respond to nearby demyelination by proliferating, thereby impairing their relationship with small caliber axons subserving pain sensation.11 Taken together, these observations call into question which cell type (that is, myelinating or non-myelinating glial cells or neurons) is the most appropriate target for therapy in diseases of myelin.

Treatment of the most common myelin disorders is limited to anti-inflammatory therapies, which can have notable adverse effects.12 Where can we find other therapeutic targets beyond inflammation? Remyelination occurs in both the CNS and PNS after damage and might provide adjunctive targets for therapy that is aimed at limiting destruction of myelin and axons.13 In MS, remyelination occurs in the CNS after initial myelin damage, but fails after multiple episodes of demyelination. This failure of remyelination subsequently leads to augmented axonal degeneration and progressive disability.[13] Thus, early intervention with treatments that promote remyelination might slow the progression of MS. Proliferation and migration of oligodendroglial precursor cells (OPCs) near MS plaques has been the focus of much work,[13] but failure of differentiation in OPCs has also been recognized. This inability to differentiate might reflect inhibitory influences, or destruction or dysfunction of axons—an important source of differentiation signals for OPCs.13

A starting point for consideration of how differentiation of OPCs might be promoted during repair is the assumption that remyelination depends on signals that are similar to those that occur in developmental myelination. After all, oligodendrocyte precursors or immature Schwann cells very likely recapitulate differentiation as they remyelinate axons.[13] Here, we review the various signals that are crucial to the axon–glia interaction (Figure 1). As the molecular basis of glial support of axons is poorly understood,[4] the main focus of this Review is on the axon to glia signaling pathways that might promote differentiation and, hence, the formation or maintenance of myelin. We also discuss the extent to which developmental studies can guide remyelination therapies, and provide an overview of the limited number of preclinical and clinical trials that have tested such treatments.

Axolemma-based signaling pathways

Glial myelination can be influenced by axonal contact,[14-17] diameter[18-20] or electrical activity.[21-25] These findings suggest that signals originating from the axonal membrane regulate myelination (Box 2; Figure 2, Figure 3).

Neuregulins and ErbB receptors

In the PNS, the neuregulin (NRG) family of proteins and their receptors, which belong to the ErbB family of tyrosine kinase receptors (heterodimeric ErbB2–ErbB3 in Schwann cells), have emerged as important regulators of most aspects of Schwann cell development (Figure 2).[26] The NRGs are encoded by at least four genes, of which *Nrg1* is the best characterized.

[27, 28] *Nrg1* encodes multiple isoforms, all containing an epidermal growth factor-like 1 domain. Most isoforms are shed, whereas others remain anchored in the membrane [28]. In the PNS, NRG1 type III, which is expressed on the axonal membrane, is a key determinant of whether a Schwann cell will form myelin and, if so, how thick the myelin sheath becomes. [29, 30] The potency of NRG1 type III for inducing myelination was demonstrated in cultured superior cervical ganglion neurons, which normally exhibit small-diameter unmyelinated axons. When this NRG was ectopically expressed in such neurons, the associated Schwann cells responded by changing fate and myelinating axons, even if the axons retained their small diameter.[30]

In the CNS, the role of NRGs in myelination remains controversial. Several studies have reported that rodents deficient in either NRG1 or ErbB receptors had impaired oligodendrocyte differentiation, proliferation, survival and myelination.[31-35] These findings suggested that NRGs might be important in CNS myelination. Brinkmann *et al.*, however, reported that mice lacking all NRG1 isoforms or both ErbB3 and ErbB4 receptors showed normal CNS myelination during development. Interestingly, these researchers also showed that NRG1 overexpression in the CNS of transgenic mice induced precocious formation and increased thickness of myelin.[36] Taken together, these data suggest that multiple parallel signals probably control myelination in the CNS, and that the regulation of myelination in CNS and PNS differs.[35, 36] In support of these assertions, animals with mutations in genes encoding neuronal growth factors,[35, 37] extracellular matrix components[38] or intracellular mediators[39, 40] displayed region-specific myelination in the CNS.

NRG-induced signals are clearly important for myelination during development; however, the involvement of these signals in myelin maintenance, damage or remyelination is unclear. The partial reduction of ErbB2 function in Schwann cells of adult transgenic mice had no effect on myelin, suggesting that neuregulin signals might be dispensible for maintenance of the myelin sheath in the PNS.41 Activation of the ErbB receptors in either myelinating neuron-Schwann cell co-cultures, or upon nerve injury, induced demyelination,42,43 although activation might involve shed NRG1 ligands (not membrane-anchored NRG1 type III). Interestingly, and in agreement with a possible role for ErbB receptors in CNS remyelination, overexpression of ErbB1 in a mouse model of demyelination promoted oligodendrocyte differentiation and remyelination.[44]

The secretases

In general, the number of growth factors is much lower than the number of biological functions that these molecules regulate. Diversification of function is achieved by altering ligands or downstream signals. For example, a growth factor presented in a soluble or membrane-bound form can activate different signaling pathways. Secretases, like the alpha disintegrin and metalloproteases (ADAM), β-secretase (β-site amyloid precursor protein cleaving enzyme 1; BACE1), or the γ-secretase complex all change the presentation of membrane-associated growth factors.

Many myelin-related growth factors (for example, NRG1) and growth factor receptors (for example, p75 neurotrophin receptor ($p75^{NTR}$) and Notch-1, undergo proteolytic cleavage at the axonal membrane, suggesting that this process is a common way of regulating these myelin-associated signaling molecules. Identification of the secretases involved in such cleavage and determination of their mechanisms of action could reveal important therapeutic targets for promoting remyelination.

Several ADAMs have been implicated in myelination.[45] One study showed that *Adam22*-null mice exhibited hypomyelinated nerves,[46] while data from another study suggested that ADAM22 might bind leucine-rich glioma-inactivated proteins,[47] which had been previously

implicated in PNS myelination.[48] In addition, ADAM19 was upregulated following axonal injury and *Adam19*-null mice had delayed remyelination.[49]

Research has revealed that NRG1 type III can be cleaved by BACE1, and that this process is probably regulated by the zinc metallopeptidase nardilysin.[50] In agreement with these findings, either BACE1 or nardilysin-null mice showed marked hypomyelination in the PNS[50, 51] and in the CNS[52, 50]. The level of hypomyelination was similar to that observed in mice with a 50% reduction in NRG1 type III expression.[29, 30] Taken together, the results from these studies indicate that BACE1 promotes myelination. The data from one study have also revealed that loss of BACE1 impairs remyelination during regeneration after a crush injury to sciatic nerve in mice.[53]

Inhibition of γ-secretase, enhances the onset and the amount of myelin formed by cultured rat oligodendrocytes.[54] Moreover, data from various studies have suggested that γ-secretase might cleave NRG1,[55, 56] $p75^{NTR}$[57] or Notch-1[58], potentially activating multiple intracellular signaling pathways in neurons. Regulated intramembrane proteolysis, a highly conserved method for intracellular communication, appears to be an important control of downstream signals in both axons and glial cells.

The secretases have generated strong interest from pharmaceutical companies as potential therapuetic targets, because these proteases are accessible and multiple secretase inhibitors are already available. Therapeutic strategies that target these proteases must consider the problem of specificity and, hence, collateral effects, as single secretases target multiple molecules. For example, BACE1, a potentiator of myelination, cleaves not only neuregulins, but also amyloid precursor protein, generating the amyloid beta42 peptide, that is probably pathogenetic in Alzheimer disease.

The Akt-1 signaling pathway

Akt-1 is emerging as an integrator of various signals that increase myelination in both the CNS and PNS (Box 3). Studies have shown that, Akt-1 is phosphorylated by phosphatidylinositol 3-kinase (PI3K) in response to growth factors that promote myelination, in particular NRG1 type III,[30] insulin growth factor 1 (IGF-1)59, 60 and steroids.61 In addition, the expression of constitutively active Akt-1 in mouse oligodendrocytes led to an increase in myelination.62 PI3K–Akt-1 augments myelination through activation of the kinase mammalian target of rapamycin (mTOR), as inhibition of mTOR *in vivo* was shown to limit myelination in the developing mouse brain.63 mTOR activation induces formation of both mTORC1 and mTORC2 complexes, which in turn regulates the terminal differentiation of oligodendrocytes, and myelin protein and lipid expression.64 The transcription factors targeted by the Akt-1/mTOR signals have yet to be fully elucidated in oligodendrocytes,65 although studies have identified two possible candidates, namely myelin regulatory factor (MRF)[66] and zinc finger protein 191 (ZFP191).67

In Schwann cells, most signals that promote myelination act through the transcription factors POU3F1 (POU domain class 3 transcription factor 1; Oct6), EGR2 (early growth response protein 2; Krox20) and SOX-10.[68] For example, in cultured mouse Schwann cells, Akt-1 upregulated EGR2-activated myelin protein zero (P0) expression after IGF-1 stimulation.49 Conversely, EGR2 repressed, and was repressed by, dedifferentiation factors, including SOX2 and Notch.69–71 Such reciprocal inhibition permits active, rapid dedifferentiation of Schwann cells after nerve injury, which is necessary for facilitating myelin regeneration.[70, 71] Furthermore, these data suggest that the NRG1 type III–Akt-1 intracellular signaling pathway could be a therapeutic target in demyelinating neuropathies where dedifferentiation genes are inappropriately expressed.[6, 72].

The calcineurin–NFAT signaling pathway

A study in mice lacking expression of the calcineurin B1 subunit showed that, in Schwann cells, the calcineurin–NFAT (nuclear factor of activated T cells) pathway is involved in activating myelination [73]. This signaling pathway is downstream of NRG1 and independent of PI3K. NFAT signal transduction pathways have important roles in multiple tissues, including the developing nervous system, where these pathways promote axonal growth and guidance.74 Nonetheless, the conditional deletion of the gene encoding calcineurin B1 only in Schwann cells in motor roots or only in sensory neurons but not Schwann cells each suggest that NFAT promotes myelination autonomously in Schwann cells 73. These experiments suggest a model in which activation of ErbB2–ErbB3 heterodimers increases intracellular Ca^{2+} levels via phospholipase Cγ, and, thus, activates calcineurin. In turn, calcineurin activation promotes nuclear translocation of NFATc4, where it complexes with SOX-10 to upregulate EGR2 transcription and myelin gene transcription.[73]

Nectin-like proteins

Nectin-like proteins (NECLs), now known as cell adhesion molecules (CADMs), comprise five members and have been implicated in axon–glia interactions and myelination. The roles of CADMs 1–4 have been investigated in the rodent nervous system. Studies have reported that heterophilic interactions between NECL1 (CADM3) on the axolemma and NECL4 (CADM4) on Schwann cells might participate in myelination in the PNS.[75, 76] Schwann cells must polarize with axonal and basal lamina surfaces to form myelin, and NECLs have been previously implicated to have a role in this process.77 Thus, NECL4 might cooperate with the PAR (partitioning defective) polarity complexes, previously implicated in PNS myelination.78 Surprisingly, transgenic mice lacking expression of NECL1 displayed a mild phenotype, with no effect on PNS myelination and a developmental delay in CNS myelination.79 Functional compensation by other NECLs,[76, 80] or unrelated adhesion proteins, might have accounted for this lack of phenotype.

Notch

Notch signaling is fundamental for glial cell development and myelination in both the CNS and PNS. Notch receptors comprise four members, all of which are type I transmembrane proteins. Upon ligand binding, notch receptors are cleaved intracellularly by secretases. The γ-secretase complex generates an intracellular fragment, the notch intracellular domain (NICD), which translocates to the nucleus to activate gene transcription. The ligand engaged on the Notch receptor determines whether the canonical (mediated by the CBF1/Su(H)/Lag-1 proteins, also known as RBPJ) or non-canonical (mediated by Deltex) signaling pathway is activated [81].

In the CNS, canonical Notch1 ligands, which comprise members of the Delta or Serrate/Jagged family, are expressed by neurons at early developmental stages.[82] Notch1 is only expressed by oligodendrocytes[83]. *In vitro* and *in vivo* studies have shown that binding of Jagged-1 to Notch1 inhibits OPC differentiation and myelination,[83–86] and that such inhibition is mediated via activation of the transcription factor HES-5 (hairy and enhancer of split 5)[82]. These findings suggest that Notch1 is important for correct spatial and temporal differentiation of OPCs.

Jagged-1 expression is downregulated in retinal ganglion cells after birth; however, in MS, this protein has been reported to be re-expressed in reactive astrocytes surrounding plaques [82]. This finding suggested that the failure of OPCs to mature near such lesions could be the result of reactivation of the Notch inhibitory pathway. In support of this assertion, HES-5 expression has been detected in oligodendrocytes near to MS plaques.[82] How much of this protein is found in the nuclei of these cells, however, remains controversial [87].

Surprisingly, in adult mice that were exposed to chemicals that induce demyelination, conditional ablation of *Notch1* in oligodendrocytes did not produce a marked effect on remyelination.[88] Nevertheless, one study has shown that if *Notch1* is inactivated in mice during development, oligodendroglial differentiation is accelerated.[86] Ultimately, adult myelin was normal in these animals. In this case, remyelination after a chemical lesion was accelerated. Taken together, these results suggest that the timing of Notch 1 inhibition might be critical for achieving remyelination, and that Notch could be a therapeutic target in myelin disorders.

Contactin-1 (also know as neural cell surface protein F3) is a putative non-canonical ligand of Notch that is expressed on axons later than the canonical ligands in development. This Notch ligand has been suggested to promote rather than inhibit OPC differentiation and the expression of myelin genes in the CNS.[89] However, the observation that myelination and remyelination are accelerated and are eventually normal in mice lacking Notch in oligodendrocytes argues against this idea 86, 88.

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HES-5 and histone deacetylases

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Molecules of axoglial junctions

A series of protein and lipid complexes mediate the interaction between axons and myelinating glia. These complexes organize proteins in the axonal and glial membrane (for example, ion channels), and maintain cell vicinity to facilitate the transmission of signals that promote myelination.[1] One role for such complexes is in the segregation of voltage-gated sodium (Nav) and potassium channels (Kv) at nodes of Ranvier and adjacent paranodal and juxtaparanodal regions. Such segregation is crucial for proper conduction of axonal impulses (Figure 1).

The combination of functional importance and accessibility renders the axoglial apparatus a prime pathogenetic—but also pharmacological—target. Indeed, early disaggregation of Nav , gliomedin,95 neurofascin96 and contactin-associated protein [97] clusters precedes demyelination in experimental allergic encephalitis (EAE) in rodents or in MS and disaggregation of clusters is also found at the edges of chronic plaques in MS brains.[97] Disruption of ion channel clusters at nodes can produce conduction block and early functional disability that accompanies demyelination.95 Furthermore, reclusterization is the first event that marks the onset of remyelination.98 Finally, nodal and paranodal proteins are altered in genetic neuropathies,99 emphasizing the importance of cluster disruption as a general pathogenetic mechanism.

Interestingly, axoglial complexes contain autoantigens that are targeted by the immune system in MS, chronic inflammatory demyelinating neuropathy and Guillan–Barré syndrome. Autoantibodies against neurofascin 186, gliomedin95 and GM1100 (all nodal components), as well as neurofascin 155[101] (a paranodal component) have been detected in patients or animal models , and in some cases shown to be pathogenetic. Human autoantibodies against both axonal and glial neurofascins directly inhibited axonal conduction when applied to rodent tissue slices.101 Moreover, when transferred together with pathogenetic T-cells, these autoantibodies exacerbated the severity of EAE in rats in a complement-dependent manner.101 Similarly, anti–GM1 antibodies bound complement proteins and disrupted Nav channel clusters in rabbit nerves.100 Thus, therapeutic strategies aimed at limiting attacks on the axoglial apparatus or promoting its reformation could protect the role of this apparatus in myelination and impulse conduction.

Extracellular modulators

In addition to molecules in the axolemma, secreted extracellular molecules modulate myelination, either independently or in concert with NRGs or other axonal signals (Box 3).[6, 13] The accessibility of these extracellular molecules and their potential role in promoting myelination make them appealing therapeutic targets in myelin diseases. Interesting examples of such molecules are laminins, semaphorins and netrins—all secreted molecules involved in axonal guidance during development.

Laminins

Seminal work from the Bunge laboratory showed that high concentrations of laminin could induce myelination in Schwann cell–neuron cultures in the absence of the myelination–promoting factor ascorbate.[102, 103] Integrin and dystroglycan receptors mediate the effects of laminin on the survival and differentiation of oligodendrocytes and Schwann cells.[104-106] In both cell types, laminin might cooperate with axonal NRG1 to promote survival and differentiation. Adhesion of integrin α6β1 to laminin effectively switches the response of oligodendrocytes to NRG1 from promoting proliferation to promoting differentiation, and allows survival only in those cells in which differentiation is induced [106, 107]. In Schwann cells, the absence of laminin impairs the phosphorylation of ErbB2 and the activation of Akt-1 , normally associated with differentiation [104, 105].

Whether the cooperation in survival and differentiation described between laminin receptors and NRG1 extends to myelination is still unclear. In the CNS, laminin and integrin β1 have been shown to activate the pro-myelinating signals Akt-1,[38] Fyn[108-110] and p38 MAP kinase,[111] while a laminin deficiency in dystrophic mice caused regional hypomyelination.[38, 109] Whether integrin β1 mediates these effects is controversial, as expression of a dominant-negative integrin β1 in mice delayed the myelination of small-caliber axons,[112] but oligodendrocytes lacking integrin β1 have been reported to either cause hypomyelination,[113] or myelinate and remyelinate normally.[114] In the PNS, myelin thickness is not obviously reduced in laminin-deficient dystrophic mice, nor in the absence of the laminin receptors dystroglycan or integrin α6β4.[115-117] The absence of integrin β1 arrests development before myelin formation, precluding its analysis.[118]

Interestingly, laminin receptors might also protect against demyelination, as acute demyelination has been described in mice lacking both integrin α6β4 and dystroglycan in Schwann cells.[117]

Semaphorins

Factors other than laminins promote glial recruitment and differentiation, and might be modulated to improve remyelination.[13] Among these factors are secreted semaphorins, whose co-receptors are present on myelinating glia.[119, 120] Semaphorins 3A and 3F have been found around active, but not chronic, demyelinated plaques in both patients with MS and mouse models of this disease. These observations led Lubetzki and colleagues to suggest that semaphorins might have a role in remyelination.[121] Myelinating oligodendrocytes also express semaphorin 4D after injury,[122] which limits oligodendrocyte number[123] and promotes process collapse.[124] Finally, in Neurofibromatosis 1, loss of semaphorin 4F in Schwann cells impairs axon–glia communication, and restoring semaphorin 4F expression normalizes this interaction *in vitro*; thus, suggesting a means of reducing neurofibroma formation and promoting myelination.[125]

Netrins

The netrins are a recently described family of factors present on myelinated axons. Netrin-1 and its receptor, DCC (deleted in colorectal cancer), are present on axons and myelinating oligodendroglia, respectively,[119, 126] and might be involved in glial recruitment and myelination.[119, 127] The absence of *Dcc* and *Netrin* in mice did not preclude myelination, but caused disruption of paranodal junctions.[128] Since these junctions are disrupted early in demyelinating diseases, activation of netrin-1 could be protective.

Evidence exists for functional or physical interactions between laminins, semaphorins and netrins.[129] Thus, these molecules might represent common pharmacological targets for combined therapies that aim to promote both remyelination and axonal regeneration.

Orphan receptors and signals

A few molecules have been shown to modulate myelination and probably mediate axon glia interactions, but their relationship to established ligands, receptors or signaling pathways is unclear.

Serum response factor

In the nervous system, serum response factor (SRF)—an immediate early gene response transcription factor—is important for axonal pathfinding[130] and NGF-dependent innervation of sensory neurons.[131] Surprisingly, ablation of SRF exclusively in neurons of mice led to hypomyelination (primarily around large caliber axons), impairment of

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- Glial cell-derived neurotrophic factor probably binds Ret on axons, and promotes myelination of nociceptive neurons in the PNS
- In the CNS, nerve growth factor binds TrkA on axons and inhibits myelination

Merlin

- Merlin is encoded by the tumor suppressor gene *NF2* (implicated in neurofibromatosis type 2 tumors) and is important for the formation of axon–glial junctions[174]

Lgi4

- Lgi4 is an orphan molecule secreted by Schwann cells that regulates PNS myelination, perhaps through effects on axons[48]

Myelin-associated glycoprotein

- Myelin-associated glycoprotein is located on the membrane of glial cells facing axons
- The absence of myelin-associated glycoprotein promotes axonal degeneration[2]

Erytropoietin

- Erytropoietin is released by Schwann cells after injury and reduces axonal degeneration[175]

Sirtuin2

- Sirtuin 2 in oligodendrocytes is a putative mediator of axonal degeneration in spastic paraplegia 2 due to mutations in proteolipid protein
- lack of proteolipid protein is associated with absence of sirtuin 2 from CNS myelin [176]

Fibroblast growth factor receptors

- Fibroblast growth factor receptor activation prevents degeneration of unmyelinated sensory axons in the PNS and CNS[177]

Gliomedin and neurofascin 155

- Gliomedin and neurofascin 155 are glial components of the node of Ranvier and paranodal axoglial junctions[1]
- Alteration of gliomedin and neurofascin 155 clusters precedes demyelination in experimental allergic neuritis[1, 95]

Abbreviation: Trk, tropomyosin receptor kinase.

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Does myelination model remyelination?

Many signaling molecules have been characterized in myelin formation during development; however, only a few of these have been evaluated in the context of remyelination. Thus, it is too early to judge whether a molecule's function in development predicts its utility in myelin repair. In some cases, the effect was not found, or paradoxically was opposite of that expected. As Franklin and ffrench-Constant have reviewed,[13] these findings might suggest that the intrinsic mechanisms underlying myelin formation in development and after damage differ. Alternatively, the contrasting effects of the signaling molecules might stem from the specific inhibitory effects of the demyelinating environment (for example, inflammatory cytokines), which are not present during development. Moreover, such effects might be explained by making comparisons between diverse processes in myelination and remyelination (for example, comparison of the effects on migration in development with effects on differentiation in demyelination) or, more simply, experimental issues (for example, genetic ablation of one gene might induce compensatory expression of other genes).

One example of a molecule that behaves differently in development and remyelination is Olig1 (oligodendrocyte transcription factor 1), which is a transcription factor that is important for the production and differentiation of OPCs, as well as myelination. Studies in *Olig1*-null mice suggested that Olig1 is much less important in developing oligodendrocytes than in remyelinating oligodendrocytes.[143] Xin and colleagues, however, found that Olig1 was crucial for normal oligodendrocyte development and myelination.[144] This discrepancy is probably a consequence of different gene targeting strategies. In the first study [143], the strategy that was used might have activated transcription from the nearby *Olig2* gene, which encodes another transcription factor that is essential for oligodendrocyte development and myelination.[144] Thus, multiple strategies to assess function, as well as tests in various models of demyelination will be required to validate putative potentiators of myelin repair.[145]

Preclinical and clinical trials

Two types of therapeutic strategies related to myelination signals have been proposed. The first involves reducing the interference to myelination signals. As noted above, interference of such signals is posited to occur at all levels of signaling pathways, ranging from extracellular ligand–receptor interactions[101, 146] to transcription.[6] Genetic proof of principle experiments are underway in mouse models of hereditary neuropathies to limit expression of 'dedifferentiation' genes such as *cJun* or *Sox2* in Schwann cells; no results have been published thus far.

The second approach is to restore or augment normal myelination signaling during remyelination. The first preclinical and clinical trials along these lines have appeared in the last 6 years. For example, 70% of hereditary demyelinating neuropathies are caused by overexpression of peripheral myelin protein 22 (PMP22) in Charcot-Marie-Tooth disease type 1A [CMT1A]. Therefore, reducing myelination signaling in order to normalize *PMP22* expression might be of therapeutic benefit. Preclinical trials of ascorbic acid, which might lower levels of cAMP,[147, 148] and the progesterone inhibitor onapristone[149, 150] have demonstrated that, in principle, the reduction of PMP22 levels improves myelination in animal models of CMT1A. As a result, several clinical trials with ascorbic acid in patients with CMT1A are underway[151–153] and a search is ongoing for less toxic progesterone inhibitors than onapristone.154 Thyroid hormone T4 promotes oligodendrocyte differentiation and myelination in development, and has been shown to promote myelin repair in a preclinical trial in acute EAE.155 IGF-1 also augments myelination in the CNS,[59]

and a phase II pilot study of the tolerability and efficacy of subcutaneously administered recombinant human IGF-1 (CEP-151) in patients with MS has been completed, although no results have been reported yet.

Conclusions

Continuous reciprocal dialogue between axons and myelinating glia is important during development and maintenance of the myelin sheath. Various types of molecules, acting at diverse regulatory levels, mediate these events. Some of these signaling molecules normally promote myelination, and might be perturbed in myelin diseases, whereas others normally inhibit myelination and can be inappropriately active in such disorders. On the basis of these signals, several therapies are being examined in preclinical trials, with the first clinical trials now underway.

The search for signaling molecules that promote remyelination holds promise for eventually developing combinatorial therapies, including existing anti-inflammatory interventions, for demyelinating diseases. Future research into the development of such treatments will need to address two additional issues. First, for the various diseases of myelin, the initial site of damage (glia or axons) needs to be resolved, as this location might have consequences for where the drug is delivered. Indeed, the cell bodies of neurons and glia that form one myelin–axon unit sometimes reside in different parts of the nervous system (e.g. Schwann cells in peripheral nerve versus motor neurons in the spinal cord), which are characterized by differing pharmacological barriers. Second, as evidence mounts for the important role of axonal injury in disability, signals originating in glia that mediate axonal support need to be identified and characterized. Surprisingly little is known about these signals currently (Box 4).

Box 1 | Myelin formation in mammals

Rodents

- Before birth, oligodendroglial precursors emerge from the ventral and dorsal neural tube and migrate throughout the forming brain and spinal cord.
- Perinatally, these cells differentiate and mostly after birth, myelinate segments of multiple axons.[158]
- Before birth, Schwann cell precursors arise from the neural crest and migrate out with extending neurites in forming embryonic peripheral nerves.
- Perinatally, immature Schwann cells differentiate, and mostly after birth, Schwann cells begin to form myelin, but unlike oligodendrocytes only myelinate a single segment of one axon.[5]

Human

- Before birth, oligodendroglial and Schwann cell precursors follow a similar developmental pattern as observed in rodents.
- Perinatally, myelination has already begun in both the PNS (spinal roots) and CNS (primarily in spinal cord, brainstem and cerebellum, but sparsely above the subcortical nuclei),[159], but myelin formation is significantly more protracted in human than in rodents, extending over the first several years of life.

Box 2 | Axonal signals to glia

Neuregulins and ErbBs

- are key regulators of Schwann cell development and myelination [26, 160]
- Secretases proteolytically cleave neuregulins on axons to regulate their levels [51-53]

Notch-1

- In the PNS, promotes Schwann cell maturation, but inhibits myelination[71]
- In the CNS, Notch-1 inhibits oligodendroglial maturation, as well as myelination[83-86]

Neurotrophins

See [161] for an overview of the role of neurotrophins in myelination

- BDNF binds to glial $p75^{NTR}$ and promotes myelination[78]
- After myelination, BDNF binds to truncated TrkB molecules on Schwann cells to limit myelination[162]
- *In vitro*, neurotrophin 3 promotes Schwann cell migration but inhibits myelination. *Ntf3*-null mice are hypomyelinated[163]
- BDNF binds to full length TrkB molecules on oligodendrocytes and promotes differentiation.
- Neurotrophin 3, like platelet-derived growth factor, promotes oligodendrocyte migration, proliferation, survival and differentiation

Neural cell adhesion molecule

- might function in neurite outgrowth, cell adhesion and maintenance of axon–glial interactions in the CNS and PNS[2, 164]

Nectin-like proteins

- mediate axon–glia interaction and promote PNS myelination[75, 76]

PSA-NCAM

- PSA-NCAM inhibits CNS myelination[165]
- PSA-NCAM is expressed by reactive astrocytes in MS plaques and is present on demyelinated, but not newly remyelinated, axons[164]

ATP purinergic signaling

- Adenosine binds glial purinergic receptors and promotes oligodendroglial differentiation [23]
- Electrically active neurons (axons) release ATP, which stimulates the production and release of leukemia inhibitory factor from astrocytes, thereby augmenting myelination by oligodendrocytes[166]
- ATP binds glial P2Y receptors and inhibits Schwann cell proliferation and differentiation [167]
- Adenosine also binds $A2_A$ receptor, which inhibits Schwann cell proliferation but not myelination [168]

Neurofascin 186 and contactin-associated protein

Axonal components of the nodes of Ranvier and paranodes could be targets of autoantibodies in MS patients[1, 97]

Aboreviations: BDNF, brain-derived neurotrophic factor; PSA-NCAM, polysialic acid neural cell adhesion molecule; Trk, tropomyosin receptor kinase

Box 3 | Non-axonal membrane signals to glia

Laminins, integrins and dystroglycan

- Components of the extracellular matrix (laminins) and their receptors (integrins and dystroglycan) are required in Schwann cells for radial sorting and ensheathment of axons, and myelination [169].

Insulin-like growth factor 1

- Insulin-like growth factor 1 promotes oligodendrocyte differentiation and survival, as well as myelin integrity and function[59]
- Administration of exogenous recombinant human insulin-like growth factor 1 to rats with experimental autoimmune encephalomyelitis closed the disrupted blood–brain barrier, reduced the number and severity of demyelinating lesions, and improved neurological function[170]

Progesterone

- Progesterone promotes myelin gene expression by Schwann cells and myelination in peripheral nerves[149]
- In the CNS, a short treatment with progesterone, following rat spinal cord injury, promotes oligodendrocyte proliferation and differentiation[171]

Thyroid hormone

- As for platelet-derived growth factor, thyroid hormone is an instructive signal for oligodendrocyte development and maturation[172]
- Administration of thyroxine (T_4) in animal models of demyelination and remyelination, such as experimental autoimmune encephalomyelitis or in cuprizone treated animals, has proven beneficial for remyelination[155, 173]

Semaphorins

- In the PNS, semaphorin 4F is required for correct axon–glial communication [125]
- In the CNS in MS, semaphorins3A and 3F are upregulated, and modulate oligodendrocyte recruitment and differentiation [121].

Netrins

- Netrins are secreted molecules that are important for axonal pathfinding
- In the CNS, the netrin receptor DCC promotes membrane extension[127]

Box 4 | Glial signals to axons

Neurotrophins

Glia release neurotrophins to axons, which in response modulate myelination[161]

- In the PNS, brain-derived neutrophic factor is bound to axonal $p75^{NTR}$ and promotes myelination; however, this neutrophic factor, when bound to full length TrkB, inhibits myelination
- Nerve growth factor binds TrkA on axons and promotes myelination in the PNS

- Glial cell-derived neurotrophic factor probably binds Ret on axons, and promotes myelination of nociceptive neurons in the PNS
- In the CNS, nerve growth factor binds TrkA on axons and inhibits myelination

Merlin

- Merlin is encoded by the tumor suppressor gene *NF2* (implicated in neurofibromatosis type 2 tumors) and is important for the formation of axon–glial junctions[174]

Lgi4

- Lgi4 is an orphan molecule secreted by Schwann cells that regulates PNS myelination, perhaps through effects on axons[48]

Myelin-associated glycoprotein

- Myelin-associated glycoprotein is located on the membrane of glial cells facing axons
- The absence of myelin-associated glycoprotein promotes axonal degeneration[2]

Erytropoietin

- Erytropoietin is released by Schwann cells after injury and reduces axonal degeneration[175]

Sirtuin2

- Sirtuin 2 in oligodendrocytes is a putative mediator of axonal degeneration in spastic paraplegia 2 due to mutations in proteolipid protein
- lack of proteolipid protein is associated with absence of sirtuin 2 from CNS myelin[176]

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Figure 1.
The myelin–axon unit. The development and maintenance of the myelin–axon unit, here illustrated in the PNS, is dependent on reciprocal signaling between glia and axons. a | Schwann cells sit at the edge of a bundle of axons in prenatal nerves and provide trophic support to neurons (1). In turn, axons and other sources (for example, extracellular matrix) transmit signals that promote the survival and differentiation of glia, as well as myelination (2). b | Eventually, Schwann cells myelinate a segment of one axon. Once the myelin–axon has formed, glia transmit signals that promote axonal health (3), while signals originating from the axon and extracellular matrix promote myelin maintenance (4). Reprinted from *Neuron*, 40, Salzer, J. L., Polarized domains of myelinated axons, 297–318 © 2003, with permission from Elsevier.

Figure 2.

Axon to glia signaling pathways in myelination. Schematic representation of the main signaling pathways that regulate the onset of myelination during CNS development. The figure depicts an oligodendrocyte just before wrapping. The pathways shown are not comprehensive, but focus on signaling that converges on the glial cell nucleus. Regulatory feedback loops are not displayed. For some molecules, the localization shown is putative. Note that, as oligodendrocytes must integrate signals from multiple axons, with different caliber and electrical activity, a layer of local peri-axonal regulation is likely to be superimposed on nuclear regulation of myelination. Dashed lines indicate signals with uncertain targets. Abbreviations: Nrg: Neuregulin; EGF: Epidermal Growth Factor; IGF1: Insulin-like Growth Factor 1; Sema: Semaphorin; Fyn: Fyn kinase; GPR17: G protein-coupled receptor 17; P2: Purinergic Receptors 2; NICD: Notch-1 intracellular domain: PI3K: phosphatidylinositol-3 kinase; Akt: serine/threonine-specific protein kinaseAkt/PKB; mTOR: mammalian target of rapamycin (mTOR) signaling complexes; HDAC: Histone deacetylase; YY1: YIN-YANG-1; Sox: SRY-box containing transcription factor; Zfp: zinc finger protein; MRF: Myelin gene regulatory factor; Tcf: T-cell factor 4 transcription factor; Hes: hairy and enhancer of split 5 transcription factor; Id: Inhibitor of differentiation transcription factor; β-cat: β-catenin; Wnt: Wingle wingless-related mouse mammary tumor virus integration site protein.

Figure 3.

Axon to glia signaling in myelination. Schematic representation of the main pathways that regulate myelination during PNS development. The figure depicts a promyelinating Schwann cell. The pathways shown are not comprehensive, but focus on signaling that converges on the nucleus. Regulatory feedback loops are not displayed. For some molecules, the localization shown is putative. Dashed lines indicate signals with uncertain targets. NFkB (nuclear factor κB); Pou (Pituitary Octamer Unc-86) 3F1, also known as Tst-1/Oct6/ SCIP, Egr (Early growth response) 2, also known as Krox-20; Sox (SRY-box containing) 10 and SREBP (Sterol Regulatory Element Binding Protein) are transcription factors that activate PNS myelination[68, 156]. Mutations in these genes are associated with CMT neuropathies9, Waardenburg-Hirschsprung disease and central dysmyelination65, [157]. *Sox* (SRY-box containing) 2 and 4, Id (Inhibitor of differentiation)2, Pax3 and c-Jun-of the activator protein 1 (AP-1 complex, are inhibitory transcription factors active before myelination. Their inappropriate activation might be harmful in neuropathies[6]. Nrg: Neuregulin; IGF1: Insulin-like Growth Factor 1; Cadm: cell adhesion molecules, also known as IGSF4, SynCAM, Necl, TSLC; PAR: Partition defective 3; GPR: G protein-coupled receptor; P2: Purinergic Receptors 2; NICD: Notch-1 intracellular domain: PI3K: phosphatidylinositol-3 kinase; PLC γ: phospholipase γ; Sema: Semaphorin; NFAT c4: Nuclear factor of activated T-cells, cytoplasmic 4.

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Special Focus: Synapse-Glia Interactions

Cell adhesion molecules in the central nervous system

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Key words: synapses, cell adhesion molecules, cadherin superfamily, immunoglobulin superfamily, nerve tissue proteins, axons

Cell-cell adhesion molecules play key roles at the intercellular junctions of a wide variety of cells, including interneuronal synapses and neuron-glia contacts. Functional studies suggest that adhesion molecules are implicated in many aspects of neural network formation, such as axon-guidance, synapse formation, regulation of synaptic structure and astrocyte-synapse contacts. Some basic cell biological aspects of the assembly of junctional complexes of neurons and glial cells resemble those of epithelial cells. However, the neuron specific junctional machineries are required to exert neuronal functions, such as synaptic transmission and plasticity. In this review, we describe the distribution and function of cell adhesion molecules at synapses and at contacts between synapses and astrocytes.

Introduction

The cell-cell adhesion system is involved in many aspects of neuronal development, including neuronal cell migration, axon-bundle formation, synapse formation and formation of complex of glial networks which surround axons and synapses. These adhesion systems are important for brain morphology and highly coordinated brain functions, such as memory and learning.[1-3] Like epithelial junctions, cell-cell junctions in the nervous systems contain a variety of transmembrane proteins, cytoskeletal elements and signaling complexes.

During early development of the nervous systems, differentiated neurons migrate to their proper positions and elongate their axons towards their targets. Growing axons are guided by various attractive or repulsive target-derived cues.[4] After reaching their target areas, axonal growth cones still need to recognize their appropriate target cells for the formation of synapses. Then, initial contacts are formed between axons and dendrites, and signaling through homophilic and heterophilic receptors induces differentiation of the synaptic specialization.[5] Most of these interactions and recognitions have been shown to be mediated by cell surface proteins. Various cell surface proteins have been identified and characterized as important regulators of axon-guidance and synapse formation (Table 1). These molecules interact with each other between the cells to activate various signaling pathways and bring the apposed cell membranes into contact. Some of these molecules are defined as adhesion molecules and others as signaling molecules. However, as certain adhesion molecules are known to have signaling functions and the signaling molecules often promote cell-cell adhesion, it might not be so easy to distinguish these membrane proteins specifically involved in adhesion or signaling.

In addition to neuron-neuron interactions, astrocyte-synapse interactions are also known to play important roles in the formation of neural networks. Astrocyte-synapse communication participates in synapse formation, synaptic transmission and axonal conduction, and perhaps modulates the activity of neuronal networks during development and throughout adult life.[6] Recent advances have clarified the molecular compositions of astrocyte-synapse interfaces, and have provided new insight into astrocyte-synapse communication. To date, a few adhesion molecules have been identified at the astrocyte-synapse contacts. Here, we briefly summarize the key cell adhesion molecules involved in the synapse formation and in the astrocyte-synapse interactions.

Synapses

Synapses are a specialized form of intercellular junctions where the axon terminal of a neuron comes into functional contact with a target cell (Fig. 1A and B). Specificity and plasticity of synapses provide neurons with a structural and functional basis for the formation of the neuronal network system. Synapses are highly asymmetrical junctions formed between two different neurons, and early ultrastructural studies showed that the synaptic junctional areas contain at least two types of adhesion structure (Fig. 1C).[7,8] One type of adhesion structure is the transmitter release zone associated with synaptic vesicles, termed synaptic junctions (SJs), and the other is a symmetrical junction, termed puncta adherentia junctions (PAJs), defined by the two criteria of symmetric paramembranous dense materials and the lack of association with synaptic vesicles (Fig. 1B). SJs are regarded as sites for neurotransmission. They are associated with presynaptic active zones containing Ca^{2+} channels and numerous neurotransmitter-filled synaptic vesicles which are docked on the presynaptic membrane by a complex of proteins, and postsynaptic densities where the specific neurotransmitter receptors and structural scaffolding and signaling proteins are localized. PAJs are regarded as mechanical adhesion sites between axon

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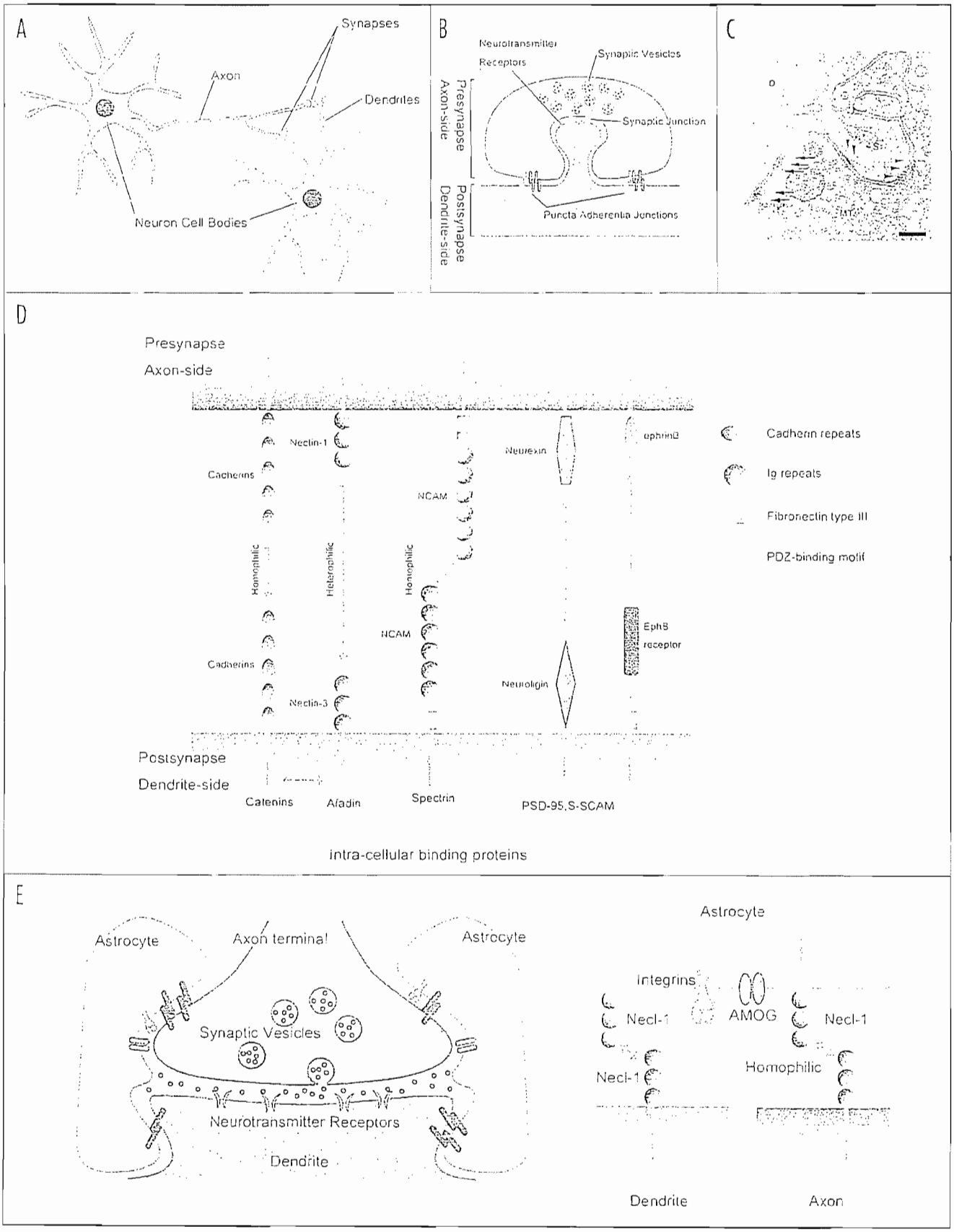
A
Synapses
Axon
Dendrites
Neuron Cell Bodies
B
Neurotransmitter Receptors
Synaptic Vesicles
Presynapse Axon-side
Synaptic Junction
Postsynapse Dendrite-side
Puncta Adherentia Junctions
C
D
Presynapse
Axon-side
Nectin-1
Cadherins
Homophilic
Heterophilic
NCAM
Homophilic
NCAM
Cadherins
Necta-3
Neurexin
ephrinB
EphB receptor
Neuroligin
Postsynapse
Dendrite-side
Catenins
Afadin
Spectrin
PSD-95,S-SCAM
Cadherin repeats
Ig repeats
Fibronectin type III
PDZ-binding motif
intra-cellular binding proteins
E
Astrocyte
Axon terminal
Astrocyte
Synaptic Vesicles
Neurotransmitter Receptors
Dendrite
Astrocyte
Integrins
Necl-1
AMOG
Necl-1
Necl-1
Homophilic
Dendrite
Axon

Figure 1 (see previous page). (A) Synapses are formed at the contact points between axons and dendrites of their target neurons. (B) At synapses, at least two types of intercellular junctions, synaptic and puncta adherentia junctions, have been recognized. Synaptic junctions are regarded as sites of neurotransmission, associated with synaptic vesicles at the presynaptic active zone where Ca^{2+} channels localize, and postsynaptic densities (PSDs), where neurotransmitter receptors localize. Puncta adherentia junctions, which are not associated with synaptic vesicles or PSDs, appear to be ultrastructurally similar to adherence junctions of epithelial cells. (C) Electron microscopic morphology of the synapses between the mossy fiber terminals and the dendrites of pyramidal cells in the CA3 area of the hippocampus. Arrows indicate PAJs. Arrow heads indicate SJs. D: dendrite. S: dendritic spine. MT: mossy fiber terminal. Scale bar, 200 nm. (D) Molecular composition of the synapse. Many of these adhesion molecules possess a binding motif that binds to PDZ proteins. These interactions associate with each other and lead to the formation of a multi-molecular scaffold beneath both the pre- and post-synaptic membranes. (E) Astrocytes have many characteristic processes and ensheath synaptic junctions in the brain, but do not form myelin. Necl-1 localizes at the contact sites between axon terminals and glia cell processes and interacts homophilically.

Table 1 **Lists of the neuron-neuron and neuron-glia interactions in the nervous systems**

Classification	Adhesion molecules	Localization
Cadherin Super Family	Classic cadherins	Synapse (PAJs), Neuron-Glia
	Proto-cadherins	Synapse (?)
Ig-like Molecules	Nectins	Synapse (PAJs), Neuron-Glia
	Nectin-like molecules (Necls)	Neuron-Glia
	NCAM	Synapse
	Syg-1, Syg-2	Synapse
	Sidekicks	Synapse
	Integrins	Synapse, Neuron-Glia
Others	Neuroligins, neurexins	Synapse (SJs)
	Eph receptors, ephrins	Synapse (SJs)

terminals and their targets, although their exact functions remain unknown. However, PAJs are morphologically similar to adherens junctions (AJs) formed in epithelia, and several important molecular constituents of neuronal synapses are common to both neurons and epithelial cells. Thus, some basic cell biological aspects of the assembly of junctional complexes may be shared between these two cell types.[9,10] During development, specific neuronal circuits are generated by synapse formation between the appropriate pre- and postsynaptic partners. Initial contacts between synaptic partners are frequently established between axonal growth cones and dendritic filopodia extending from dendrites in vitro.[11,12] Once initial axon-target interactions develop, various molecules can engage in bidirectional signaling to coordinate the differentiation of synaptic membrane specializations and stabilize the synaptic contact. Several factors that may be involved in these processes are summarized below. Although electrical synapses are formed at narrow gaps between the pre- and postsynaptic neurons known as gap junctions, we describe chemical synapses.

Cadherins

Cadherins are Ca^{2+}-dependent cell-cell adhesion molecules that constitute a superfamily comprised of more than 100 members in vertebrates, and are grouped into subfamilies that are designated as classic cadherins and protocadherins.[13] Classic cadherins are single-pass transmembrane proteins and have five extracellular cadherin repeat (EC) domains (EC1 to EC5). All classic cadherins are homophilic adhesion molecules that function with their cytoplasmic (CP) partners, catenins (Fig. 1D).[14] Catenins are cadherin-binding proteins that connect cadherins to the actin cytoskeleton. These include α-catenin, β-catenin and p120 catenin. The cadherin-catenin complexes are known to regulate actin polymerization, a property important for maintaining the cell-cell adhesion. Cadherins and their associated catenins have been observed in many neuronal populations in the central nervous systems (CNS). At the ultrastructural level, these proteins were found in synaptic junctions of most regions of the nervous systems, forming a symmetrical adhesion structure in the PAJs.[9] During development, the cadherin-catenin complexes accumulate at early axo-dendritic filopodial contacts, and are retained in many of the mature synapses.[9,15-18] A fragment of N-cadherin lacking its extracellular region serves as a dominant negative mutant of cadherins and inhibits their cell-cell adhesion activity. Expression of this mutant results in the appearance of filopodia-like spines, an increase in the spine length, and a decrease in the spine head width, and affects the organization of synapses in the cultured hippocampal neurons.[18,19] Despite the evidence that cadherins are involved in the formation of synapses, they are not sufficient to form them in vitro, because expression of N-cadherin in non-neuronal cells fails to induce pre-synaptic differentiation in axons at the sites of contact.[20] Recent studies also implicate catenins in the control of spine structure and synaptic organization in cultured hippocampal neurons. Deletion of β-catenin affects localization of synaptic vesicles along the axon,[21] and loss of p120 catenin affects Rho-family small G-protein signaling, which results in a reduced spine density.[22] A remarkable feature of classic cadherins is its binding specificity and region-specific distribution. In the brain, many subtypes of classic cadherins are expressed by restricted groups of functionally connected nuclei and laminas.[23] Whether cadherin-mediated adhesion contributes to the formation of selective inter-neuronal connections during neural network formation remains unknown.

Protocadherins

Protocadherins are a group of transmembrane proteins that belong to the cadherin superfamily, and have varying numbers of the EC domains but divergent cytoplasmic domains that do not appear to signal through catenins.[24,25] Various protocadherins are expressed in the nervous systems, and some of them are localized at synapses. Multiple α- and γ-protocadherin isoforms are highly expressed in distinct, although partially overlapping, sets of neurons and concentrated at synapses. The complex genomic organization and alternative splicing of protocadherins have led to the speculation that their diversity underlies synaptic specificity.[26] γ-protocadherins are required for survival of specific neuronal types[27] and arcadlin is required for activity-dependent synaptic morphogenesis.[28] However, the biological functions of most protocadherins are unknown.

Nectins

Nectins represent a family of Ca^{2+}-independent immunoglobulin (Ig)-like cell-cell adhesion molecules, which consist of four members (Fig. 1D).[29] At the CA3 region of hippocampus, nectin-1 and nectin-3 asymmetrically localize at the pre- and post-synaptic sides, respectively, of the PAJs, but not at SJs.[10] Nectins form homo- or hetero-*trans*-dimers in a Ca^{2+}-independent manner, where heterotypic binding leads to stronger adhesion than homotypic binding.[30-32] In epithelial cells in culture, nectins first form cell-cell adhesion and then recruit cadherin to the nectin-based cell-cell adhesion sites to cooperatively form AJs.[33,34] Afadin, an actin-filament binding protein that connects nectins to the actin cytoskeleton, is also present at PAJs. Disruption of nectin-based cell-cell adhesion in cultured hippocampal neurons decreases the size of synapses but increases their number,[10] and a nectin-1 mutant causes human cleft lip/palate-ectodermal dysplasia, Margarita island ectodermal dysplasia, and Zlotogora-Ogür syndrome, characterized by mental retardation, cleft lip/palate, syndactyly and ectodermal dysplasia.[35] In both nectin-1 and -3-deficient mice, the number of PAJs at the synapses between the mossy fiber terminals and the dendrites of the CA3 pyramidal cells in the hippocampus is reduced. In addition, the abnormal mossy fiber trajectory is observed, suggesting that nectins are involved in the formation of PAJs, which maintain the proper mossy fiber trajectory in the CA3 region of the hippocampus.[36] In afadin-deficient mice, perforated synapses in the hippocampus are observed. Reduction in the number of PAJs is likely to be further enhanced in afadin-deficient mice than in nectin-1 or -3-deficient mice. The observation of loss of PAJs in the nectin and afadin-deficient mice suggests the possibility that the localization of the cadherin/catenin complex is regulated by the nectin/afadin system, as for epithelial adherens junctions. The recruitment of afadin (AF-6) to postsynapse is regulated by small G-protein Rap1, and is involved in spine formation.[37]

The axon-biased localization of nectin-1 and its *trans*-interaction with nectin-3 in cooperation with the cadherin machinery is critical for the ordered association of axons and dendrites.[38] However, the sorting signal of nectin-1 to axons has not been identified.[39] The genetic deletion of nectin-1 loosens the contacts between axons and dendritic spines, while the overexpression of nectin-1, causing mislocalization of nectin-1 to dendrites, induces atypical dendro-dendritic as well as excessive axo-dendritic contacts. These actions of nectins require cadherin-catenin complexes suggesting that the two adhesion systems cooperate.[38] These data suggest that localized cadherin activity may be achieved by cooperative heterophilic nectin interactions. It is also likely that mechanisms work for restricting adhesion activity at specific cell-cell contact sites. These data are consistent with those obtained in epithelial cells, suggesting that nectins form initial cell-cell adhesion and recruit cadherins to the nectin-based cell-cell adhesion sites to form AJs, and suggest that nectins play similar roles in the formation of PAJs.

Other Ig Superfamily CAMs

Other Ig superfamily CAMs, which have varying numbers of Ig-like domains, have been identified at synapses and have been shown to be involved in synaptic formation and plasticity. For example, neural cell adhesion molecule (NCAM), which contains five Ig-like domains and two fibronectin type III repeats, is engaged in homophilic and heterophilic interactions with a variety of ligands at synapses, such as fibroblast growth factor receptor (FGFR), L1, TAG-1/axonin-1 and heparan sulfate proteoglycans (Fig. 1D).[40,41] NCAM is widely expressed in the developing and adult brains and plays crucial roles in migration, pathfinding of axons, and synaptic plasticity. It is involved in both early synaptogenesis and subsequent synaptic maturation.[42,43] NCAM is unique among adhesion molecules in that it carries a large amount of the negatively charged sugar, polysialic acid (PSA) (Bonfanti et al., in this issue). Poor axonal fasciculation is observed in the hippocampus of NCAM-deficient mice, resulting in an impaired synapse formation in the CA3 region.[44] Mossy fibers also appear defasciculated in mice with the NCAM-180 isoform.[45] These functions of NCAM appear to be mediated by primarily by presence of the PSA moiety.[46] Neurofascin 186 (NF186), an L1 family Ig-like cell adhesion molecule, is implicated in the subcellular organization of GABAergic synapses between basket interneurons and Purkinje cells in the cerebellum.[47]

SYG-1/SYG-2

SYG-1/SYG-2 are specific adhesion molecules that determine synaptic specificity in a lock-and-key manner. SYG-1, a four Ig-like domain-containing protein, and SYG-2, a seven Ig-like domain- and one fibronectin type III repeat-containing protein, were isolated in a genetic screen for *C. elegans* mutants that exhibit defective synaptic positioning.[48,49] Interactions between SYG-1 and SYG-2 induce formation of synapses at appropriate synaptic targets. The Drosophila orthologues of *roughest(rst)* have been implicated in axon fasciculation and layer targeting in the fly visual system.[50] Moreover, SYG-1 and SYG-2 share significant homology with the mice and human proteins NEPH and Nephrin, which are expressed in the CNS,[51] although their roles in the CNS remain unknown.

Sidekicks

Sidekicks, which have six Ig-like domains and thirteen fibronectin type III repeats, have been implicated in selective synapse formation in the chicken retina.[52] Sidekick-1 and -2 are differentially expressed among subsets of retinal ganglion cells in a non-overlapping manner. Sidekicks act as homophilic adhesion molecules in vitro, and are highly concentrated at synapses of restricted regions in vivo. Ectopic expression of Sidekick in Sidekick-negative cells induces mistargeting. These data suggest that sidekick interactions may promote lamina-specific connectivity.

Neuroligin

Neuroligin is an esterase-like domain-containing protein and localizes at the post-synaptic side of SJs, whereas β-neurexin is a laminin-globular-domain-containing protein and localizes at the pre-synaptic side of SJs. These two molecules interact with one another and this interaction induces the formation of synapses in vitro (Fig. 1D). The neurexin family was first identified as receptors for alpha-latrotoxin, which acts presynaptically to release neurotransmitters from sensory and motor neurons.[53] More than 1,000 neurexin isoforms are generated by alternative splicing, which are differentially expressed in the nervous systems.[54] Neuroligins is a β-neurexin binding partner.[55,56] β-Neurexin binds neuroligins *trans*-synaptically and induces formation of glutamatergic and GABAergic presynaptic specializarions in vitro.[20,57,58] However, neuroligins are

indispensable for synapse maturation and synaptic transmission, but not for triggering initial synapse formation from the phenotypes of knockout mice.[59]

Necl-2

Necl-2 was previously characterized as a tumor suppressor gene, and is also termed TSLC1/SgIGSF/RA175/IGSF4/SynCAM1. Necl-2 is a homophilic adhesion molecule, but also shows heterophilic cell-cell adhesion activity with Necl-1 and nectin-3.[60] Necl-2 is widely expressed in various tissues and localizes at the basolateral plasma membrane in epithelial cells, not in the specialized cell–cell junctions such as AJs, Tjs and desmosomes.[61] Necl-2 localizes at synapses and induces presynaptic differentiation and stabilization, at least in vitro.[62]

Eph Receptor

Eph receptor tyrosine kinases and their ephrin ligands are grouped into two families: ephrinA ligands are tethered to the plasma membrane by a GPI linkage and bind to EphA receptors, whereas ephrinB ligands are transmembrane proteins that bind preferentially to EphB receptors (Fig. 1D).[63] EphB receptors localize to synapses, where they can bind the NMDA-type glutamate receptor subunit NR1 via the extracellular domain.[64] Stimulation of EphB receptors by ephrin ligands results in increased synaptic density and in NMDA receptor-mediated calcium influx and gene expression.[65] EphBs multiple mutant mice develop abnormal spines in the hippocampus both in vitro and in vivo.[66] However, the molecular mechanisms of many ephrin/Eph-related synaptic functions and their roles in the initial steps of synapse assembly are still largely unknown.

Axon-Astrocyte Contacts

Astrocytes are characteristic star-shaped glial cells, and their many processes ensheath synaptic junctions in the brain, but do not form myelin (Fig. 1E). Astrocytes are also known to regulate synaptic transmission by uptake of neurotransmitters, such as glutamate, ATP and GABA, from the synaptic cleft through membrane transporters, and release of glutamate upon reversal of the transporter.[6] Other substances released by astrocytes can strengthen synaptic transmission by co-activating NMDA receptors in the postsynaptic membrane (e.g., D-serine) or can reduce it by binding to neurotransmitters.[67,68] Synapse formation may also be regulated by factors produced by astrocytes.[69] The co-culture of purified neurons with astrocytes can facilitate synaptogenesis.[70] For example, an astrocyte-derived factor induces the maturation of retinal ganglion cells [71]; this diffusible factor has been identified as cholesterol complexed with apolipoprotein E-containing lipoproteins.[69] A recent study showed that thrombospondin-1 and -2, astrocyte-secreted proteins, promote CNS synaptogenesis in vitro and in vivo.[72] On the other hand, integrins localize at contacts between neurons and astrocytes promote synaptogenesis.[73] The interaction between neurons and astrocytes is important for synaptogenesis. However, a few adhesion molecules have been identified at contacts between neurons and astrocytes.

AMOG (Adhesion Molecule on Glia)

AMOG (Adhesion molecule on glia), the β2-subunit of the Na^+/K^+ ATPase, is a membrane glycoprotein and localizes at contacts between neurons and astrocytes. AMOG is implicated in neurite outgrowth and neuronal migration.[74-76] AMOG associates with the catalytic α-subunit of the Na^+/K^+ ATPase and forms a functional ion channel.[77] This unique molecule serves as a cell adhesion molecule and a subunit of ion channel. AMOG is firstly expressed in the brain shortly before granule cell migration. The expression level of AMOG increases during early postnatal development and reaches the highest expression level in adult.[78] AMOG-deficient mice present motor incoordination and paralysis in early postnatal life and die shortly after birth.[79] The exact functions of AMOG still remain elusive.

Integrins

Integrins are cell surface receptors that interact with the extracellular matrix (ECM) and transduce the signal from the ECM to the cell. Integrins consist of two distinct chains, α- and β-subunits. Integrins at contacts between neurons and astrocytes activates protein kinase C (PKC) signaling and promotes synaptogenesis in vitro.[73] However, the involvement of the integrins-dependent PKC signaling in synaptogenesis still remains elusive in vivo.

Necl-1/TSLL1/SynCAM3

Necl-1/TSLL1/SynCAM3, which has a domain structure similar to those of nectins, localizes at axon-astrocyte contacts (Fig. 1E).[80] Necl-1 shows Ca^{2+}-independent homophilic cell-cell adhesion activity and heterophilic cell-cell adhesion activity with Necl-2, nectin-1 and nectin-3, but not Necl-5 or nectin-2. Necl-1 does not bind afadin, but binds Dlg3/MPP3, a membrane-associated guanylate kinase family member, Pals2 and CASK. Necl-1 is specifically expressed in neural tissue, and localizes to contact sites along axons, nerve terminals, glial cell processes, axon bundles and myelinated axons. However, the exact functions of Necl-1 remain unknown.

Conclusions and Perspectives

Herein, we described the roles of the various adhesion molecules in synapse formation, and neuron-glia interactions. Functional studies of individual cell adhesion molecules have provided a wealth of information on their roles in synapse assembly, spine morphogenesis and synaptic plasticity. Although the various adhesion systems can mediate adhesive interactions, individually, they probably control specific aspects of synapse formation. Because multiple systems appear to cooperate at individual synapse, it will be of great interest to determine whether they act in a parallel or in a hierarchical manner. For most of the functions of neuron-glia contacts, we still lack sufficient information on their functions at both cellular and molecular levels. Future research on the mechanisms of neuron-glia interactions will lead to greater insight into the mechanisms underlying the formation of complex neural circuitries.

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Neuroligins and neurexins link synaptic function to cognitive disease

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The brain processes information by transmitting signals at synapses, which connect neurons into vast networks of communicating cells. In these networks, synapses not only transmit signals but also transform and refine them. Neurexins and neuroligins are synaptic cell-adhesion molecules that connect presynaptic and postsynaptic neurons at synapses, mediate signalling across the synapse, and shape the properties of neural networks by specifying synaptic functions. In humans, alterations in genes encoding neurexins or neuroligins have recently been implicated in autism and other cognitive diseases, linking synaptic cell adhesion to cognition and its disorders.

The brain integrates and processes sensory inputs to generate motor outputs appropriate for the survival of the organism. This transformation of information is carried out by cascades of synapses, assembled in overlapping neural circuits[1]. All processing of information in the brain involves synapses, and almost all abnormalities in brain function have a direct or indirect effect on synaptic function.

Synapses are specialized intercellular junctions dedicated to the transfer of information from a neuron to a target cell, usually another neuron[1] (Fig. 1a). Synaptic transmission of information is fast, dynamic, efficient and tightly regulated (Box 1). Synapses have many of the same properties as intercellular junctions in non-neural tissues, but they differ from all other such junctions because they are inherently asymmetrical, transmit information by an extremely fast mechanism, and are highly plastic. Moreover, synapses have diverse properties that are specified by both the presynaptic neuron and the postsynaptic neuron (for example, see ref. 2).

Enormous progress has recently been made in understanding synaptic transmission; much is now known about the machinery and functional properties of synapses. However, the molecular mechanisms underlying synapse formation and the specification of synapse diversity are less clear, as are the processes mediating the assembly of synapses into neural circuits[3]. For appropriate neural-circuit function, the formation and specification of synapses is immensely important. The input–output properties of a neural circuit depend on both its pattern of synaptic connectivity (referred to as its wiring diagram) and the diverse properties of individual synapses in the circuit[4]. The pattern of connectivity in a circuit is no more important than the properties of the individual synapses comprising the circuit. Use-dependent changes in synaptic strength (that is, synaptic plasticity) can completely alter the relative contributions of different synapses in a circuit, thereby sometimes even reversing its input–output properties as a function of previous use without a change in the wiring diagram (for example, see ref. 5).

Synapse formation and the specification of synaptic diversity are intricately linked and probably depend on the actions of synaptic cell-adhesion molecules[3]. The diversity of synapses is partly due to differences in the composition of their neurotransmitter release and receptor machineries but seems to be based largely on differences in the organization of these machineries. Synapse formation and specification probably involves three steps: initial recognition of the target cell by the neural growth cone, formation of synaptic junctions with recruitment of synaptic components, and maturation of synaptic junctions with specification of circuit-specific properties. Functional assays for synapse formation and tests of specific molecules are difficult to carry out (Box 2), hindering identification of the molecular mechanisms involved. These difficulties are confounded by the fact that many candidate molecules, such as cadherins and WNTs, carry out essential functions during earlier development, in addition to their presumptive role in synapse formation[6,7].

Vertebrate neurexins (NRXNs) and neuroligins (NLGNs) are arguably the best-characterized synaptic cell-adhesion molecules, and they are the only ones for which a specifically synaptic function has been established[8,9]. Here I describe the role of NRXNs and NLGNs as synaptic cell-adhesion molecules that function in an unexpected manner. I suggest that these molecules are required for synapse function but not for synapse formation, that they affect trans-synaptic activation of synaptic transmission but are not essential for synaptic cohesion of the presynaptic and postsynaptic specializations, and that their dysfunction impairs the properties of synapses and disrupts neural networks without completely abolishing synaptic transmission[10–12]. Because they are cell-adhesion molecules, NRXNs and NLGNs probably function by binding to each other and by interacting with intracellular proteins (most notably with PDZ-domain proteins), but the precise mechanisms involved and their relationship to synaptic transmission remain unclear. The importance of NRXNs and NLGNs for synaptic function is, however, evident from the marked deficits in synaptic transmission in mice lacking NRXNs or NLGNs.

The role of NRXNs and NLGNs in synaptic function almost predestines them for a role in cognitive diseases, such as schizophrenia and autism spectrum disorders (ASDs), the mechanisms of which have proved difficult to ascertain. One reason for the difficulties in understanding cognitive diseases is that they may arise from subtle changes in a subset of synapses in a neural circuit, as opposed to a general impairment of all synapses in all circuits. As a result, the same molecular alteration can produce different circuit changes and neurological symptoms, which are then classified

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as distinct cognitive diseases. Indeed, recent studies have identified mutations in the genes encoding NRXNs and NLGNs as a cause of ASDs, Tourette's syndrome, learning disability and/or schizophrenia; sometimes family members with the same mutation have different cognitive disorders[13–27]. Viewed as a whole, current results thus identify NRXNs and NLGNs as trans-synaptic cell-adhesion molecules that mediate essential signalling between presynaptic and postsynaptic specializations. This signalling is central to the brain's ability to process information and is a key target in the pathogenesis of cognitive diseases. I discuss the idea that identification of mutations in NRXNs and NLGNs in patients with cognitive diseases, especially ASDs and schizophrenia, supports the notion that these diseases are caused, at least in part, by abnormalities in synaptic transmission in a subset of neural circuits.

Neurexins are polymorphic synaptic receptors

The venom of the black-widow spider contains a vertebrate-specific toxin called α-latrotoxin. α-Latrotoxin is a large protein that binds to presynaptic receptors and induces a massive release of neurotransmitters[25]. NRXNs were discovered as receptors for α-latrotoxin[29]. NRXNs are type I membrane proteins and can be classified into two types: α-NRXNs and β-NRXNs. α-NRXNs are larger than β-NRXNs; the two types of NRXN contain different amino-terminal extracellular sequences but identical carboxy-terminal transmembrane regions and cytoplasmic tails (Fig. 1b). Extracellularly, α-NRXNs have six LNS domains (laminin, NRXN, sex-hormone-binding globulin domains) with three intercalated epidermal growth factor (EGF)-like domains, whereas β-NRXNs have a single LNS domain. In addition to α-NRXNs and β-NRXNs, neurons express NRXN-related proteins called CASPRs (contactin-associated proteins), which resemble α-NRXNs but contain additional extracellular domains that are not found in α-NRXNs[30]. CASPRs also function as cell-adhesion molecules, like NRXNs, but are mainly involved in neuron–glia interactions outside synapses[31].

The mammalian genome contains three NRXN genes (*NRXN1*, *NRXN2* and *NRXN3*), each encoding an α-protein and a β-protein from independent promoters[32]. Furthermore, extensive alternative splicing of the encoded proteins at five canonical positions generates thousands of NRXN isoforms[33] (Fig. 1c). Conceptually, these isoforms could specify a 'code' of interactions at synapses. Consistent with this idea, alternative splicing of NRXNs is regionally regulated and altered by activity in neurons[33,34]. Splice sites 1 to 4 (SS 1 to SS 4) involve short sequences (30 residues or fewer), are located in or adjacent to LNS domains and are conserved in all three NRXNs. Splice site 5 in *NRXN1* involves the insertion of only three residues. But in *NRXN2*, splicing at this site inserts 191 residues, and in *NRXN3* it creates a huge diversity of sequence inserts that include multiple variants with in-frame stop codons and therefore encode secreted NRXNs[33,35].

Using *in situ* hybridizations, messenger RNAs encoding the different α-NRXNs and β-NRXNs were shown to be coexpressed in the same class of neuron, but each type of NRXN was found to be differentially distributed between different classes of neuron[33]. Immunofluorescence studies, subcellular fractionations and the function of NRXNs as α-latrotoxin receptors indicate that NRXNs are located on presynaptic terminals[29,36–38]. It remains unclear, however, whether NRXNs are

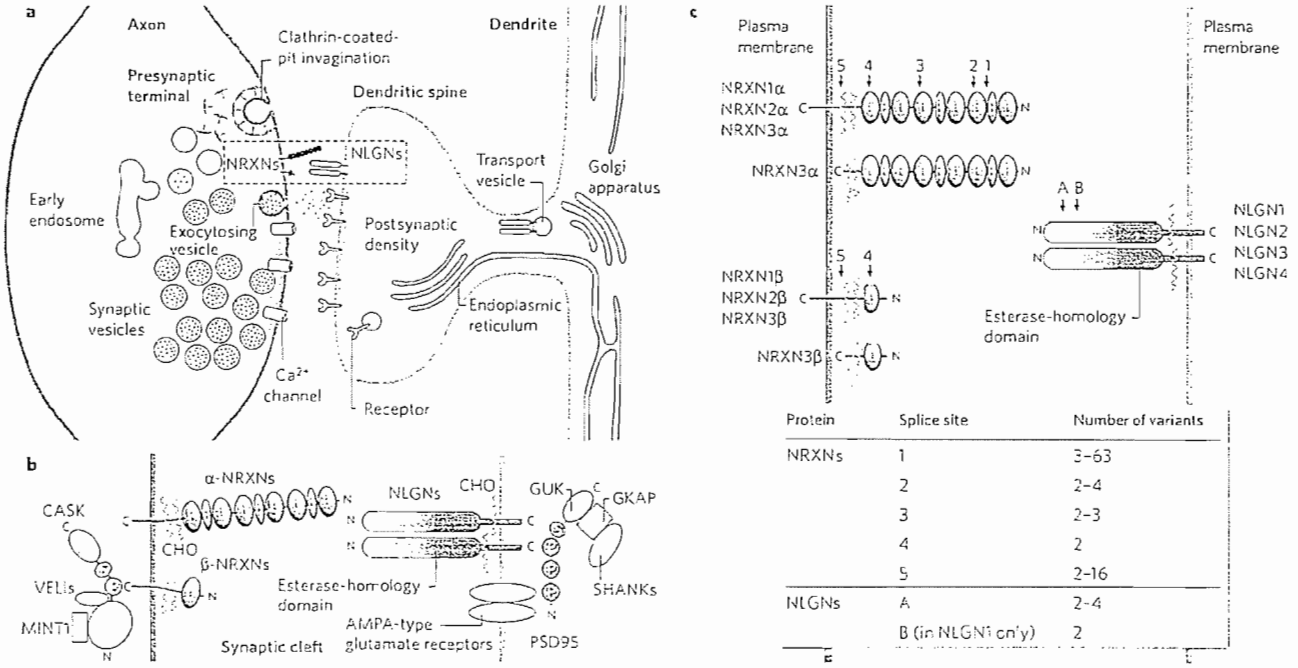
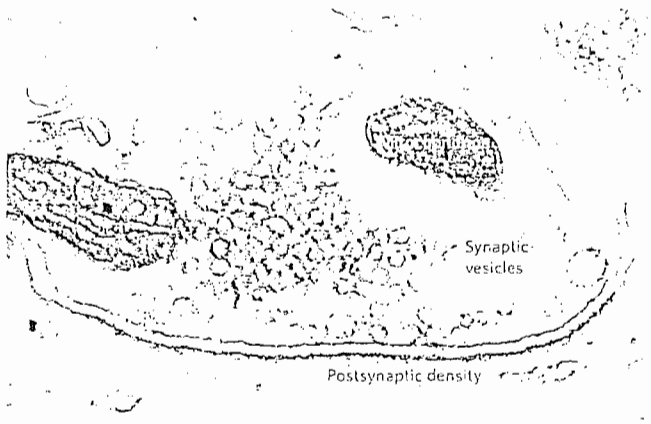
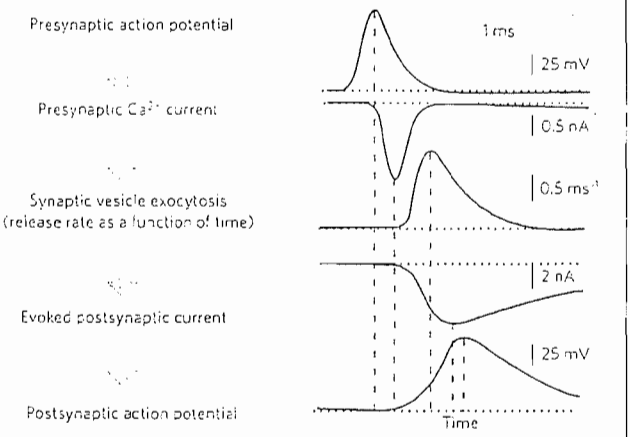


Figure 1 | Architecture of the trans-synaptic neurexin–neuroligin complex. a, The structure of an excitatory synapse and the putative locations of neurexins (NRXNs) and neuroligins (NLGNs) in the synapse. A presynaptic varicosity containing synaptic vesicles is shown on the left, and a postsynaptic spine with a postsynaptic density containing neurotransmitter receptors on the right. b, The NRXN–NLGN junction, including selected presynaptic and postsynaptic binding proteins: CASK, VELIs and MINTs on the presynaptic side[62]; and PSD95 (which binds to AMPA-type glutamate receptors through its first PDZ domain, and to NLGNs through its third PDZ domain[64]), GKAP and SHANKs on the postsynaptic side. Note that a proportion of NRXNs and CASK could be also postsynaptic, and that SHANKs might also be presynaptic. C, carboxyl terminus; CAMK, Ca^{2+}/calmodulin-dependent protein kinase domain of CASK; CHO, carbohydrate-attachment sequence; E, epidermal growth factor (EGF)-like domain; GUK, guanylate-kinase domain; L, LNS (laminin, NRXN, sex-hormone-binding globulin) domain; N, amino terminus; P, PDZ domain; S, SH3 domain. c, Alternative splicing of NRXNs and NLGNs. α-NRXNs contain five canonical splice sites (1 to 5), and β-NRXNs contain two (4 and 5). Splice site 1 is C-terminal to the first EGF-like domain. Splice sites 2, 3 and 4 are at similar positions in the second, fourth and sixth LNS domains, respectively. Splice site 5 is between the glycosylated CHO sequence and the transmembrane region. Most alternative splicing involves the insertions of small evolutionarily conserved sequences, except for at splice site 5. Splicing at this site in NRXN2 involves a large insertion (191 residues), and in NRXN3 it involves at least 16 variants, some of which include stop codons and thus produce secreted isoforms of NRXN3 (ref. 35). NLGNs contain only two sites of alternative splicing, and one of these sites, site B, is present only in NLGN1.

Box 1 How a synapse works





At a synapse, a presynaptic terminal containing abundant synaptic vesicles contacts a postsynaptic cell, usually a neuron, as shown in the electron micrograph of a synapse in the left panel above. When an action potential invades the presynaptic terminal, Ca^{2+} channels open, and the inflowing Ca^{2+} triggers fusion of synaptic vesicles with the presynaptic plasma membrane, thereby emptying the neurotransmitters in the vesicles into the synaptic cleft[90]. The neurotransmitters then bind to postsynaptic receptors to complete the information transfer. The overall process is incredibly rapid, with each of the main steps (presynaptic vesicle fusion and postsynaptic signal reception) initiating in less than 1 ms. The time course of synaptic transmission as measured electrophysiologically is shown in the right panel above. The five sequential steps are indicated, as deduced from measurements in the rat calyx of Held synapse[90]. Vertical dashed lines identify the time points when the five steps described reach their peak; horizontal dashed lines identify baselines. In addition to this classic mode of synaptic transmission, synapses have other types of signalling that operate on a longer timescale and function to regulate the synaptic transmission. Structurally, synapses are characterized by coats that line the intracellular face of the presynaptic plasma membrane (referred to as the active zone because synaptic vesicles undergo fusion here) and the postsynaptic plasma membrane (referred to as the postsynaptic density). Presynaptic and postsynaptic plasma membranes are always precisely aligned, and they are separated by a synaptic cleft of about 20 nm. The cleft contains an undefined proteinaceous material in the middle and is presumably bridged by synaptic cell-adhesion molecules such as neurexins (NRXNs) and neuroligins (NLGNs) that align the presynaptic and postsynaptic elements and mediate trans-synaptic signalling. (Left panel courtesy of X. Liu, University of Texas Southwestern Medical Centre, Dallas. Right panel modified, with permission, from ref. 91.)

confined to the presynaptic side, because deletion of genes encoding α-NRXNs also has postsynaptic effects[39] and a proportion of NRXNs has been shown to be present on postsynaptic sites[40].

Neuroligins are neurexin ligands

NLGNs are endogenous NRXN ligands[41]. They are type I membrane proteins, like NRXNs, but have a simpler domain structure and are less diverse (Fig. 1b, c). In addition to NLGNs, neurexophilins (neuropeptide-like proteins) and dystroglycan (a cell-adhesion molecule involved in many types of junction) are also NRXN ligands[42,43]. In contrast to NLGNs, however, no functional effect of binding of neurexophilin or dystroglycan to NRXNs has been observed so far.

The extracellular sequences of NLGNs are composed of a single domain that is homologous with acetylcholinesterases, but it lacks crucial residues in the active site, which is thus disabled (Fig. 1b, c). NLGNs form constitutive dimers through this domain, which is connected to the single transmembrane region by a glycosylated linker sequence. Mammals express four genes encoding NLGNs, with *NLGN3* and *NLGN4* in humans localized to the X chromosome. In humans, *NLGN4* is complemented on the Y chromosome by a similar gene, *NLGN5*. All NLGNs are alternatively spliced at a single canonical position (referred to as SS A); in addition, NLGN1 is alternatively spliced at a second position (called SS B)[44,45]. Most NRXNs and NLGNs are evolutionarily conserved in vertebrates and have more distant relatives in invertebrates[32,46]. It is of interest that the gene encoding NLGN4 diverged rapidly in rodents, suggesting that at least some genes encoding NLGNs are subject to less evolutionary constraint[47]. Sequence comparisons indicate that NLGN1, NLGN3 and NLGN4/NLGN5 are more similar to each other than to NLGN2. All NLGNs are enriched at postsynaptic densities, as judged by subcellular localization. Immunocytochemistry revealed that NLGN1 and NLGN2 are exclusively localized to excitatory and inhibitory synapses, respectively, whereas NLGN3 might be present in both[48–51].

NLGNs bind to both α-NRXNs and β-NRXNs with nanomolar affinities; binding involves the sixth LNS domain of α-NRXNs, which corresponds to the only LNS domain of β-NRXNs[52]. The binding affinities differ characteristically between various pairs of NLGNs and NRXNs, and they are controlled by alternative splicing of both NRXNs and NLGNs[45,52,53] (Fig. 1c). SS B of NLGN1 is a master switch for NRXN binding — the inclusion of only eight residues in this site restricts the binding of NLGN1 to those β-NRXNs that lack an insert in SS 4, whereas exclusion of these eight residues allows the binding of both α-NRXNs and β-NRXNs independently of SS 4 (ref. 45). The NLGN1 splice variant containing an insert in SS B predominates, indicating that most NLGN1 is specific for β-NRXNs lacking an insert in SS 4, whereas all other NLGNs bind to both α-NRXNs and β-NRXNs. SS A of all NLGNs also regulates NRXN binding, but the effect is smaller[52]. In NRXNs, SS 4 (which is located in the last LNS domain) not only controls the binding of β-NRXNs to NLGN1 containing an insert in SS B (discussed earlier) but also modulates the affinity of α-NRXNs and β-NRXNs for NLGNs lacking an insert in SS B. Thus, the current data suggest that NRXN–NLGN binding is governed by a hierarchical code that depends on which principal isoforms are expressed and which splice variants are used.

The trans-synaptic neurexin–neuroligin complex

NRXNs and NLGNs are thought to form a trans-synaptic complex that is coated on both sides by PDZ-domain-containing proteins (Fig. 1b). The crystal structure of the NRXN1–NLGN1 complex (without inserts in SS 4 of NRXN and SS B of NLGN1) revealed that the NRXN LNS domain attaches with a large contact area to the lateral sides of the NLGN esterase-homology domain, opposite to the position of the crippled active site[54–56] (Fig. 2). In the structure of crystals that were grown in the presence of Ca^{2+}, two fully occupied Ca^{2+}-binding sites were found that are coordinated by ligands from both proteins[55]. Mapping of the alternative splicing sites into the structure shows that SS B

Box 2 Analysis of synaptic cell-adhesion molecules and synapse formation

Studying synaptic cell-adhesion molecules functionally has turned out to be extremely difficult. This box lists the assays used to study these molecules and summarizes the advantages and the disadvantages associated with each assay.

Gain-of-function approaches

Cell-adhesion assay

This type of assay uses non-neuronal cells expressing cell-adhesion molecules to test whether these molecules can mediate stable cell–cell interactions (for example, between NRXNs and NLGNs) but provides no functional read-out.

Artificial synapse formation assay

In this assay, neurons are cultured together with non-neuronal cells expressing a cell-adhesion molecule. The assay tests whether the cell-adhesion molecule induces the neurons to form stable junctions with synapse-like properties with the non-neuronal cells[35,50,67,68]. Many molecules promote synapse formation in the artificial synapse formation assay.

Neuronal transfection assay

This assay uses neurons overexpressing a cell-adhesion molecule. The assay measures the synapse density on the transfected neurons by microscopy[53] and the synapse function by electrophysiology[12]. The assay allows a better functional analysis of the effects of a cell-adhesion molecule than the artificial synapse formation assay; however, neither directly measures synapse formation, and both are subject to overexpression artefacts.

Loss-of-function approaches

RNA interference

This type of experiment uses cultured neurons or cultured slices and tests whether a cell-adhesion molecule is essential for synapse formation or synapse function. When paired with rescue controls, RNA interference (RNAi) is ideal, but it has three potential limitations. First, it is difficult to target multiple proteins simultaneously with RNAi, and it is therefore difficult to address redundancy. Second, for many targets, RNAi is simply inefficient; that is, it achieves less than 75% suppression when measured quantitatively (and not by densitometry of blots). Even successful RNAi is never complete — it does not achieve more than 95% suppression. Third, compensatory changes are as likely during RNAi-mediated knockdown experiments as during gene-knockout experiments.

Constitutive genetic manipulation

Constitutive genetic manipulation in gene-targeting experiments permanently deletes or alters the expression of a gene to test its overall importance. In addition to the problems listed for RNAi, this approach has the potential to cause developmental alterations, but it allows the complete elimination of expression and makes organismal analyses possible.

Conditional deletion

Conditional deletion by means of gene targeting allows spatially and/or temporally regulated deletion or changes in a cell-adhesion molecule, and usually involves Cre-recombinase-mediated genetic changes. This is a powerful approach, but it is labour intensive and is limited by the paucity of mouse lines with reproducible, tight and robust expression of Cre recombinase.

Pharmacological inhibition

This type of inhibition of a cell-adhesion molecule can be used to cause an acute disruption of function. Potentially the best approach, it is limited by lack of availability of effective agents for almost all cell-adhesion molecules, and by the side effects of many of the agents that do exist.

Overall evaluation

Gain-of-function approaches for analysing synapse formation are more sensitive but are harder to interpret. Loss-of-function approaches have greater validity but are technically more difficult, and they can be limited by functional redundancy between multiple genes. Note that both gain-of-function approaches and loss-of-function approaches, including RNAi and overexpression experiments, suffer from the potential problem of compensatory changes in the expression, localization and/or stability of other proteins.

is included in the binding interface, and that SS A of NLGN1 and SS 4 of NRXN1 are close by, providing an explanation for the effect of alternative splicing of these sites on the NRXN–NLGN binding affinity. Indeed, direct comparison of the crystal structures of β-NRXN LNS domains containing and lacking inserts in SS 4 supports this conclusion by revealing major conformational changes induced by this alternative splicing event[57,58].

The shape of the NRXN–NLGN complex suggests that it forms an interaction layer in the centre of the synaptic cleft, with the C-terminal sequences emerging from the complex in opposite directions (Fig. 2). This interaction layer — which might contribute to the electron-dense material observed in the synaptic cleft by electron microscopy — is separated from the presynaptic and postsynaptic plasma membranes by the glycosylated linker sequences that are present in NRXNs and NLGNs just outside the membrane. These glycosylated sequences could function as a 'cuff' that creates a distance between the interaction layer and the plasma membranes, and forces the extracellular domains to project into the synaptic cleft away from the membrane.

The cytoplasmic sequence of NRXNs contains a C-terminal binding site for class II PDZ domains that binds to the PDZ domain of CASK and related proteins, and a membrane-proximal binding site for protein 4.1 (refs 59, 60). CASK is a MAGUK protein (for membrane-associated guanylate kinase protein) containing a PDZ domain, an SH3 domain and a guanylate-kinase domain. CASK is an unusual MAGUK, however, because the PDZ, SH3 and guanylate-kinase domains account for only its C-terminal half; its N-terminal half is occupied by a Ca^{2+}/calmodulin-dependent protein kinase (CAMK) domain that is absent from other MAGUKs. The CASK CAMK domain contains substitutions in canonical residues that coordinate Mg^{2+} in CAM kinases, suggesting that it may be catalytically inactive. Unexpectedly, however, recent evidence indicates that CASK is catalytically active, and may be the first described kinase that phosphorylates proteins, including NRXN1, independently of Mg^{2+} (ref. 61). In addition, CASK nucleates the assembly of actin on the cytoplasmic sequence of NRXN by simultaneously binding to protein 4.1 (ref. 60). Finally, CASK interacts with VELI proteins (also known as MAL proteins, which are homologues of *Caenorhabditis elegans* LIN-7) and with MINT proteins (also known as X11 proteins) to form a tight trimeric complex[62,63]. In addition to NRXNs, CASK binds to other cell-surface proteins, including CASPRs, and probably carries out analogous functions. Deletion of *Cask* in mice causes a lethal phenotype that includes synaptic abnormalities, indicating that CASK is an important molecule[64]. CASK is probably a component of a signal transduction cascade that translates extracellular interactions of cell-surface proteins into an intracellular response by modulating the actin cytoskeleton and phosphorylating target proteins.

Like NRXNs, NLGNs bind to intracellular PDZ-domain proteins, but in contrast to NRXNs, NLGNs bind to class I PDZ domains such as those contained in PSD95, a postsynaptic MAGUK protein[65]. PSD95 and its homologues are centrally involved in recruiting glutamate receptors at postsynaptic sites[66]. Similarly to CASK, PSD95 binds to intracellular adaptor proteins, and especially to GKAP (a protein that binds to the guanylate-kinase domain of PSD95), which, in turn, binds to SHANK proteins (Fig. 1b). A possible role of these interactions is to recruit postsynaptic adaptor proteins to the site of synaptic junctions. As a result of their binding to PDZ-domain proteins, the junction formed by NRXNs and NLGNs resembles the architecture of tight junctions, but it differs from them in that the NRXN–NLGN junction is asymmetrical in all of its components.

Function of neuroligins and neurexins

Initial evidence that NLGNs function at synapses came from ingenious experiments showing that NLGNs expressed in a non-neuronal cell can induce co-cultured neurons to form presynaptic specializations onto the non-neuronal cell[67] (Box 2). This finding was amplified by complementary experiments showing that NRXNs, when expressed in a non-neuronal cell, can induce the formation of postsynaptic specializations in co-cultured neurons[50,68]. Moreover, direct overexpression of NLGNs in transfected neurons caused an increase in synapse numbers on these neurons[69].

Taken together, these studies indicated that NLGNs and NRXNs may induce synapse formation. However, analysis of gene-knockout mice surprisingly revealed that NLGNs and α-NRXNs are essential for synaptic function, but not for synapse formation[10–12]. Mice lacking NLGN1, NLGN2 and NLGN3 die at birth, but they have nearly normal synapse numbers with an apparently normal ultrastructure. Electrophysiological analyses in sections cut from brains of the mutant mice and analysed immediately without culturing showed that these mice have a severe impairment of synaptic transmission[11]. Although mice that lack the gene encoding either NLGN1 or NLGN2 are viable and fertile, electrophysiological analysis also uncovered significant synaptic dysfunctions in these mice[12]. In agreement with the localizations of NLGN1 and NLGN2 to excitatory and inhibitory synapses, respectively, excitatory synapses showed impairments in *N*-methyl-D-aspartate (NMDA)-receptor signalling in *Nlgn1*-knockout mice, whereas *Nlgn2*-knockout mice had deficits in inhibitory synaptic transmission[12].

The gene-knockout analysis seems to contradict the *in vitro* assays showing that NLGNs induce synapses in the artificial synapse formation assay and the neuronal transfection assay (see Box 2, which explains the various approaches). However, the assays with cultured neurons do not directly measure synapse induction — rather, they measure an increase in synapse numbers after a particular manipulation. In these assays, the molecules tested could simply function by inducing signal transduction events that stabilize otherwise transient, tentative synaptic contacts. In support of this interpretation, and in agreement with the results from gene-knockout experiments, the ability of NLGNs to increase the number of synapses in a transfected neuron can be decreased by the inhibition of synaptic activity, which has no effect on the expression and localization of the transfected NLGNs[12]. More conclusively, paired recordings from inhibitory neurons in the somatosensory cortex of either *Nlgn1*- or *Nlgn2*-knockout mice demonstrated that deletion of the *Nlgn1* and *Nlgn2* did not decrease the number of synaptic connections (Fig. 3). Instead, deletion of *Nlgn2* (but not *Nlgn1*) selectively lowered the strength of GABA (γ-aminobutyric acid)-utilizing inhibitory synapses formed by fast-spiking, parvalbumin-containing interneurons, but not of GABA-utilizing synapses formed by somatostatin-containing interneurons. Together, these data suggest that NLGNs have a function in the maturation of synaptic junctions with the specification of circuit-specific properties, but not in the initial formation of synaptic junctions. This conclusion is consistent with the finding that a partial knockdown of the mRNAs encoding NLGNs in cultured neurons produced a partial decrease in synapse numbers that could have been a secondary consequence of a decrease in synaptic function[70].

The activities of NRXNs have been more difficult to characterize than those of NLGNs. The lack of high-affinity antibodies, the complexity of the NRXN isoforms, and the challenges in analysing presynaptic function have contributed to this difficulty. At present, it even remains uncertain whether NRXNs are exclusively presynaptic, or whether at least some NRXNs are postsynaptic. Analysis of mice that lacked all α-NRXNs but still had β-NRXNs uncovered a phenotype that is similar to that of the *Nlgn*-knockout mice described above (note that knockout mice lacking β-NRXNs or both α-NRXNs and β-NRXNs have not yet been analysed). Deletion of the genes encoding individual α-NRXNs causes only moderate increases in mortality in mice, but deletion of two of the three genes encoding α-NRXNs increases postnatal mortality markedly. Deletion of all three genes invariably leads to neonatal fatality in mice[10]. Again, synapse numbers and their ultrastructure

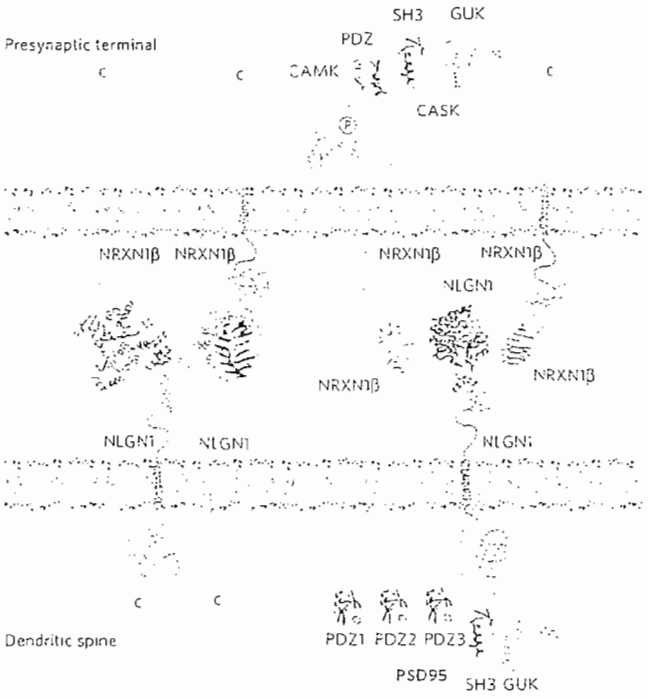
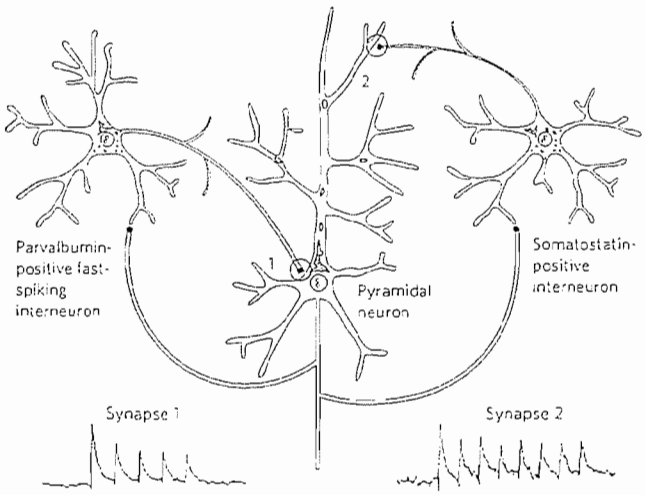


Figure 2 | Atomic model of the trans-synaptic complex formed by NRXN1β and NLGN1. The NRXN1β–NLGN1 complex is shown in two orientations: left, en face with the NRXN1β LNS domain on top of the NLGN1 esterase-like domain, to illustrate the NLGN1 dimer; right, in a 90° rotation to illustrate the sideways attachment of the NRXN1β LNS domains to the NLGN1 esterase-like domains. The two orientations of the NRXN1β–NLGN1 complex illustrate the spatial arrangement and relative sizes of the NRXN LNS domains and the NLGN esterase-like domain in a synaptic cleft. Other NRXN and NLGN isoforms for which no full structure is available, including α-NRXNs, would presumably have a similar arrangement except that the additional LNS domain in α-NRXNs would occupy a larger space in the synaptic cleft. (Images were generated from the following Protein Data Bank files: NRXN–NLGN, 3BIW; PSD95 SH3 GUK, 1JXO; PSD95 PDZ, 1BE9; CASK CAMK, 3C0I; CASK GUK, 1KGD; CASK PDZ, 1KWA. Diagram courtesy of D. Arac and A. Brunger, Stanford University, California.)

are nearly normal in mice that lack α-NRXNs, but synapse function is severely impaired. This impairment is both presynaptic and postsynaptic but is most significantly observed in action-potential-driven neurotransmitter release, which is severely depressed, largely because of a loss of presynaptic Ca^{2+}-channel function[71]. Postsynaptically, deletion of α-NRXN-encoding genes caused a decrease in synaptic responses dependent on NMDA receptors but not on AMPA receptors, similarly to the deletion of *Nlgn1* (ref. 39). Overall, analysis of mice in which α-NRXN-encoding genes have been knocked out indicates that lack of these genes results in disorganized synapses. These findings characterize α-NRXNs, like NLGNs, as synaptic cell-adhesion molecules that are essential for the proper assembly of synapses into a fully functional unit but not for the initial formation of synapses. NRXNs may also be required globally for the organization of secretory systems because mice that lack α-NRXNs show an additional major impairment in neuroendocrine secretion[72].

How, precisely, do NRXNs and NLGNs function in synapses? A plausible hypothesis is that trans-synaptic cell adhesion mediated by NRXNs and NLGNs — either by binding to each other or by binding to other ligands — triggers presynaptic and postsynaptic signal transduction events that activate synaptic function and specify synaptic properties. Without this activation, synapses assemble but do not work properly. The activation is clearly not a simple yes-or-no switch. Instead, NRXNs and NLGNs shape synaptic efficacy and plasticity. Moreover, a synaptic



Knockout mouse	Synapse 1		Synapse 2	
	Connectivity	IPSC amplitude	Connectivity	IPSC amplitude
Nlgn1	↔	↔	↔	↔
Nlgn2	↔	↓	↔	↔
Nlgn1 and *Nlgn2*	↔	↓	↔	↔

Figure 3 | Differential effects of deletion of the gene encoding NLGN1 and NLGN2 on inhibitory synapses in the somatosensory cortex. Connections of parvalbumin-positive fast-spiking interneurons (blue) and of somatostatin-containing interneurons (green) with excitatory pyramidal neurons (pink) are shown schematically. The connectivity (measured in paired recordings as percentage success) and amplitude of the inhibitory synapses of the interneurons onto the pyramidal neuron (IPSC amplitude) was measured for *Nlgn1*-knockout mice, *Nlgn2*-knockout mice, and *Nlgn1* and *Nlgn2* double-knockout mice; the table shows how these values differ from the connectivity and amplitude in wild-type mice (J. Gibson, K. Huber and T.C.S., unpublished observations). Horizontal arrow, no change compared with wild-type mice; downward arrow, lower than in wild-type mice.

transmission-specific element is involved, at least for NLGNs. How this synapse activation may occur is unclear. Binding of NLGN to NRXNs does not induce the dimerization of NRXNs, in contrast to other receptor-dimerization-dependent signalling cascades, because the crystal structure reveals that the two NRXNs bound to a NLGN dimer are distant monomers[54–56] (Fig. 2). The most parsimonious mechanism for this activation would be that NRXNs and NLGNs recruit 'coats' to the junction, coats that might consist of PDZ-domain proteins or actin filaments, or might also involve other types of interaction. For example, binding of NLGN to NRXNs might stimulate the CASK-dependent phosphorylation of NRXNs and other substrates; however, no direct evidence for this mechanism exists.

Neuroligins and neurexins in autism

ASDs are common and enigmatic diseases. They comprise classical idiopathic autism, Asperger's syndrome, Rett's syndrome, and pervasive developmental disorder not otherwise specified[73,74]. Moreover, several other genetic disorders, such as Down's syndrome, fragile X mental retardation, and tuberous sclerosis, are frequently associated with autism. Such syndromic forms of autism and Rett's syndrome are usually more severe because of the nature of the underlying diseases. The key features of ASDs are difficulties in social interactions and communication, language impairments, a restricted pattern of interests, and/or stereotypic and repetitive behaviours. Learning disability (in about 70% of cases) and epilepsy (in about 30% of cases) are frequently observed; in fact, the observation of epilepsy in patients with ASDs has fuelled speculation that autism may be caused by an imbalance of excitatory and inhibitory synaptic transmissions. In rare instances, idiopathic autism is associated with specialized abilities, for example in music, mathematics or memory. The relationship of ASDs to other cognitive diseases such as schizophrenia and Tourette's syndrome is unclear. As is the case for phenotypes caused by mutations in NLGNs and NRXNs (discussed later), the boundaries between the various disorders may not be as real as the clinical manifestations suggest.

A key feature of ASDs is that they typically develop before two or three years of age[73,74]. They therefore affect brain development relatively late, during the time of human synapse formation and maturation. Consistent with this time course is the fact that few anatomical changes are associated with ASDs[75]. An increase in brain size has repeatedly been reported[76] but is not generally agreed on[75]. Thus, similarly to other cognitive diseases, ASDs are a disorder not of brain structure but of brain function. Among cognitive diseases, ASDs are the most heritable (about 80%), suggesting that they are determined largely by genes and not by the environment. ASDs show a male-to-female ratio of about 4:1, indicating either that ASDs involve the X chromosome directly or that the penetrance of pathogenic genes is facilitated in males[73,74].

Mutations in many genes have been associated with familial ASDs. A consistent observation emerging from recent studies is the discovery of mutations in the genes encoding NRXN1, NLGN3 and NLGN4. Specifically, seven point mutations, two distinct translocation events and four different large-scale deletions in the *NRXN1* gene were detected in patients with autism[13–18]. Ten different mutations in the *NLGN4* gene were observed (two frameshifts, five missense mutations and three internal deletions), and a single mutation in the *NLGN3* gene (the Arg451Cys substitution)[21–24]. Besides these mutations, five different larger deletions of X-chromosomal DNA that includes the *NLGN4* locus (referred to as copy-number variations) were detected in patients with autism[18,25–27].

In addition to the NRXN–NLGN complex, mutations in the gene encoding SHANK3 — an intracellular scaffolding protein that binds indirectly to NLGNs through PSD95 and GKAP (ref. 66; Fig. 1) — may also occur frequently in ASDs. An astounding 18 point mutations were detected in the *SHANK3* gene in patients with autism, in addition to several cases containing copy-number variations that cover the gene[18,77–82]. Indeed, terminal 22q deletion syndrome is a frequent occurrence that shows autistic features that have been correlated with the absence of *SHANK3*, which is normally localized to this chromosome. SHANK3 is particularly interesting because it not only interacts indirectly with NLGNs but also binds directly to CIRLs (latrophilins), which, in turn, constitute α-latrotoxin receptors similar to NRXNs, suggesting a potential functional connection between SHANK3 and NRXNs[83].

Overall, the description of the various mutations in the NRXN–NLGN–SHANK3 complex seems to provide overwhelming evidence for a role of this complex in ASDs, given the fact that, in total, these mutations account for a significant proportion of patients with autism. It should be noted, however, that two issues give rise to scepticism with regard to the role of this complex in ASDs.

First, at least for some of the mutations in this complex, non-symptomatic carriers were detected in the same families in which the patients with the mutations were found. Whereas the *NLGN3* and *NLGN4* mutations seem to be almost always penetrant in males, and even female carriers with these mutations often have a phenotype, the *SHANK3* point mutations in particular were often observed in non-symptomatic siblings[77,78]. Thus, these mutations may only increase the chance of autism rather than actually causing it.

Second, the same mutations can be associated with different phenotypes in different people. For example, a microdeletion in *NLGN4* was found to cause severe autism in one brother but Tourette's syndrome in the other[26]. This raises the issue of whether the 'autism' observed in patients with mutations in these genes is actually autism, an issue that could also be rephrased as the question of whether autism is qualitatively distinct from other cognitive diseases, as opposed to being a continuum of cognitive disorders. In support of the latter idea, two different deletions in NRXN1 that are in the region encoding NRXN1α but not NRXN1β have also been observed in families with schizophrenia[19,20],

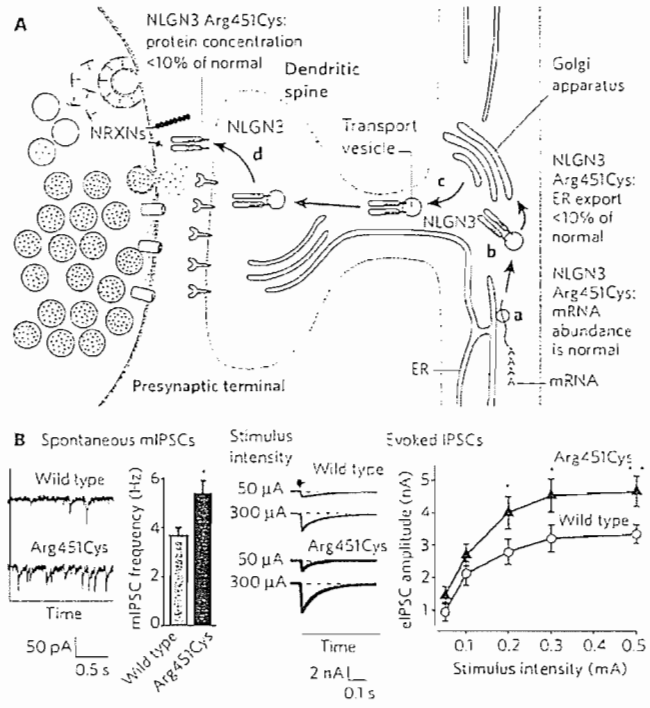


Figure 4 | The Arg451Cys substitution in NLGN3 impairs NLGN3 synthesis but enhances inhibitory synaptic transmission. **A**, The effect of the Arg451Cys mutation on NLGN3 synthesis. The mutation does not alter the amount of *Nlgn3* mRNA (a) but decreases the export of NLGN3 from the endoplasmic reticulum (ER) (b)[85]. As a result, the concentration of Arg451Cys mutant NLGN3 that is exported from the Golgi apparatus (c) and inserted into synapses (d) is less than 10% of the wild-type NLGN3 concentration[86]. **B**, Despite resulting in a decrease in the concentration of NLGN3, the Arg451Cys mutation produces a synaptic gain-of-function effect in inhibitory synapses in the somatosensory cortex. The figure illustrates, with two examples, an increased inhibitory synaptic activity in Arg451Cys mutant mice: by measurements of spontaneous 'miniature' synaptic events (mIPSCs; left) and by measurements of evoked synaptic responses (eIPSCs; right). Each example depicts representative electrophysiological traces and a summary graph. Asterisks indicate statistical significance (*P <0.05; **P <0.01). Note that NLGN3-deficient synapses do not show this phenotype[86]. IPSC, inhibitory postsynaptic current. (Panel modified, with permission, from ref. 86.)

indicating that there is a continuum of disorders that involve dysfunctions in synaptic cell adhesion and are manifested in different ways. Conversely, markedly different molecular changes can produce similar syndromes, as exemplified by the different mutations that are associated with ASDs[84].

At present, the relationship between the NRXN–NLGN synaptic-cell-adhesion complex and ASDs is tenuous. On the one hand, many of the mutations observed in familial ASD are clearly not polymorphisms but are deleterious, as demonstrated by the effect of these mutations on the structure or expression of the corresponding genes, and by the severe autism-like phenotypes observed in *Nlgn3* and *Nlgn4* mutant mice[85–87]. On the other hand, the nonlinear genotype–phenotype relationship in humans, evident from the only 70–80% heritability and from the occasional presence of mutations in non-symptomatic individuals, requires explanation. Work to explain the underlying mechanisms for this incomplete genotype–phenotype relationship is a promising avenue to insight into the genesis of autism. Furthermore, in addition to the link between schizophrenia and mutations in the gene encoding NRXN1α[19,20], linkage studies have connected NRXN3 to different types of addiction[88,89]. It is possible that, because of the nature of their function, mutations in genes encoding NRXNs and NLGNs constitute hotspots for human cognitive diseases.

Dissecting autism in mouse models

One way to address the question of whether the mutations in NRXNs and NLGNs observed in human patients are directly associated with ASDs is to test whether the same mutations elicit a significant phenotype in an animal. Such experiments were carried out in mice for two mutations in NLGNs: the NLGN3 Arg451Cys substitution and the NLGN4 loss-of-function mutation[86,87].

The Arg451Cys knock-in mouse has a striking phenotype with some, but not all, of the same features (as far as is possible to analyse) as human patients with ASDs. Behaviourally, the mice show normal motor and anxiety behaviours, a moderate impairment in social interactions, and a large increase in spatial learning capability[86]. Although this behavioural phenotype is somewhat satisfying because it is reminiscent of the 'savant' variant of autism and indicates that the Arg451Cys substitution did not impair cognitive function in the mice, the phenotype is also puzzling because human patients with the Arg451Cys substitution suffer from learning disabilities[21]. Electrophysiologically, the Arg451Cys mutant mice showed an increase in inhibitory synaptic transmission in the somatosensory cortex, which is consistent with the idea that a change in the excitatory–inhibitory balance contributes to the phenotype (Fig. 4). The Arg451Cys mutation seems to be a gain-of-function and not a loss-of-function mutation because *Nlgn3*-knockout mice did not have any of the phenotypes associated with the Arg451Cys knock-in mice[86]. This is surprising, because the Arg451Cys mutation depressed NLGN3 protein abundance in the knock-in mice by about 90%; thus, it is the remaining 10% of the mutant protein that produced a marked change in synaptic transmission (Fig. 4).

The gain-of-function action of the Arg451Cys mutation differs from that of the *Nlgn4* deletion, which also caused an autism-like phenotype in knockout mice[87], but it is clearly a loss-of-function mutation. These observations may provide an explanation for the finding of multiple mutations in *NLGN4* in patients with autism, but only a single mutation in *NLGN3*, despite the fact that both genes are on the X chromosome. It seems likely that only a loss-of-function mutation of *NLGN4*, but not of *NLGN3*, produces autistic symptoms, and that the Arg451Cys mutation in NLGN3 was an accidental gain-of-function mutation that occurred in only a single family. Moreover, these observations provide further support for the idea that NLGNs and NRXNs are activators of synapse function, not simply building blocks of synapses, in which small changes in NLGN function can induce massive changes in the neural network.

Perspective

Discovery of the NRXN–NLGN cell-adhesion system opened up new avenues to the understanding of synapses and cognitive disease, but it also raised many new questions. For example, do NRXNs and NLGNs function only by binding to each other — in fact, do they actually function by binding to each other at all? Do different NRXNs — either different principal isoforms, or different splice variants — have distinct functions? α-NRXNs and β-NRXNs cannot be functionally redundant because the deletion of genes encoding α-NRXNs causes a massive phenotype — a phenotype that cannot be compensated for by the remaining β-NRXNs[16] — so what else do α-NRXNs do? Uncovering answers to these and many other questions will provide insight not only into the fundamental mechanisms of synaptic cell adhesion but also into the molecular determinants of neural-circuit properties. Moreover, the apparent involvement of NRXNs and NLGNs in different cognitive diseases begs the question of whether these diseases are distinct entities or form a continuum of mental dysfunctions. With the emerging findings on the genetics of cognitive diseases, a molecular nosology of cognitive diseases may become possible. Furthermore, if a participation of NRXNs and NLGNs in cognitive diseases is confirmed in more extensive studies, new diagnostic and therapeutic possibilities may emerge, for example by selectively modulating the NRXN–NLGN interaction. Again, much more work will be required to explore these possibilities, but the present results are encouraging in this direction as well.

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Homeobox genes in the genetic control of eye development

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ABSTRACT Vertebrate eye formation is a complex process which involves early specification of the prospective eye territory, induction events, patterning along the polarity axes and regional specification, to bring about the proper morphogenetic movements, cell proliferation, cell differentiation and neural connections allowing visual function. The molecular machinery underlying such complex developmental events is presently under an intense research scrutiny and many associated genetic factors have been isolated and characterized. These studies produced striking knowledge in the field, especially with respect to uncovering the role of key genes and their possible evolutionary conservation. Presently, a major task is to define the complex interactions connecting the multiplicity of molecular players that regulate eye development. We recently identified two homeobox genes, *Xrx1* and *Xvax2*, and studied their function by using the *Xenopus* embryo as a developmental model system. *Xrx1* and *Xvax2* control key aspects of eye development. In particular, *Xrx1* appears to play a role in the early specification of anterior neural regions fated to give rise to retina and forebrain structures, and in promoting cell proliferation within these territories. On the other hand, *Xvax2* is involved in regulating the eye proximo-distal and/or dorso-ventral polarity, and the morphogenetic movements taking place during formation of the optic stalk and cup. Here we review the experimental results addressing the roles of *Xrx1* and *Xvax2* and their vertebrate orthologues, and discuss their relationship with other molecules also playing a related function in eye development.

KEY WORDS: *eye field, eye polarity, rx1, vax2, homeobox genes.*

Introduction

Eye morphogenesis occurs with similar sequential steps in all vertebrate embryos (Land and Fernald, 1992). It begins at the end of neurulation, when the optic vesicle, a bulge-like structure that will originate the neural structures of the eye, evaginates from the ventro-lateral wall of the forebrain. Subsequently, the lens primordium becomes evident in the ectoderm overlaying the distal-most part of the optic vesicle. At the same time, the distal vesicle invaginates around the lens primordium forming the optic cup, a two-layered structure, whose external layer will differentiate into the pigmented epithelium, while the internal layer, closer to the lens, will give rise to the neural retina. On the other hand, the proximal part of the optic vesicle narrows and elongates forming the optic stalk, a transient tubular structure that collects the axons of the retinal ganglion cells into the optic nerve and allows the blood vessels to reach the eye. Invagination of the optic vesicle around the lens begins in its ventral part, and, at the end of the invagination process, a narrow opening, known as optic fissure, remains in the ventral-most part of the cup. The invagination process and formation of a ventral fissure occur not only in the optic vesicle, but also in the optic stalk. The optic fissure allows both the retinal axons to enter the optic stalk and the blood vessels to reach the retina. Both the optic stalk and the optic fissure are only transient structures; during late developmental stages, the optic fissure is closed by fusion of its retinal lips, while the optic stalk disappears and its cells differentiate into the astrocytes that will populate the body of the mature optic nerve.

Molecular processes that regulate this complex and coordinated sequence of morphogenetic events start much earlier, around late gastrula/early neurula stages of development, than the first morphological appearance of the eye bud at the end of neurulation. It is now established that molecular control of the very first events of eye formation involves two different and temporally distinct steps. In a first phase, a wide presumptive eye-forming region is identified as a continuous crescent shaped region in the anterior neural plate. In a second phase, this initially single eye field is subdivided in two distinct bilateral eye anlagen by prechordal

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plate signals which induce ventral diencephalic developmental fates into the antero-medial neural plate (Li *et al.*, 1997). At the same time, the eye anlage begins to be patterned so that the prospective ocular structures become regionally specified within this territory (McDonald *et al.*, 1995; Chiang *et al.*, 1996).

We recently isolated and characterized in *Xenopus* two homeobox genes, *Xrx1* and *Xvax2*, that play important roles both in the early specification of the eye field and in the patterning events of the eye, respectively (Casarosa *et al.*, 1997; Mathers *et al.*, 1997; Andreazzoli *et al.*, 1999; Barbieri *et al.*, 1999; Ohsaki *et al.*, 1999; Schulte *et al.*, 1999). We summarize below the experimental results supporting such roles, and we discuss the possible functions of these two genes with respect to the genetic hierarchies governing development of the vertebrate eye.

Genetic control of the eye field

Recent lineage tracing experiments in *Xenopus*, refining classical embryology observations, have identified the earliest region fated to become retina as a continuous area in the anterior-most part of the early neural plate (Eagleson *et al.*, 1995). Genes involved in early decisions in eye development would be expected to be expressed in the eye field when this is first defined. This is indeed the case for *pax6* (Fig. 1A), a *paired* class homeobox gene expressed in the eye field at early neurula stage that has been proposed to be at the top of the genetic cascade governing eye development. In fact, in *Drosophila*, targeted misexpression of

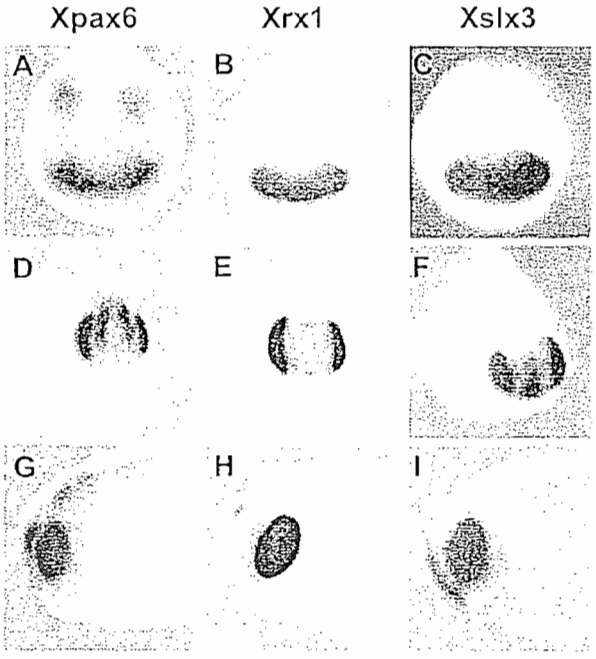

Fig. 1. Expression of *Xpax6*, *Xrx1* and *Xsix3* in the prospective eye field and in the optic vesicle. *Whole mount in situ hybridizations showing expression of Xpax6* (A,D,G), *Xrx1* (B,E,H) *and Xsix3* (C,F,I), *at early neurula (A-C) or tailbud (D-I) stage (st.) of Xenopus development. (A-F) Frontal view. (G-I) Lateral view. All three genes are expressed in a crescent shaped region of the anterior neural plate, including the prospective eye field (A-C) and later on, througnout the developing optic vesicle (D-I). At tailbud stage, specific expression domains within the forebrain are also evident (D-I).*

murine *Pax6* or its fly homologue *eyeless* (*ey*) leads to initiation of a complete eye developmental program in ectopic imaginal discs, while mutations in *ey*/*pax6* cause malformation or lack of eyes in *Drosophila*, mouse and humans (Hill *et al.*, 1991; Ton *et al.*, 1991; Quiring *et al.*, 1994). However, the identification in *Drosophila* of other genes whose mutations cause a failure in eye development has led to the idea that more than one gene is required to achieve the formation of a normal eye. In particular, functional studies on *sine oculis* (*so*), *dachshund* (*dac*) and *eyes absent* (*eya*) have shown that eye development is due to the interactions between these genes and *ey* rather than to the action of *ey* alone (see Desplan, 1997). Moreover, the ability to induce ectopic eye development does not appear to be a prerogative of *ey*, since both *eya* and *dac* can elicit eye formation, although with a much lower efficiency than *ey*. Furthermore, different combinations of *ey*, *eya*, *so* and *dac* induce eye development with a higher frequency and in a wider range of tissues than *ey*, *eya* or *dac* alone. Nonetheless, the recent isolation of *twin of eyeless* (*toy*), a second *pax6*-like gene in *Drosophila*, has restored the idea of the existence of a single master gene controlling eye development (Czerny *et al.*, 1999). In fact *toy*, whose structure and expression pattern are more closely related to vertebrate *pax6* than *ey*, acts upstream of *ey*, does not require feedback by *so*, *eya* and *dac* and its targeted expression induces ectopic eyes. The idea of a master eye gene has recently been strengthened by the observation that *pax6* misexpression is able to induce ectopic eyes also in a vertebrate, as shown by microinjection experiments in *Xenopus* (Chow *et al.*, 1999). This remarkable conservation of *pax6* function across species, has also led to propose a monophyletic origin for the eye (Gehring and Ikeo, 1999). The role of *pax6* in vertebrates has also been assessed by analysis of mice carrying a *Pax6* loss-of-function mutation (*Small eye*: *Sey*, see Callaerts *et al.*, 1997, and Treisman, 1999, and references therein). *Sey* mice, beside displaying defects in brain and nose development, show abnormally shaped optic vesicles and a complete absence of the lens. Interestingly, while in these mice a lens placode never forms, the optic vesicles initially undergo a normal evagination and make contact with the overlaying ectoderm. Although this contact is not maintained, probably due to the lack of a lens placode, and a proper optic cup does not form, *Sey* optic vesicles have been shown to display at least some aspects of proximo-distal patterning. Thus, even though *pax6* plays a crucial role in eye development, being sufficient to induce a complete eye under overexpression conditions, and being necessary for lens and late retina development, its function does not seem to be absolutely required for the initial steps of optic vesicle formation. This process is therefore likely to involve other genes during embryo development. The possibility that a second vertebrate *pax6* gene might play a specific (or redundant) function in these early events seems to be ruled out by the results of a search for *pax6* genes in various species. These results may suggest that a duplication of the *pax6* gene has only occurred in the genomes of holometabolous insects (Czerny *et al.*, 1999).

Obvious candidate partners for *pax6* are the vertebrate homologues of *so*, *eya* and *dac*. The search for the *so* homologue has revealed the existence of several vertebrate *so*-related genes, the *six* gene family. Paradoxically, the members more closely related to *so*, *Six1* and *Six2* are not expressed in the eye primordium (Oliver *et al.*, 1995b). On the other hand, two other members of this gene family, namely *Six3* (Oliver *et al.*, 1995a) and *Optx2* (Toy *et al.*, 1998), both of which are expressed early in eye territories, have

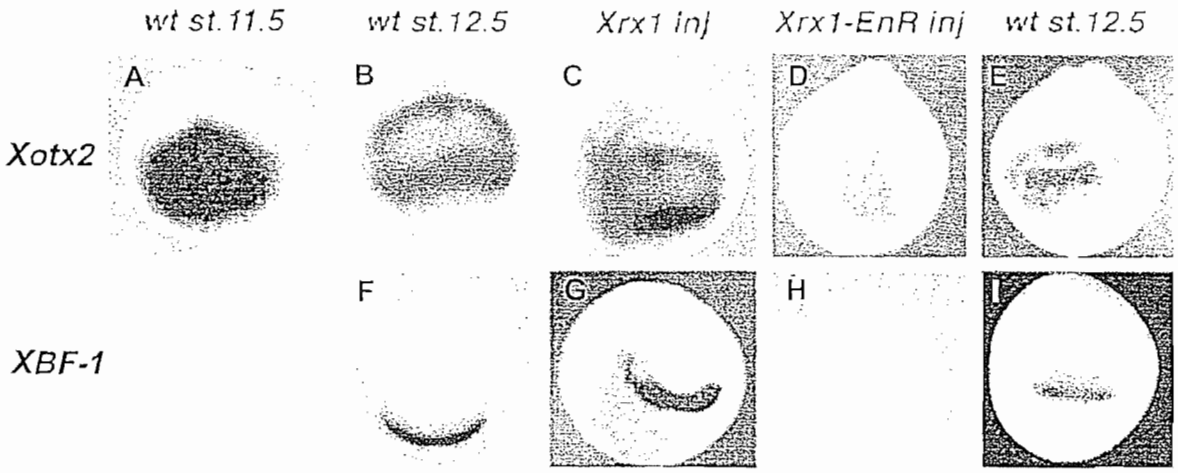

Fig. 2. Interactions between *Xrx1*, *Xotx2* and *XBF-1* within the early *Xenopus* neural plate. (A,B) *Expression of Xotx2 in the anterior neuroectoderm in wild-type embryos at midgastrula (st. 11.5, (A)) and late gastrula (st. 12.5, (B)) stages. At st. 12.5 (B) Xotx2 is downregulated in the prospective eye field.* (F) *Expression of XBF-1 in the presumptive telencephalon of a wild type embryo at late gastrula (st. 12.5) stage.* (C,G) *Expression of Xotx2 (C) and XBF-1 (G) at early neurula stage (st. 13) in embryos unilaterally injected with full-length Xrx1 RNA. Distribution of the injected RNA is detectable as magenta staining, while expression of the gene of interest is detectable as blue staining. Xotx2 expression is repressed, while the XBF-1 domain is expanded on the injected side.* (D,H) *Expression of Xotx2 (D) and XBF-1 (H) at early neurula stage (st. 13) in embryos bilaterally injected with Xrx1-EnR chimeric RNA. Both genes appear to be strongly downregulated.* (E,I) *Double in situ hybridizations on late gastrula (st. 12.5) wild type embryos with Xrx1 (magenta staining in (E,I)) and either Xotx2 (blue staining in (E)) or XBF-1 (blue staining in (I)), showing that at this stage, Xrx1 expression is complementary to that of Xotx2 and overlaps with the XBF-1 positive domain. st. = stage.*

now been shown to be the orthologues of another *Drosophila* gene, *optics*, whose function in the fly has not yet been assessed. (*Optx2* is also known as *Six6* in mouse and *Six9* in chick: Jean *et al.*, 1999; Lopez-Rios *et al.*, 1999). However, in *Xenopus*, *Xsix3* is co-expressed with *Xpax6* in the early eye field (Andreazzoli *et al.*, 1999; Fig. 1C), and microinjection experiments in fish have shown that *six3* is able to induce ectopic retina and lens, as well as an enlargement of the forebrain (Kobayashi *et al.*, 1998; Loosli *et al.*, 1999). *six3* function in brain and eye formation has also recently been underscored by the finding that mutations in human *SIX3* are the cause of a form of holoprosencephaly (Wallis *et al.*, 1999). *Xoptx2* is also expressed in the *Xenopus* eye field, even though at a later stage than *Xsix3*, and it has been shown to control cell proliferation thus determining the final size of the eye (Zuber *et al.*, 1999). Vertebrate homologues of *eya* and *dac* expressed in different eye regions have also been described (Xu *et al.*, 1997; Hammond *et al.*, 1998); however at the moment their role in eye development is unclear and awaits functional studies.

Besides looking for the vertebrate homologues of known *Drosophila* genes, other approaches have also led to the isolation of novel vertebrate transcription factors expressed in the eye field. In particular, this was the case for *Xrx1* (Casarosa *et al.*, 1997), a *paired*-like homeobox gene whose expression in *Xenopus* is activated in the eye field in concomitance with that of *Xpax6* and *Xsix3* (Fig. 1B) and that plays a role in retinal and anterior brain proliferation (see below).

Since the establishment of the eye field seems to involve several players, in order to understand the mechanisms of eye development in vertebrates it becomes now of primary interest to identify the interactions occurring between all these genes. Recent overexpression experiments have begun to address this issue. Thus, misexpression studies using *Xpax6* and *Xrx1* in *Xenopus* and in medaka, have shown that each of these genes is able to activate the expression of the other two, although with a different timing (Andreazzoli *et al.*, 1999; Chow *et al.*, 1999; Loosli *et al.*, 1999). *Xoptx2*, when overexpressed, can also activate *Xpax6* and *Xrx1*, while co-injection with *Xpax6* has been shown to potentiate the effects of *Xoptx2* on eye and brain enlargement (Zuber *et al.*, 1999). Altogether, the present data point to the existence of a complex set of regulatory interactions between the genes involved in vertebrate eye development.

Xrx1 and the eye-brain field

The *paired*-like homeobox gene *Xrx1* was isolated in our laboratory during a screening designed to look for homologues of the *Orthopedia* gene (Simeone *et al.*, 1994; Casarosa *et al.*, 1997). *Xrx1* shares indeed sequence similarity with *Orthopedia* both in the encoded homeodomain and in a novel motif denominated OAR (Furukawa *et al.*, 1997) which in Orthopedia has been shown to work as a transactivation domain. *Xrx1* was also independently isolated by Jamrich and coworkers during a screening for genes induced in *Xenopus* animal caps by ammonium chloride treatment, which is known to trigger anterior ectodermal fate (Mathers *et al.*, 1997). *Xrx1* transcripts are first detected at early neurula stage in the anteriormost part of neural plate including presumptive retina, diencephalic and telencephalic territories (Fig. 1B). Later on, *Xrx1* expression becomes restricted to the evaginating eye vesicles, diencephalon floor, pituitary and pineal gland (Fig. 1 E,H). When optic cups are formed, both presumptive neural retina and pigmented epithelium express *Xrx1*, while no expression is detected in the forming lens at any stage. Interestingly, *Xotx2*, a homeobox gene of the *bicoid* class, is expressed in the whole prospective anterior neuroectoderm already at the end of gastrulation, before the onset of *Xrx1* transcription (Pannese *et al.*, 1995; Fig. 2A). However, less than two hours later in development, *Xotx2* expression becomes

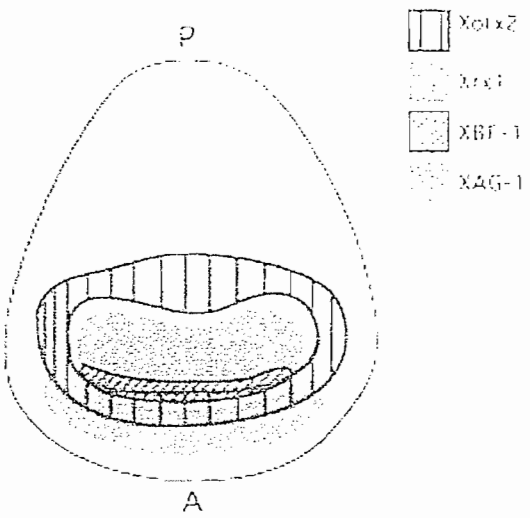

Fig. 3. Schematic representation of *Xotx2, Xrx1, XBF-1* and *XAG-1* expression in the early *Xenopus* neural plate. *Xrx1 (magenta) overlaps with both Xotx2 (green) and XBF-1 (blue) in the presumptive telencephalon, while it is complementary to Xotx2 in the presumptive diencephalon and retina territories, where it identifies the prospective eye field. XAG-1 expression (orange) identifies the presumptive cement gland and delimits the anterior border of the neural plate. A, anterior; P, posterior.*

repressed in most of the area where *Xrx1* is activated (Fig. 2 B,E). This is one of the earliest described events in anterior neural plate patterning, and comparison between the expression domain of *Xrx1* and those of other anterior genes indicates that the neural plate is indeed regionalized in specific territories already at the very beginning of neurulation (Andreazzole *et al.*, 1999; Fig. 3). Early *Xrx1* expression was found to overlap, although not perfectly, with that of *Xpax6* and *Xsix3*. *Xotx2* expression becomes almost completely complementary to *Xrx1*, except ventrally where *Xrx1*, *Xotx2* and *XBF-1* expressions overlap (Fig. 2 E,I; Fig. 3). Ventrally *Xrx1* borders, without overlapping, to the adjacent cement gland territory, marked by *XAG-1* expression. Thus, the combination of expression of different homeobox genes seems to correlate with a very early patterning of the neural plate (Fig. 3). The region expressing *Xrx1*, *Xpax6* and *Xsix3*, but not *XBF-1* and *Xotx2*, may correspond to retina and diencephalic territories, while the area coexpressing *Xrx1*, *XBF-1* and *Xotx2* may correspond to presumptive telencephalic areas. Consistent with the idea of an involvement of *Xrx1* in these early patterning events are the results from *Xrx1* overexpression studies. In fact, misexpression of *Xrx1* in anterior-dorsal territories results in the repression of *Xotx2* and *XAG1* and in the ectopic activation of *XBF-1* at early neurula stage (Fig. 2 C,G). It is tempting to think that the same interactions might occur during normal development, thus explaining how the different expression domains, and therefore the early neural plate patterning, are established.

The main feature of the *Xrx1* overexpression phenotype is the occurrence of hyperproliferation in the neural tube, neural retina and retinal pigmented epithelium (Mathers *et al.*, 1997; Andreazzoli *et al.*, 1999; Fig. 4 A,B). Expression analysis of eye-brain markers in tailbud injected embryos has shown that *Xpax6*, *Xotx2* and *Xsix3* are activated in the over-proliferating tissue (Fig. 4 C,D). On the other hand, *Xrx1* overexpression strongly represses *En2* and *Xpax2* expression in the midbrain-hindbrain boundary and reduces *Krox20* expression in the hindbrain. Thus, *Xrx1* appears to possess a proliferating activity which is linked to the promotion of anterior fate. Interestingly, the anterior neural plate and the optic vesicles, where *Xrx1* is expressed, are characterized by a high rate of proliferation and by a delay in neuronal differentiation when compared to the rest of the neuroectoderm (Eagleson *et al.*, 1995; Papalopulu and Kintner, 1996). Given its activity, *Xrx1* might be one of the genes that confer anterior proliferative properties to this region of the neuroectoderm. Other candidate genes for this function are transcription factors similarly expressed in the anterior neural plate, like *Six3* and *Xopix2*, whose overexpression triggers over-proliferation in eyes and brain (Kobayashi *et al.*, 1998; Loosli *et al.*, 1999; Zuber *et al.*, 1999). *Xrx1* and other anteriorizing-proliferative genes in the early eye-brain field might also antagonize posteriorizing signals involved in promoting neuronal differentiation (cf. Bourguignon *et al.*, 1998; Papalopulu and Kintner, 1996). Future experiments will specifically address the role of *Xrx1* in counteracting the range of action of posteriorizing signals.

Loss-of-function experiments performed both in mouse and in *Xenopus* have stressed the necessity of *Xrx1* function for normal eye and brain development. Targeted disruption of the mouse *Rx1* gene leads to the absence of optic cups and forebrain as determined by histological analysis (Mathers *et al.*, 1997). A very similar effect is observed expressing in *Xenopus* embryos *Xrx1-EnR*, a *Xrx1* dominant repressor construct where the putative transactivation OAR domain was substituted by the *engrailed* repressor domain (Andreazzoli *et al.*, 1999). Expression analysis of anterior genes in *Xrx1-EnR* injected embryos showed a remarkable reduction of eye-brain presumptive territories already at early neurula stage (Fig. 2 D,H), which resulted in the absence of telencephalon, eye vesicles and ventral diencephalon at tailbud stage. Further analysis of *Xrx1-EnR* injected embryos has shown that early anterior neural plate cells undergo apoptosis. Together with the unchanged expression domains of hindbrain markers, these data suggest that the anterior deletions described in these embryos are due to an early loss of cells constituting the eye-brain field regions rather than to a posteriorization of the anterior CNS. Thus, *Xrx1* seems to play roles in cell survival, cell proliferation and anterior specification. All these functions might actually be strictly related and could be required by cells of the eye-brain field. In fact, these cells, which are specified as anterior, might have as first thing to avoid programmed cell death in order to enter a proliferative phase.

The function played by *Xrx1* is likely to be conserved through evolution, as suggested by the similarity between mouse and *Xenopus* loss-of-function phenotypes and also by the isolation of *Xrx1* homologues in several species, which share a similar expression pattern. In mouse only one homologue has been isolated (Furukawa *et al.*, 1997; Mathers *et al.*, 1997), while in zebrafish three genes of the *rx* family - *Zrx1*, *Zrx2* and *Zrx3* -, have been described (Mathers *et al.*, 1997; Chuang *et al.*, 1999). The three zebrafish genes are initially all expressed in the anterior neural plate but later *Zrx1* and *Zrx2*, which are closely related to each other, are only expressed in the optic vesicles, while *Zrx3* becomes expressed predominantly in the ventral forebrain and, at a low level, in the optic vesicles. The presence of a higher number of

homologues and the splitting of expression patterns between them seems to be a general characteristic of the zebrafish genome. Nevertheless, the recent isolation in chick of two *rx* homologues, namely *cRaxL*, which is closer to *Zrx1* and *Zrx2*, and *cRax*, which shares higher homology with *Xrx1* (Ohuci *et al.*, 1999), suggests that the *rx* gene family may be larger than previously thought and that some members have probably not been identified yet. A common characteristic to all the vertebrate *rx* genes is their expression in the anterior neural plate first and optic vesicles later. Interestingly, this does not completely hold true for the only identified *Drosophila* homologue which is expressed in the protocerebrum, the anteriormost part of the fly brain, but neither in the eye primordia nor in the larval eye imaginal discs (Eggert *et al.*, 1998). Unless a second *rx* homologue with an eye specific expression exists in *Drosophila*, these data seem to indicate that the most evolutionary conserved function of *rx* genes is in brain development, while *rx* expression in the eye could have been a recent acquisition of vertebrates. On this issue, it is worth to notice that while in *Drosophila ey* is required for eye development, its vertebrate homologue *Pax6* seems to be necessary mostly for lens development. Since both the vertebrate lens and the whole *Drosophila* eye derive from an ectodermal sensory placode it has been proposed that the lens could be the only vertebrate eye region sharing a common origin with the *Drosophila* eye (Treisman, 1999). The lack of *rx1* expression both in the vertebrate lens and in *Drosophila* eyes gives further support to this hypothesis.

Regulation of eye polarity

The vertebrate eye is a complex polarized structure where the developmental fate of its cells is highly dependent on their position within the prospective eye territory. Eye polarity is commonly described in terms of proximo-distal (P-D), dorso-ventral (D-V), and nasal-temporal axes of the ocular structure, that extend parallel to the latero-medial, dorso-ventral and antero-posterior axes of the body, respectively. P-D polarity of the eye becomes evident during the transition from the optic vesicle to the optic cup, when cells nearer to the brain contribute to the optic stalk, while cells located in distal positions with respect to the neuraxis give rise to the retina. At the same time, D-V polarity of the eye also becomes apparent, since cells located in different positions with respect to the dorso-ventral axis of the eye undertake different morphogenetic movements and different programs of proliferation, differentiation and axonal projections. The optic fissure is the most evident morphological marker of the D-V polarity of the developing eye: its specific occurrence in the ventral retina and optic stalk reflects the ventro-dorsal progression of the invagination movements of the optic vesicle around the lens to form the optic cup. Furthermore, a spatio-temporal gradient of cell differentiation has been detected in the retina, with cells in ventral positions proliferating longer and differentiating later with respect to dorsal retinal cells (Zuber *et al.*, 1999). Regional differentiation of specific cell types also occurs along the retinal D-V axis, since, for example, middle- and shortwave cone photoreceptors dominate dorsal and ventral retinal regions, respectively (Szel *et al.*, 1992). Finally, D-V polarity of the neural retina is also represented by the neuritic organization of the visual system, since ventral and dorsal retinal ganglion cells project their axons specifically to the dorsal and ventral tectum, respectively (Crossland *et al.*, 1974). A similar reversed organization array in retinotectal projections has also been detected along the antero-posterior axis of the retina and optic tectum, and constitutes the main feature of the nasal-temporal polarity of the eye (Crossland *et al.*, 1974).

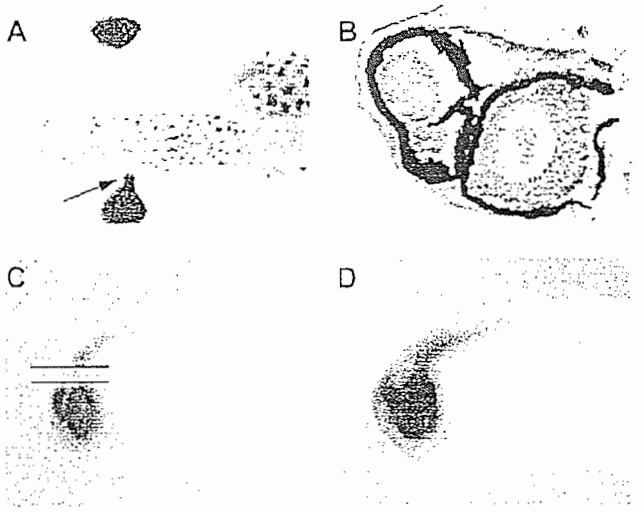

Fig. 4. Effects of *Xrx1* overexpression on eye development. (A) *Dorsal view of a swimming tadpole Xenopus embryo unilaterally injected on the left side with Xrx1 full-length RNA, showing the presence of ectopic pigmented retina extending from the eye to the diencephalon (arrow).* (B) *Transverse section through the eye of an Xrx1-injected embryo at the swimming tadpole stage, showing complete duplication of the optic cup.* (C,D) *Expression of Xpax6 in a tailbud stage Xenopus embryo unilaterally injected with Xrx1 RNA.* (C) *Lateral view from the control side. A gap of Xpax6 expression is detectable in the midbrain (area between lines).* (D) *Lateral view from the injected side. Xpax6 expression is ectopically expanded in the midbrain region. Distribution of the injected RNA is detectable as magenta staining in the embryo, while blue staining indicates Xpax6 expression.*

In the last few years our understanding of the molecular machinery regulating eye polarity has been greatly improved thanks to the use of animal models apt to both genetic and embryological studies. In particular, the zebrafish embryo has allowed the identification of several secreted or trasmembrane molecules involved in P-D and D-V patterning of the eye. The genes *cyclops* (Rebagliati *et al.*, 1998; Sampath *et al.*, 1998), coding for a nodal-related factor of the TGF-ß superfamily of secreted proteins, and *one-eyed pinhead* (Zhang *et al.*, 1998), encoding a putative EGF receptor protein apparently working as an essential extracellular cofactor for *nodal* signaling molecules (Gritsman *et al.*, 1999), have recently been identified. *cyclops* and *one-eyed pinhead* are expressed in anterior midline tissues and are responsible for the homonym zebrafish mutations, which are characterized by the striking occurrence of a single cyclopic eye and the lack of the ventral forebrain. The absence of the optic stalk, as assayed by detailed morphological and molecular analyses of these mutants, suggests the existence of a molecular link between the subdivision of the initially continuous eye field into two separate bilateral eye anlagen and the proximo-distal patterning of the eye (McDonald *et al.*, 1995; Hammerschmidt *et al.*, 1996). In particular, in the well characterized *cyclops* mutant, the *paired*-like homeobox gene *pax2* - a specific marker of the optic stalk and the ventral-most part of the

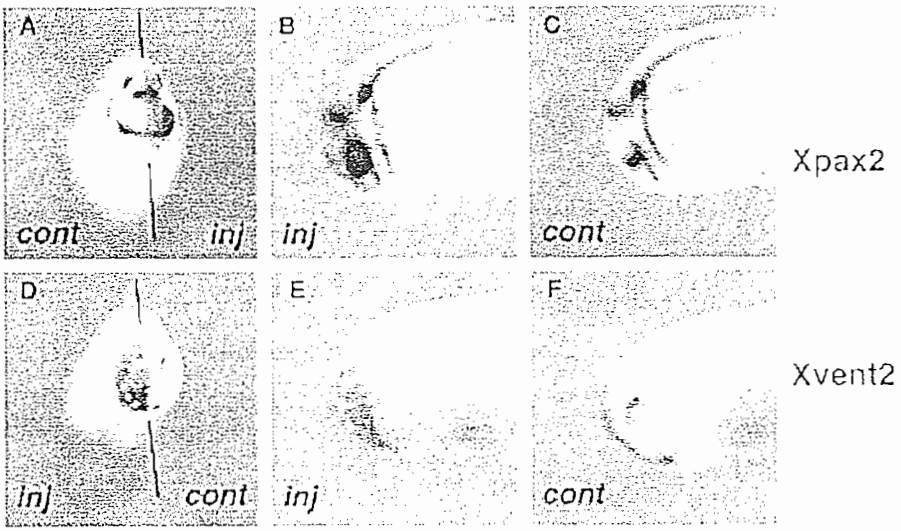

Fig. 5. *Xvax2* overexpression affects D-V patterning of the eye. *Expression of Xpax2 (A-C) and Xvent2 (D-F) genes in tailbud Xenopus embryos, unilaterally coinjected with Xvax2 and ß-galactosidase RNAs. The gene of interest is detectable as a dark blue staining, while the distribution of the injected RNA is visualized by the ß-galactosidase enzymatic reaction (light blue staining).* **(A,D)** *St. 23 injected embryos, frontal view. On the injected side (inj, right in A, left in D), the Xpax2 expression domain, normally confined to the ventral developing eye, is expanded dorsally (A), while Xvent2 expression, which is dorsal in the control side (cont), is strongly reduced (D).* **(B,E)** *St. 23 injected embryos laterally viewed from the injected side.* **(C,F)** *The same embryos as in (B,E) laterally viewed from the control side.*

retina - is almost completely suppressed in the mutant cyclopic eye. Reciprocally, expression of *pax6*, normally confined to the prospective retinal territory, is ubiquitous within the cyclopic eye (McDonald *et al.*, 1995).

The *sonic hedgehog* (*shh*) gene, isolated in several vertebrate species by its homology to *Drosophila hedgehog*, codes for a secreted signaling molecule that is involved in the developmental patterning of various structures, such as the neural tube, the limb bud, the somitic mesoderm, the tooth and the gut (Echelard *et al.*, 1993; Riddle *et al.*, 1993; Hebrok *et al.*, 1998; Borycki *et al.*, 1999; Hardcastle *et al.*, 1999). Early in development, *shh* is expressed in midline tissues. Within the nervous system, it plays a pivotal role in regulating development of the ventral neural tube, being both necessary and sufficient for differentiation of ventral neuronal cell types all along the antero-posterior axis of the embryo (Echelard *et al.*, 1993; Roelink *et al.*, 1994; Ericson *et al.*, 1995; Chiang *et al.*, 1996). *shh* also appears to play a role in establishing the ventral forebrain and proximal eye identity, since its expression in *cyclops* and *one-eyed pinhead* mutant embryos is strongly downregulated (McDonald *et al.*, 1995; Hammerschmidt *et al.*, 1996). Indeed, *shh* knockout in the mouse results in cyclopia and deletion of proximal eye and ventral forebrain structures (Chiang *et al.*, 1996). On the other hand, *sonic hedgehog* misexpression both in fish and mouse embryos activates ectopic expression of ventral forebrain and optic stalk markers, such as *nkx2.1*, *nk2.2* and *pax2*, while repressing expression of the retinal marker *pax6*. Simultaneously, an expansion of the optic stalk and a concomitant reduction of the optic cup are induced (Barth and Wilson, 1995; McDonald *et al.*, 1995; Shimamura and Rubenstein, 1997). These data suggest a role for *shh* as a morphogen, controlling specification of the latero-medial polarity of the anterior neural plate, which results in subsequent P-D and D-V regionalization of the eye and brain.

Retinoic acid (RA), a signaling molecule crucial in a number of developmental processes (Maden, 1999), also plays a role in establishing eye polarity. Studies performed in zebrafish have in fact pointed out a major role for RA in D-V patterning of the eye. RA synthesis is higher in the ventral than in the dorsal retina (McCaffery *et al.*, 1992, 1999; McCaffery and Drager, 1993). Moreover, RA treatments of zebrafish embryos induce an enlargement of the optic stalk and an expansion of the *pax2* expression domain in the eye. On the other hand, transcription of the homeobox gene *msh[c]*, a marker of the dorsal retina, is strongly downregulated following exposure of the embryos to RA. Furthermore, localized treatments by implantation of beads soaked in RA result in formation of supernumerary optic fissures near the site of implantation (Hyatt *et al.*, 1996). Finally, treatments of zebrafish embryos with inhibitors of RA synthesis result in ablation of the ventral retina, thus indicating that RA is both necessary and sufficient for development of ventral ocular structures (Marsh-Armstrong *et al.*, 1994).

The relationships among the *nodal*, *shh* and RA pathways in eye patterning are presently unclear and represent a central topic for further investigation. Nevertheless, as shown by either gain- or loss- of-function approaches, all these pathways appear to converge, directly or indirectly, on the *pax2* gene, that may therefore work as an intracellular determinant of ventro-proximal ocular fates. However, knockout studies of *pax2* in both mouse and fish brought to a reassessment of the relevance of the *pax2* role (McDonald *et al.*, 1995, 1997). The knock-out mutants in fact display an ectopic extension of the pigmented epithelium in the optic stalk territory and non-closure of the optic fissure, thus suggesting that, while *pax2* is important for correct development of proximal and ventral eye structures, it is not absolutely required for their initial specification.

Recently, a novel homeobox gene, *vax1*, was found to be specifically expressed in the ventral forebrain and in ventro-proximal structures of the developing eye, namely the optic stalk and the optic disk (Hallonet *et al.*, 1998). Functional studies have addressed the *vax1* role in eye development in both frog and mouse (Hallonet *et al.*, 1999). Knock-out of *Vax1* in the mouse results in an ocular phenotype very close to that observed in *Pax2-/-* mutant mice, i.e. non-closure of the optic fissure and ectopic extension of the pigmented epithelium into the optic stalk. Furthermore, both *vax1* and *pax2* appear to be necessary for glial differentiation of the optic stalk cells, which is similarly deficient in both *vax1-/-* and *pax2-/-* mutants (McDonald *et al.*, 1997; Bertuzzi *et al.*, 1999). Interestingly, both *Pax6* and *Rx* are ectopically expressed in the optic stalk of *Vax1-/-* mutant mice. Conversely, *Xvax1* overexpression in *Xenopus* embryos leads to a strong

repression of *Xrx1* expression. Moreover, overexpression in *Xenopus* embryos of either *shh* or *banded hedgehog*, another member of the *hedgehog* family, induces a striking expansion of *Xvax1* and a concomitant repression of *Xpax6* in the retinal territory. On these notions, it is possible to speculate that *pax2* and *vax1* could play a permissive role, downstream of *shh*, in allowing proper development of proximal eye structures, by repressing *pax6* and *rx* expression in such territories (Hallonet *et al.*, 1999; Fig. 7B). However, the possibility that *Pax6* expression in the *Vax1-/-* optic stalk could be due to the abnormally open optic fissure allowing for the unimpeded migration of retinal cells into the nerve, cannot be ruled out (Bertuzzi *et al.*, 1999). Surprisingly, *Pax2* expression is maintained in the optic stalk of *Vax1-/-* mutant mice, and *Vax1* transcription is still detectable in *Pax2-/-* mutant mice (Hallonet *et al.*, 1999), thus suggesting that the two genes may work in parallel pathways, being necessary for closure of the optic fissure and glial differentiation in the optic nerve. Nevertheless, it is clear that neither *pax2* nor *vax1* are absolutely required for the initial formation of the optic stalk and the ventral retina. While asking for a *Pax2/Vax1* double mutant mouse, the present knowledge also leaves the question open of whether other genes may exist that play a role in the early specification of proximo-ventral eye territories.

The role of the *vax2* gene

Starting from an Expressed Sequence Tag (EST) database screening, aimed at the identification of novel homologues of *Drosophila* mutant genes, we recently isolated in mouse, human and *Xenopus* a gene closely related to *vax1*, that we accordingly named *vax2* (Barbieri *et al.*, 1999). The same gene was independently identified by other groups through RT-PCR based screenings for novel ocular genes (Ohsaki *et al.*, 1999; Schulte *et al.*, 1999). The vax1 and vax2 proteins share an almost identical homeodomain, which is closely related to that found in the emx and not transcription factors. Remarkably, mapping studies in mouse and human assigned a close chromosomal location to *vax2* and *emx1* and to *vax1* and *emx2*, respectively, thus suggesting a possible common origin for these genes by tandem duplication events (Hallonet *et al.*, 1998; Barbieri *et al.*, 1999; Ohsaki *et al.*, 1999; Schulte *et al.*, 1999). However, up to now only one *vax* gene, named *cVax*, was identified in the chick (Schulte *et al.*, 1999). While more similar to *vax1* in terms of protein sequence, *cVax* shows a spatial expression pattern covering both *vax1* and *vax2* expression domains in other species, thus suggesting that it may encompass the functions of both *vax1* and *vax2* in the chick. Mouse *Vax2* is strongly transcribed in the ventral developing retina, while only faint expression levels were detected in the optic stalk and the ventral forebrain, where *Vax1* is instead highly expressed (Hallonet *et al.*, 1998; Barbieri *et al.*, 1999). On the other hand, both *cVax* and *Xenopus Xvax2* are significantly expressed in the ventral retina, in the optic stalk and in brain regions, particularly in the ventral telencephalon, suggesting that *vax1* and *vax2* gene functions may have been segregated to distinct regional domains during evolution (Barbieri *et al.*, 1999; Schulte *et al.*, 1999; our unpublished results; Fig. 6D). Also noteworthy are the spatio-temporal relationships between *vax2* and *pax2* expression in the eye. At early developmental stages, soon after evagination of the optic vesicle, *pax2* and *vax2* are coexpressed in the proximo-ventral part of the vesicle, fated to give rise to the optic stalk and the ventral retina. However, after formation of the optic cup, while *pax2* becomes almost exclusively

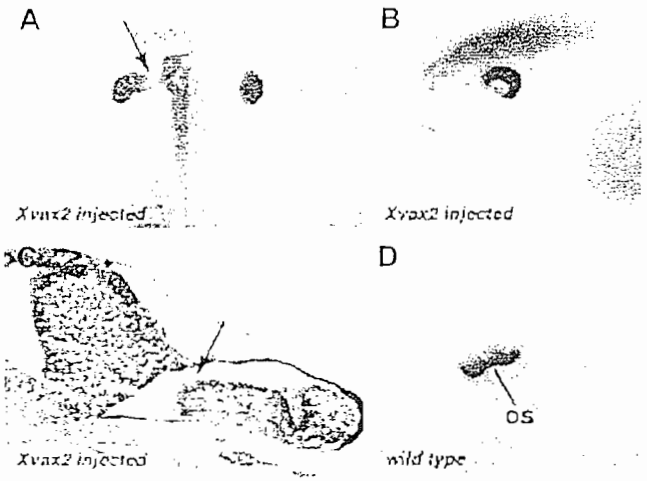

Fig. 6. *Xvax2* is expressed in the ventral retina and induces an aberrant morphogenesis of the eye. *(A-C) Effects of Xvax2 overexpression on eye development.* **(A)** *Dorsal view of a swimming tadpole Xenopus embryo unilaterally coinjected with Xvax2 and ß-galactosidase RNAs, showing that, on the injected side (left), the optic cup and the forebrain are connected by a giant optic stalk (arrow).* **(B)** *The same embryo shown in (A), laterally viewed from the injected side. The optic fissure remains widely open.* **(C)** *Transverse section of a swimming tadpole embryo injected with Xvax2 RNA. A wide optic stalk-like structure (arrow) abnormally joins the optic cup to the brain.* **(D)** *Lateral view of a wild type tadpole stage embryo hybridized with an Xvax2 probe, showing Xvax2 expression in the ventral part of the retina, in the optic stalk (os) and in the ventral forebrain.*

restricted to the optic stalk (with the exception of few retinal cells bordering the optic fissure), *vax2* is strongly maintained in the ventral half of the retina throughout development (Nornes *et al.*, 1990; Barbieri *et al.*, 1999; our unpublished results). Remarkably, prominent *VAX2* expression was also found in the developing human ventral retina, thus suggesting a *vax2* conserved role in regulating the retina D-V asymmetry (Barbieri *et al.*, 1999).

The question concerning the role played by *vax2* in eye development was independently addressed by Barbieri *et al.* (1999) and Schulte *et al.* (1999) through overexpression studies performed in frog and chick, respectively. In either species *vax2* overexpression causes ventralization of the retina, as detected by the ectopic activation in the dorsal retina of ventral retinal markers, such as *pax2*, *vax2* itself and *EphB* receptors *EphB2* and *EphB3*, and by the concomitant down-regulation of dorsal retinal markers, namely *Xvent2*, *ET*, *Tbx5*, *ephrinB1* and *ephrinB2* (Barbieri *et al.*, 1999; Schulte *et al.*, 1999; our unpublished results; Fig. 5). Remarkably, this ventralizing effect was obtained after overexpression of any of the frog, mouse and human *vax2* genes and chick *cVax*, thus again pointing to a strong evolutionary conservation of *vax2* function in controlling the retinal D-V patterning. Notably, *vax2* overexpression in the frog resulted in a wide *pax2* ectopic expression in the retina even at the optic cup stage, when *pax2* is normally excluded from the retina and restricted to the optic stalk. This result raised the question of whether *vax2* overexpression could affect proper specification of the optic stalk and the optic cup territories. Strikingly, morphological and molecular analysis of the injected *Xenopus* embryos at late developmental stages revealed the presence of a giant optic stalk abnormally connecting the eye and the

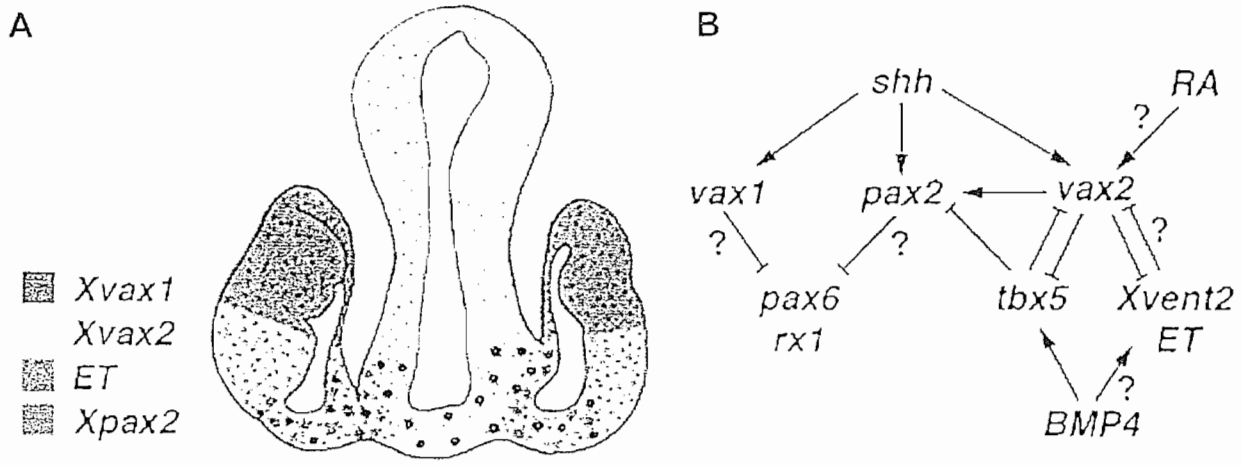

Fig. 7. Genetic regulation of eye polarity. (A) *Schematic representation of Xvax2 (yellow), ET (brown), Xvax1 (blue spots) and Xpax2 (red spots) expression in the eye and diencephalon of a late tailbud/early tadpole Xenopus embryo. At this stage, ET is expressed in the dorsal retina, Xvax2 is expressed in the ventral retina and in the optic stalk, Xvax1 expression covers the optic stalk and the ventral diencephalon, while Xpax2 is transcribed exclusively in the optic stalk.* (B) *Model for a molecular cascade involved in the regulation of proximo-distal and dorso-ventral polarity of the eye. vax1 and pax2 are induced by sonic hedgehog (shh) and may determine repression of the retinal genes pax6 and rx1 in the optic stalk. vax2, also regulated by the hedgehog pathway, is implicated in activation and/or maintenance of pax2 in proximo-ventral eye structures. A mutual antagonism between vax2 and tbx5, activated by BMP4 in the dorsal retina, is involved in regulating dorso-ventral polarity of the retina. Xvent2, ET and pax2 may also participate in this process. A possible regulation of vax2 by the retinoic acid (RA) pathway is proposed. Question marks indicate epistatic relationships still to be proved (see text for explanations).*

forebrain (Fig. 6 A,C). At the same time, morphogenesis of the optic cup was strongly affected, since the ventral retina did not encircle the lens correctly, but instead extended medially, thus resulting in a widely enlarged optic fissure (Fig. 6 B,C). Remarkably, both these morphological abnormalities correlated with ectopic *Xpax2* expression in the retina, that was detected both in dorsal retinal regions abnormally contiguous with the enlarged optic stalk and in the ventral part of the retina undertaking abnormal morphogenesis (Barbieri *et al.*, 1999). These data suggest that *vax2* overexpression may cause the observed morphological alterations by affecting the patterning of the eye along its P-D and/or D-V axis, thus supporting a *vax2* role in the regulation of eye polarity. On the other hand, proper regulation of *vax2* expression might be important in controlling the execution of the correct morphogenetic movements required for invagination of the optic vesicle and proper formation of the optic cup and stalk. Experiments performed in the chick also highlighted a pivotal role for *cVax* in regulating the establishment of a correct topographic map of retino-tectal projections along the D-V axis of the retina. In fact, following *cVax* misexpression, axonal trajectories of dorsal retinal cells appeared to be ventralized, as they never encompassed the ventral-most tectum, the area where they should normally focus, but grew in much more dorsal tectal positions (Schulte *et al.*, 1999). On the whole, these studies indicate that *vax2* may be involved in regulating eye polarity at different levels, such as regional specification of ocular structures, execution of correct morphogenetic movements and proper establishment of the D-V retinotectal projection map. Loss of function studies will help to elucidate for which of these processes *vax2* is indeed effectively required.

The possible relationships between *vax2* and other known genes involved in regulating eye polarity represent another crucial, open question. The striking effect on optic stalk development seen after *vax2* overexpression suggests that *sonic hedgehog* might represent a putative upstream regulator of *vax2*, in that it is known to play a pivotal role in the specification of proximal eye structures and to activate *vax1* expression in the eye. Indeed, we recently found that overexpression of *banded hedgehog* in *Xenopus* is able to strongly activate *Xvax2* expression throughout the optic cup (unpublished results), thus suggesting that *vax2*, *vax1* and *pax2* all lay on the *hedgehog* pathway. The partially overlapping expression domains of these three genes in the eye and ventral forebrain at the optic cup stage of frog development (Heller and Brändli, 1997; Hallonet *et al.*, 1998; Barbieri *et al.*, 1999; our unpublished results; Fig. 7A), may lead to further speculation about the existence of a morphogenetic gradient of *hedgehog* receptor stimulation. According to this view, *vax1* would be activated by high and intermediate *shh* levels in the ventral diencephalon and in the optic stalk, respectively; *vax2* would be turned on by intermediate and low *shh* levels in the optic stalk and in the ventral retina, respectively; and *pax2* would be activated by intermediate levels of *shh* in the optic stalk only. The activation of the *vax2*, *vax1* and *pax2* genes by the *hedgehog* pathway, as well as their co-expression in some territories, raises the question of their possible epistatic relationships in eye development. Results of knock-out experiments in the mouse, where *Vax1* and *Pax2* expression is maintained in *Pax2-/-* and *Vax1-/-* mutant mice, respectively, suggest that these two genes may lay on different hierarchies, independently turned on by the *hedgehog* pathway (Hallonet *et al.*, 1999). On the other hand, the strong upregulation of *pax2* following *vax2* overexpression (Barbieri *et al.*, 1999; Schulte *et al.*, 1999), may indicate that *vax2* is an upstream regulator of *pax2* in the eye. However, the occurrence of a direct activation of *pax2* by the *hedgehog* pathway, independently from *vax2*, cannot be ruled out, since transcription of *pax2* precedes *cVax* activation within the

presumptive chick ventral retina (Schulte *et al.*, 1999). Finally, the possible relationships occurring between the two *vax* genes themselves still remain to be addressed.

Besides *shh*, other signaling molecules may play a role in regulating *vax* gene expression. RA is a likely candidate to control *vax2* expression in the ventral optic vesicle. Indeed, RA treatment of fish embryos brings about phenotypic effects very close to those induced by *vax2* overexpression in the frog, namely enlargement of the optic stalk and *pax2* upregulation in the eye (Hyatt *et al.*, 1996). The signaling molecules cyclops (Rebagliati *et al.*, 1998; Sampath *et al.*, 1998) and one-eyed pinhead (Zhang *et al.*, 1998), which are involved in patterning the zebrafish eye and forebrain, could also be required for proper transcriptional regulation of the *vax* genes.

While positively acting on other genes expressed in the ventro-proximal part of the eye, as indicated by overexpression experiments *vax2* might also negatively interact with genes regulating dorsal retinal fates, thus restricting them to their appropriate functional domains (Barbieri *et al.*, 1999; Schulte *et al.*, 1999). Interestingly, *Tbx5*, a putative target of *vax2* repression (Schulte *et al.*, 1999), has recently been shown to play a somewhat complementary role with respect to *vax2* on retinal development (Koshiba-Takeuchi *et al.*, 2000). In fact, *Tbx5* overexpression in the chick is able to repress ventral retinal genes (including *cVax*), upregulate dorsal retinal genes and dorsalize the retinotectal projection pattern. Remarkably, the TGF-ß secreted protein BMP4 is expressed in the dorsal retina and is able to activate *Tbx5* and repress *cVax* transcription (Koshiba-Takeuchi *et al.*, 2000). It is tempting to speculate that the dorso-ventral patterning of the retina may involve the antagonistic action of the *hedgehog* and *BMP* pathways, intracellularly mediated by *vax2* and *Tbx5* in the ventral and dorsal retina, respectively (Fig. 7B). *shh* and *BMPs* are also involved in controlling dorso-ventral patterning of the brain and the spinal chord (Lee and Jessel, 1999): thus the developing eye, notwithstanding its unique features, would make use of the same molecular mechanisms that regulate the dorso-ventral polarity of the neural tube.

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REVIEW

Molecular Regulation of Vertebrate Retina Cell Fate

Massimiliano Andreazzoli*

The specification of retinal cell fate is a multistep process that begins during early development and results from the spatio-temporal coordination of cell cycle, cell differentiation, and morphogenesis. This review focuses on recent advances in understanding the molecular mechanisms underlying the distinct steps of retinal specification. Emphasis is placed on key regulatory events that control the multipotency of retinal progenitors, the generation of cell diversity, and the establishment of the clock that determines the ordered generation of retinal cell types. These basic studies have paved the way to the latest progress on the isolation and in vitro generation of retinal stem cells, which is presented in the light of possible therapeutic applications. **Birth Defects Research (Part C) 87:284–295, 2009.** © 2009 Wiley-Liss, Inc.

Key words: eye; cell fate; transcription factors; growth factors; miRNAs; stem cells

INTRODUCTION

The retina, as an experimentally accessible part of the central nervous system, has been the subject of intensive studies in several animal models. Integration of data obtained with different experimental approaches during the past decade has uncovered several of the cellular and molecular mechanisms underlying retinal development. In particular, a large body of genetic and molecular studies has revealed that genes controlling retinal cell fate are remarkably conserved among vertebrates and, in some cases, even across the animal kingdom. These genes encode mainly transcription factors and signaling molecules that act in distinct combinations to either define a certain competence/specification state of retinal progenitors or to determine retinal cell types. Factors controlling retinal cell fate have recently become of great interest for regenerative medicine as they may represent potential tools to direct stem cells to differentiate into the specific cell types that are lost in retinal degenerative diseases.

INITIAL SPECIFICATION OF RETINAL CELL FATE

Experiments aimed at defining early retinal fate were mainly performed in the frog *Xenopus* because of the accessibility of its embryos throughout development. The earliest embryonic cells destined in part to form retina have been traced back to nine animal blastomeres in *Xenopus* 32-cell stage blastula (reviewed in Zaghloul et al., 2005). However, the progeny of these blastomeres are not exclusively retinal since they make contributions to all three germ layers. A retina-specific presumptive territory becomes well defined only at the early neurula stage. This area, known as the "eye field," is comprised of a single territory located in the most anterior region of the neural plate (Fig. 1A). Lineage tracing experiments in *Xenopus* have shown that both the early retinogenic blastomeres and the cells of the eye field are not homogeneous populations, being composed of cells with different potentialities (Moody et al., 2000). For instance, individual retinogenic blastomeres are differentially biased to generate distinct subsets of amacrine cells. Similarly, eye field cells can be grouped into two classes: multipotent progenitors that will generate all the retinal cell types and progenitors with a more restricted potential, giving rise to cells of a single retinal layer (Moody et al., 2000). It is unclear whether the mixed cell population of the eye field may reflect the co-existence of progenitors in two determinative states, displaying progressively restricted potentialities, or, rather, distinct primary and sec-

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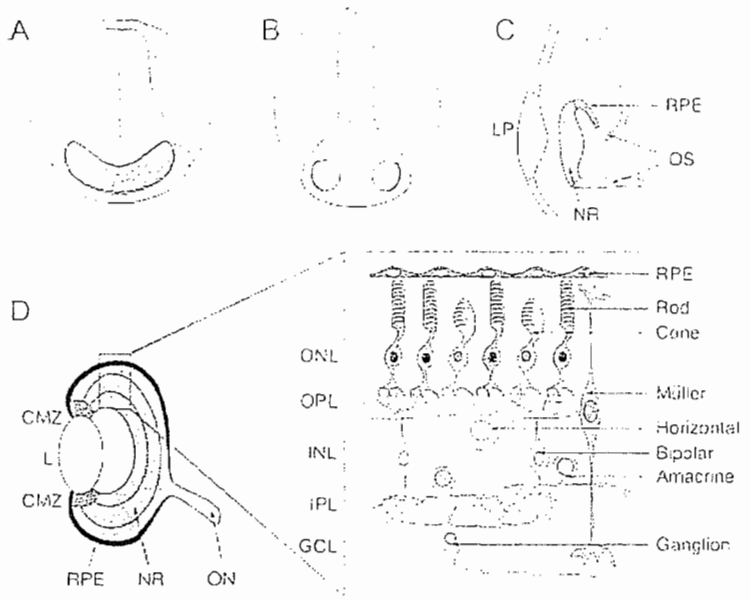

Figure 1. Main phases of vertebrate retinal development. (**A,B**) The eye field (blue) is specified as a single territory at early neurula stage (A) and, during neurulation, separates into the two retinal primordia (B). (**C**) After neural tube closure, lateral evaginations of the prosencephalon give rise to the optic vesicles, which contain the presumptive territories of neural retina (NR), retinal pigmented epithelium (RPE), and optic stalk (OS). The optic vesicles contact and exchange signals with the lens placode (LP). (**D**) Cellular organization of the mature retina. CMZ, ciliary marginal zone; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; L, lens; NR, neural retina; ONL, outer nuclear layer; OPL, outer plexiform layer; ON, optic nerve.

ondary progenitors that will give rise to the larval and the adult retina, respectively.

Signals that induce the eye field are not easily distinguishable from those that induce the presumptive forebrain. Indeed, specification of the eye field, as the remaining anterior neural plate, occurs by inhibition of ventralizing bone morphogenetic protein (BMP) and posteriorizing canonical Wnt signals, as well as by positive signals provided by fibroblast growth factor (FGF) and insulin-like growth factor (IGF) (Pera et al., 2001; Wilson and Houart, 2004; Delaune et al., 2005). In addition, maternal factors that promote endodermal and mesodermal fate in *Xenopus*, including VegT and Vg1, apparently need to be inhibited to allow retinal competence in dorsal-animal blastomeres (Yan and Moody, 2007). Recent data from our laboratory (L. Lan, A. Vitobello, M. Bertacchi, F. Cremisi, R. Vignali, M. Andreazzoli, G. C. Demontis, G. Barsacchi, S. Casarosa, Stem Cells, 2009) indicate that Noggin, a BMP inhibitor, exerts dose dependent effects, with high levels able to induce a retinal fate in ectodermal explants from *Xenopus* blastulae (animal caps). Animal caps treated with high doses of Noggin, upon transplantation in host embryos, can form a complete and layered retina and retinal pigmented epithelium (RPE), even when transplanted in the posterior neural plate. These data are consistent with previous reports suggesting that the retinal fate is specified by high levels of neural inducers (Chow et al., 1999; Kenyon et al., 2001). Moreover, presumptive retinal cells exhibit early cellular dispersal and migration into the eye field. These cell movements are promoted by ephrin B1 that acts by inhibiting FGF signaling (Moore et al., 2004). More recently, purine-mediated signaling has also been implicated as a critical player in the induction of the eye field (Massé et al., 2007). In particular, purinergic signaling by ADP, mediated by E-NTPDase2 and acting through P2Y1 receptors, was shown to be active in the anterior neural plate where it triggers the initial events of the eye development. However, this signaling pathway also affects both the eyes and the brain, indicating that specific eye-inducing signals, if they exist, remain to be identified.

Recently, progress has been made in clarifying the factors acting downstream of the known signaling pathways that induce the eye field. The factors identified so far appear to constitute a network of transcription factors ("Eye Field Transcription Factors," EFTFs: ET, Pax6, Six3, Rx1, Lhx2, tll, and Optx2) displaying slightly different expression domains that overlap in the eye field (Zuber et al., 2003). Overexpression experiments in *Xenopus* embryos indicate that Noggin and Otx2 act upstream of EFTFs, inducing a pre-pattern of the anterior neural plate and allowing their expression. When co-injected, EFTFs are able to induce ectopic eyes even outside the nervous system. Similar to a model proposed for *Drosophila* eye development (Kumar and Moses, 2001), the EFTFs function as a self-regulating feedback network that specifies the eye field. Each EFTF appears, moreover, to have specific characteristics that contribute to defining the eye field. For instance, an important feature of eye field cells is that they continue to proliferate even when cells of surrounding territories are undergoing neuronal differentiation. The extended proliferative phase is crucial to produce a sufficiently large pool of progenitors necessary to generate all the retinal cell types. An essential contribution to maintain the prolonged proliferation of the eye field is provided by the homeodomain transcription factors, Rx1, Six3, and Optx2/Six6, which display the ability to induce proliferation by controlling the expression of cell cycle genes (Zuber et al., 1999; Andreazzoli et al., 2003;

Gestri et al., 2005). In addition, Six3 was shown to directly bind Geminin, preventing its inhibition of cell cycle progression (Del Bene et al., 2004). Moreover, by actively repressing the transcription of both Wnt1 and BMP4, Six3 also contributes to maintain the low levels of these signaling factors required for eye field specification and early retinal development (Lagutin et al., 2003; Gestri et al., 2005).

MAINTENANCE AND DEVELOPMENT OF EARLY RETINAL PROGENITORS

After its initial specification, the eye field is split into the two retina territories (Fig. 1B). This is due to the prechordal plate expression of Six3, which activates the secreted factor sonic hedgehog (Shh) that reaches the neuroectoderm and converts the medial area of the eye field into ventral diencephalon (Chiang et al., 1996; Geng et al., 2008).

During neurulation, the cells of the eye field form the two optic primordia, which in turn will give rise to the optic vesicles and, upon invagination, to the optic cups (Fig. 1C; Chow and Lang, 2001; Rembold et al., 2006). The external layer of the optic cup becomes RPE, while the internal layer forms the neural retina. At these stages, the pool of retinal progenitors expands by proliferation and will subsequently generate the six types of neurons (ganglion cells, rods, cones, horizontal, amacrine, bipolar) and one type of glia (Müller glia) of the mature retina (Fig. 1D). In vertebrates, the generation of retinal cell types follows an evolutionarily conserved temporal order that occurs in partially overlapping waves. In general, ganglion cells are generated first, followed by cones and horizontal cells, amacrine cells, rods, bipolar cells and, finally, Müller glia. Lineage tracing experiments have suggested that these seven cell types derive from a common pool of retinal progenitors that pass through a series of competence states. A histogenic clock appears to control the generation of retinal cell types. In fact, the time at which a progenitor exits the cell cycle affects its cell fate (Livesey and Cepko, 2001). Early progenitors are competent to give rise to early-born cells, while late progenitors are competent to generate late-born cells. Each competence state is defined by intrinsic factors that, acting cell autonomously, control the subset of retinal cell types that a progenitor can potentially generate. Within this subset, the production of a specific cell type is influenced by extrinsic (environmental) signals. Similar to the eye field, there is evidence that the population of retinal progenitors is heterogeneous, being comprised of multipotent progenitors, capable of giving rise to all the retinal cell types, as well as of progenitors biased to generate a specific cell type. Specific genes have been shown to control proliferation and multipotency of early progenitors. A primary role is played by Notch, which is known to maintain retinal progenitor cells (RPCs) in a proliferative undifferentiated state (Jadhav et al., 2006). This activity is mediated by Hes bHLH proteins, direct downstream effectors of Notch that, acting as transcriptional repressors, inhibit the expression of proneural genes (refer Ohsawa and Kageyama, 2008). Rx1 is another essential factor for maintaining proliferation and multipotency of RPCs. This homeobox gene is initially expressed in the entire early proliferating retina and its downregulation correlates spatially and temporally with neuronal differentiation (Casarosa et al., 1997; Furukawa et al., 1997a; Mathers et al., 1997). In Xenopus, in vivo lipofection of Rx1 in early optic vesicles results in increased proliferation of RPCs, which, however, do not lose their multipotency (Casarosa et al., 2003; Zaghloul and Moody, 2007). Rx1 appears to maintain progenitor cells in a proliferative, multipotent state by repressing the expression of genes that induce differentiation, possibly through the activation of the antineurogenic genes, Hairy2 and Zic2, and by repressing the cell cycle inhibitor, p27Xic (Andreazzoli et al., 2003). A similar role has been recently proposed for Vsx2, a homeobox gene required for retinal proliferation and bipolar cell fate (Vitorino et al., 2009). Analysis of transgenic zebrafish, in which fluorescent proteins are expressed under the control of *vsx2* regulatory regions, indicates that this gene is initially expressed throughout the retina but is then gradually downregulated in all cells except for Müller glia and a subset of bipolar cells. Therefore, Vsx2-negative cells that derive from Vsx2-positive early progenitors give rise to all other retinal cell types. Vsx2 acts as a repressor to inhibit the expression of cell fate determining factors, thus maintaining early progenitors in a proliferative and multipotent condition. While Rx1 and Vsx2 maintain cells in an undifferentiated state by preventing the expression of factors that induce specific cell fates, Pax6 appears to play a positive role in promoting most of the multipotent retinogenic potential of RPCs. In fact, Pax6 conditional knock-out in mouse retinal progenitors of the optic cup results in the generation of amacrine cells only (Marquardt et al., 2001). This effect appears to be due to the ability of Pax6 to directly activate retinogenic bHLHs with the exception of NeuroD, which promotes production of amacrine cells. Another relevant gene in controlling retinal multipotency is Sox2, a SoxB1 HMG transcription factor, whose inactivation is responsible for ~10% of human cases of anophthalmia or microphthalmia. Sox2 conditional inactivation in mouse retina causes the loss of retinal progenitor ability to proliferate and differentiate (Taranova et al., 2006). Sox2 exerts these effects by directly regulating the expression of Notch. Interestingly, the observation that Rx1 is able to activate both Notch and its downstream effectors, Hes1 and hairy2 (Furukawa et al., 2000; Andreazzoli et al., 2003), suggests that the Notch pathway could rep-

resent a common target for genes controlling retinal multipotency.

INTRINSIC AND EXTRINSIC FACTORS CONTROLLING RETINAL CELL FATE

In the past 10 years, the search for intrinsic factors has led to the identification of a large number of transcription factors of the homeodomain and activator-type bHLH protein families that interact in a combinatorial way to control cell cycle exit and cell fate determination. In particular, it has been proposed that, in general, homeodomain factors may provide positional information within the retina, defining layer specificity, whereas activator bHLH proteins may determine a cell fate within the specified layer. This subject has been recently reviewed (Ohsawa and Kageyama, 2008), and an updated list of cell fate determining transcription factors is shown in Table 1.

Several cell cycle components also behave as intrinsic factors playing a crucial role in retinal cell fate (Cremisi et al., 2003; Ohnuma and Harris, 2003). For instance, p27Xic overexpression in *Xenopus* drives RPCs out of the cell cycle and induces them to become Müller glia (Ohnuma et al., 1999). This is consistent with a model in which p27Xic concentration gradually increases in dividing progenitors until it accumulates to a level sufficient to cause cell cycle exit. This threshold is reached during the last phase of retinogenesis, at the time when progenitors that exit cell cycle differentiate into Müller glia. However, when p27Xic is co-expressed with Ath5, an essential proneural inducer of ganglion cells (Kay et al., 2001), RPCs exit cell cycle early and differentiate mostly into ganglion cells, indicating that the determinative effect of proneural genes is dominant with respect to that of cell cycle inhibitors (Ohnuma et al., 2002).

The correct spatio-temporal combination of transcription factors, which seems to be the major key to retinal cell fate determination (Ohsawa and Kageyama, 2008), is not always achieved through transcriptional control. This is of particular importance for retinal progenitors, as mRNAs coding for transcription factors determining different cell fates were found to be co-expressed in single progenitors (Moore et al., 2002; Trimarchi et al., 2008). In these cases, different levels of post-transcriptional control take place to assure the correct timing of activation for each transcription factor. An example is given by the co-expression in *Xenopus* retinal progenitors of *Ath5* and *NeuroD*, two proneural genes implicated in the generation of ganglion and amacrine cells, respectively. During early retinogenesis, the function of Neuro D, but not Ath5, is inhibited by GSK3β-mediated phosphorylation. This prevents NeuroD from being active when Ath5 promotes the differentiation of the earliest born cell type. Another documented case of post-translational modification of a cell fate determining factor is represented by SUMOylation of Nr2e3. Indeed, Nr2e3, a transcription factor expressed in rod precursors and essential for their formation, promotes the expression of rod-specific genes, but when SUMOylated it is converted into a strong repressor of cone-specific genes (Onishi et al., 2009). Beside post-translational modifications, other possible mechanisms that sort out the spatio-temporal regulation of co-expressed mRNAs include control of translation and/or stability of mRNAs. MicroRNAs and RNA binding proteins are the primary regulatory molecules involved in this process. In particular, specific microRNAs are expressed in the retina and a few functional studies indicate their implication in retinal cell lamination, apoptosis, and timing of retinal cell generation (Loscher et al., 2007; Xu et al., 2007; Damiani et al., 2008; Decembrini et al., 2008; Qiu et al., 2009; Walker and Harland, 2009). On the other hand, the role of RNA binding proteins in retinogenesis has not been fully investigated, although a function has been established for Xseb4R, which displays proneural properties acting downstream of neurogenin and NeuroD (Boy et al., 2004) and Musashi, which is required for photoreceptor survival (Susaki et al., 2009).

The role of the environment in determining cell fate was addressed by classical studies based on ablation of mature retinal cells as well as co-cultures of early progenitors with postmitotic retinal cells. These works demonstrated that a particular fate of a cell depends also on neighboring cells. A typical pathway involved in the generation of neuronal diversity by cell–cell interaction is Delta-Notch signaling. In the vertebrate retina, multipotent progenitors express both Delta and Notch and inhibit each other from differentiating. When a progenitor cell increases its level of Delta, it inhibits Delta expression in surrounding cells. In this way, the cell with high Delta expression is no longer inhibited by neighboring cells and is committed to differentiate. Several secreted factors have been involved in controlling the proliferation of RPCs. These include Shh (refer below), ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), BMP, Wnt, and FGF (Yang, 2004; Wallace, 2008). Some growth factors have been shown to affect cell fate. This is the case, for instance, of CNTF and leukemia inhibitory factor (LIF), which are known to block rod differentiation (Livesey and Cepko, 2001). Moreover, it has been shown that the generation of ganglion cells, amacrine, and cones is regulated by a negative feedback. In fact, each of these cell types inhibits the additional production of the same cell type from RPCs. Some of these signals have been recently identified. Both Shh and Growth/Differentiation factor 11 (GDF11) are secreted molecules expressed by retinal ganglion cells that act as negative feedback signals to prevent overproduction of ganglion cells (Kim et al., 2005; Wallace, 2008). On the other hand, amacrine cell number is controlled by transforming growth factor βII

TABLE 1. Transcription Factors Involved in the Determination of Retinal Cell Types

Cell Type	TF	TF Class[a]	Species	Reference
Ganglion	Pax6	Hd	Mouse	Marquardt et al., 2001
	Ath5	bHLH	Mouse	Brown et al., 2001; Wang et al., 2001
			Zebrafish	Kay et al., 2001
			Xenopus	Kanekar et al., 1997
	Pou4f (Brn3)	POU	Mouse	Gan et al., 1996
			Chick	Liu et al., 2000
	Xbh1	Hd	Xenopus	Poggi et al., 2004
	Eomes	Tb	Mouse	Mao et al., 2008
Horizontal	Math3	bHLH	Mouse	Tomita et al., 2000
	Prox1	Hd	Mouse	Dyer et al., 2003
	Pax6	Hd	Mouse	Marquardt et al., 2001
	Foxn4	Fh	Mouse	Li et al., 2004
	Ptf1	bHLH	Mouse	Fujitani et al., 2006
Amacrine	Six3	Hd	Mouse	Inoue et al., 2002
	Pax6	Hd	Mouse	Inoue et al., 2002
	Foxn4	Fh	Mouse	Li et al., 2004
	Ptf1	bHLH	Mouse	Fujitani et al., 2006
	NeuroD	bHLH	Mouse	Morrow et al., 1999
	Math3	bHLH	Mouse	Tomita et al., 2000
	Bhlhb5	bHLH	Mouse	Feng et al., 2006
	Barhl2	Hd	Mouse	Mo et al., 2004
Photoreceptors	NeuroD	bHLH	Mouse	Morrow et al., 1999
	Mash1	bHLH	Mouse	Tomita et al., 1996b
	Crx	Hd	Mouse	Chen et al., 1997; Furukawa et al., 1997b
	Otx5	Hd	Xenopus	Viczian et al., 2003
	Otx2	Hd	Mouse	Nishida et al., 2003
	Rx-L	Hd	Xenopus	Pan et al., 2006; Wu et al., 2009
	Nrl	bLZ	Mouse	Mears et al., 2001
	Nr2e3	ONR	Mouse	Chen et al., 2006
Bipolar	Mash1	bHLH	Mouse	Tomita et al., 1996b
	Math3	bHLH	Mouse	Tomita et al., 2000
	Vsx2 (Chx10)	Hd	Mouse	Burmeister et al., 1996
	Vsx1	Hd	Mouse	Chow et al., 2004; Ohtoshi et al., 2004
	Otx2	Hd	Xenopus	Viczian et al., 2003
	Bhlhb5	bHLH	Mouse	Feng et al., 2006
	Bhlhb4	bHLH	Mouse	Bramblett et al., 2004
Muller	Hes1	bHLH	Mouse	Tomita et al., 1996a
	Hes5	bHLH	Mouse	Hojo et al., 2000
	Hesr2	bHLH	Mouse	Satow et al., 2001
	Rx1(Rax)	Hd	Mouse	Furukawa et al., 2000

[a]bHLH, basic Helix-Loop-Helix; bLZ, basic Leucine Zipper; Hd, homeodomain; ONR, orphan nuclear receptor; POU, POU domain; Tb, T-box; Fh, Forkhead domain; TF, transcription factor.

(TGFβII), a cytokine whose expression in amacrine cells is regulated by the zinc finger transcription factor Zac1 (Ma et al., 2007).

CELL FATE SPECIFICATION BY ASYMMETRIC DISTRIBUTION OF CELLULAR DETERMINANTS

An evolutionary conserved aspect in the generation of cell diversity is represented by asymmetric cell division (Huttner and Kosodo, 2005). Asymmetric division occurs when a mother cell divides unequally to generate two daughter cells with different fates. In an asymmetric division occurring in a neuroepithelium, the mitotic spindle axis is perpendicular to the neuroepithelium and the division takes place along the apico-basal axis. In the retina, the apical side is close to the RPE, while the basal side is close to the lens. On the contrary, symmetric cell divisions tend to give rise to identical daughter cells and are generated when the mitotic spindle is oriented along the plane of the neuroepithelium (planar divisions). In this way, spindle orientation determines an equal (symmetric divisions) or unequal (asymmetric divisions) distribution of cell fate

determinants or a differential exposure to localized signaling molecules. Even in zebrafish, where apico-basal cell divisions are not observed, divisions with different orientations are present and they appear to be linked to different cell fates (Das et al., 2003). In this case, cells divide always tangentially to the hemispheric surface of the retina, but their mitotic spindle can display either a central-peripheral or a circumferential orientation. In particular, early retinal progenitors preferentially undergo central-peripheral divisions, while late progenitors mostly divide along the circumferential axis. A clear correlation between orientation of cell divisions and cell fate was demonstrated by analyzing zebrafish transgenic lines expressing GFP under the control of the *ath5* promoter (Poggi et al., 2005). In a wild-type background, *ath5*-expressing cells divide along the circumferential axis and give rise to a ganglion cell and another cell type. On the contrary, in *lakritz* mutants, which lack ganglion cells, transplanted wild-type *ath5*-expressing cells tend to divide along the central-peripheral axis and generate two ganglion cells. Thus, the lack of the negative feedback signal from ganglion cells determines a change in spindle orientation, which results in the generation of more ganglion cells.

Inheritance of a cell fate determinant in the retina is exemplified by mammalian Numb, an asymmetrically localized factor which is thought to function, at least in part, by inhibiting Notch signaling. As a consequence of its apical localization in retinal precursors, Numb segregates to the apical daughter cell in asymmetric apico-basal cell divisions, while it is equally distributed in both daughter cells following symmetric planar divisions. Furthermore, misexpression experiments result in more homogeneous cell populations compared to control retinas, indicating that an equal distribution of Numb among daughter cells reduces cell diversification (Cayouette and Raff, 2003).

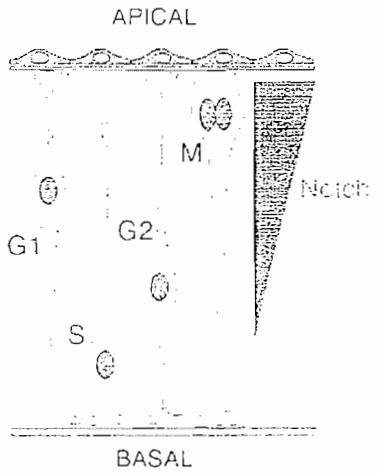

Figure 2. Interkinetic nuclear migration. Relationship between cell cycle phases, nuclei position along the apical-basal axis, and the Notch gradient.

The essential role of spindle orientation in determining retinal cell fate has been emphasized by functional studies on the mammalian *Inscutable* (*mInsc*) gene. In *Drosophila* neuroblasts, *Inscutable* acts as an essential linker between the apical complex of polarity proteins and the G protein system that is thought to directly attract and reorient one of the two spindle poles. mInsc protein is localized to the apical side of the retinal progenitors and its RNAi-mediated knockdown results in a significant reduction of apico-basal cell divisions. This effect is associated to increased proliferation, probably due to a higher frequency of symmetric divisions giving rise to proliferating daughter cells. As a consequence, progenitors that would normally differentiate into photoreceptors are maintained in proliferation for a longer time and will eventually adopt a later bipolar fate (Zigman et al., 2005).

Differential exposure to a signaling molecule has been recently shown to play a crucial role in determining retinal cell fate during interkinetic nuclear migration (INM; Del Bene et al., 2008). Retinal progenitors are elongated cells that contact both the apical and the basal surfaces of the retina. Their nuclei migrate along the apico-basal axis and the position that they occupy is strictly related to a specific phase of the cell cycle. In fact, mitosis occurs when nuclei are close to the apical surface, whereas DNA synthesis takes place near the basal surface (refer Fig. 2). Recent observations have shown heterogeneity among RPCs in the extent of nuclear migration toward the basal side. Moreover, cells displaying a reduced basal migration give rise to proliferative divisions, whereas cells whose nuclei migrate further to the basal surface tend to undergo neurogenic divisions (Baye and Link, 2007). In the zebrafish mutant *mikreoko* (*mok*), a mutation in *Dynactin-1*, a gene coding for a microtubule-motor-associated protein, results in perturbed INM. In *mok* retinas, progenitor nuclei move more rapidly and deeply toward the basal surface and more slowly in the apical direction. Baier and collaborators (Del Bene et al., 2008) also demonstrated that Notch signaling is activated according to an apical-to-basal gradient in both zebrafish and mouse retinas and that this gradient is maintained in the *mok* mutant. Consistent with the correlation between extent of basal migration and cell divisions, *mok* retinas display an overproduction of early-born ganglion cells at the expense of late interneurons and glia. Therefore, in this mutant, many cells exit the cell cycle prematurely during the initial phase of retinogenesis. Altogether, these data suggest that, during normal INM, nuclei close to the apical surface are exposed to high levels of the antineurogenic activity of Notch and give rise to proliferative daughter cells, whereas nuclei located more basally experience low levels of Notch and generate daughter cells that will become postmitotic.

UNRAVELING THE MOLECULAR MECHANISM OF THE RETINAL CLOCK

How do retinal progenitors move from one competence state to the next? How is this related to the

cell cycle and how do extrinsic and intrinsic factors coordinate to orchestrate the ordered generation of retinal cell types? These questions, which aim at uncovering the molecular mechanism of the histogenic retinal clock, have been the subject of a large number of studies. Although we are still far from understanding the big picture, these works provided several clues on how the clock may function. An important breakthrough about the way in which neural precursors can generate distinct cell types in a specific order has come from studies on *Drosophila* central nervous system. In the fly, neuroblasts divide asymmetrically, generating a series of daughter cells (ganglion mother cells, GMCs), each of which produces postmitotic neurons. The identity of these neurons is determined by their birth order and is a consequence of changes in competence of GMCs. Four transcription factors, Hunchback (Hb), Krüppel (Kr), Pdm, and Castor (Cas), were shown to be the primary intrinsic factors controlling neuroblast competence. These proteins are expressed sequentially and transiently in dividing GMCs, but their expression is maintained in the neural progeny generated at the time when each factor is expressed. The inheritance of the gene expression profile of progenitors represents an excellent way in which GMCs can keep the memory of their birth order. In particular, functional experiments have shown that Hb and Kr are necessary and sufficient to control early-born identity in a neuroblast cell lineage. The sequential expression of Hb, Kr, Pdm, and Cas requires cell cycle progression, indicating that a cell cycle clock regulates the timing of the generation of specific cell types (Isshiki et al., 2001).

The recent demonstration that in mouse, *Ikaros*, a *Hunchback* homologue, and *Castor* are both expressed in the retina (Blackshaw et al., 2004; Elliot et al., 2008), has suggested that the Hb-Kr-Pdm-Cas network might represent an evolutionary conserved pathway that controls the timing of neural cell fate generation. This hypothesis is supported by functional studies on the role of *Ikaros* during retinogenesis. *Ikaros* is expressed in early but not late retinal progenitors, and Ikaros-positive progenitor cells give rise to Ikaros-negative cells at later stages in the same lineage, thus indicating that all retinal progenitors pass through an initial temporal stage characterized by *Ikaros* expression. *Ikaros* knockout retinas display a reduced number of early-born cells, whereas no effect is observed on late-born cells. Misexpression of *Ikaros* is sufficient to confer competence to late progenitors to generate early-born retinal cell types, including ganglion, horizontal, and amacrine cells. Thus, Ikaros activity differs from the function of cell fate determining transcription factors that promote the generation of one specific cell type. In addition, clonal analysis shows that *Ikaros* misexpression does not completely prevent the generation of late-born cell types, indicating a permissive rather than an instructive role for Ikaros in promoting early-born cell fates. However, forced expression of *Ikaros* almost completely abolishes Müller glia generation, suggesting that during retinogenesis, downregulation of *Ikaros* is required by RPCs to progress to the last competence stage in which Müller glia cells are produced.

An interesting example of how an extrinsic factor can regulate the sequential activation of cell fate determining proteins is given by recent studies on the translational control of Xotx5, Xotx2, and Xvsx1. In *Xenopus*, Xotx5, a Crx homologue, promotes photoreceptor fate, while Xotx2 and Xvsx1 control specification of bipolar cells, the last neuronal cell type generated in the retina. Although the mRNAs of these three genes display a widespread and overlapping pattern of expression during early and mid-retinogenesis, the translation of the relative proteins is spatially and temporally regulated (Decembrini et al., 2006). In particular, the three proteins are sequentially translated, with Xotx5 protein detected in photoreceptors at the onset of their generation, followed by Xvsx1, translated in bipolar cells, and finally by Xotx2, which is translated in bipolar cells at late retinogenesis. Thus, a retinal clock controls the generation of different cell types by allowing the sequential translation of cell fate determining proteins. A central element of this clock appears to be the variation of cell cycle length. In fact, it is known that in neural progenitors, cell cycle length increases over time and this parallels the temporal change in competence of progenitors that generate different cell types at different times. Consequently, early and late retinal progenitors are characterized by short and long cell cycles, respectively. In particular, shortening the cell cycle of late retinal progenitors by E2F overexpression results in inhibition of Xotx2 translation and a decrease in number of bipolar cells. This suggested that a long cell cycle during the last cell divisions of a retinal progenitor is a prerequisite for Xotx2 translation and bipolar cell generation (Decembrini et al., 2006). What determines cell cycle length in the retina and how is this linked to translational control of fate determining genes? Shh is a primary candidate for the role of retinal regulator of cell cycle length. In fact, Shh was shown to control cell cycle kinetics through regulation of G1 and G2 length in retinal precursors (Locker et al., 2006). In particular, overexpression of Shh results in accelerated cell cycles with short G1 and G2 phases, whereas Shh inhibition by cyclopamine treatment decreases the rate of cell cycling by extending the duration of G1 and G2. A fast cell cycle is associated with early cell cycle exit, while a slow cell cycle is associated with late cell cycle exit. Secreted by the RPE and the ganglion cell layer, Shh is supposed to generate gradients that have been proposed to be responsible for speeding up the cell cycle of retinal stem cells, trigger-

ing their transformation into faster cycling progenitors (Locker et al., 2006). With the aim of identifying the molecules responsible for the delayed translation of Xotx2 and Xvsx1, Cremisi and collaborators (S. Decembrini, D. Bressan, R. Vignali, L. Pitto, S. Mariotti, C. Rainaldi, X. Wang, M. Evangelista, G. Barsacchi and F. Cremisi, submitted) recently showed that inhibition of Shh by cyclopamine anticipates the translation of these two genes. As the elements controlling Xotx2 and Xvsx1 translation reside in the 3'UTR, where putative target sequences for miRNAs are present, a strategy was designed to identify miRNAs activated by Shh that could potentially inhibit translation of Xotx2 and Xvsx1 in fast cycling cells. This led to the selection of four miRNAs expressed in proliferating RPCs and downregulated in slowly proliferating cells, which directly repress Xotx2 and Xvsx1 translation. In particular, a mixture of the four selected miRNAs is able to counteract the positive effects of cyclopamine on Xotx2 translation, thus indicating that these miRNAs are direct mediators of Shh activity on Xotx2 translation.

As mentioned earlier, RNA binding proteins also play a role in the post-translational control of retinogenesis. Even in the case of Xotx2 and Xvsx1, although miRNAs that directly repress the translation of their mRNA have been identified, there is indirect evidence that RNA binding proteins may contribute to the translational control. In fact, knockdown of Dicer, an essential enzyme for miRNA biogenesis, unexpectedly results in a delay in Xotx2 and Xotx5 translation (Decembrini et al., 2008). These data suggest a complex miRNA regulatory network, where, beside miRNAs directly repressing mRNA translation, it is possible to postulate the existence of miRNAs that inhibit the translation of RNA binding proteins, which are directly involved in translational repression of the target mRNA. This is actually the modus operandi of let-7 (Slack et al., 2000) and lin-4 (Moss et al., 1997), two *C. elegans* miRNAs that indirectly promote the translation of developmental genes by repressing the translation of inhibitory RNA binding proteins. It is noteworthy that a vertebrate homologue of lin-4, named miR-125, is expressed in *Xenopus* developing retina and that its inactivation significantly decreases the proportion of bipolar cells, the cell type determined by Xvsx1 and Xotx2 (Decembrini et al., 2008).

PERSPECTIVES ON RETINAL STEM CELLS, REGENERATION, AND THERAPEUTICAL APPLICATIONS

Retinal stem cells (RSCs) are present in several locations in the eyes of developing and adult vertebrates. In the retina of fishes and amphibians, RSCs are located in a mitotically active region at the peripheral margin called Ciliary Marginal Zone (CMZ; Fig. 1D), which generates all the retinal cell types throughout the lifetime of the animal. This region is spatially ordered, with the youngest and least-determined stem cells closest to the periphery, the proliferative retinoblasts in the middle, and postmitotic cells at the central edge (Wetts et al., 1989). The CMZ expresses the same genes transcribed in early embryonic retinal progenitors, with a distribution along the peripheral-central axis that recapitulates the onset of their expression during early development. A similar region is present also in birds and mammals, although there has been an increasing reduction in the neurogenic activity of the CMZ from fishes to mammals. An additional source of retinal stem cells is represented by the RPE. In amphibians, experimental removal of the retina is followed by transdifferentiation of RPE cells into retinal progenitors, which differentiate into all the retinal cell types and regenerate a complete retina. In birds, the embryonic RPE displays the same regenerative ability observed in amphibians, although this ability is lost in the adult retina. In mammals, the retinal stem cell potential of the RPE was uncovered in vitro. In fact, these cells generate spherical colonies, typical of neural stem cells, which differentiate into several retinal cell types (Tropepe et al., 2000). Finally, there is recent evidence that Müller glia may function as multipotent retinal stem cells. During normal zebrafish development, Müller glia give rise to rod progenitors, whereas in injured retina Müller glia reenter the cell cycle and regenerate the missing neurons (Bernardos et al., 2007). Chick Müller glia display a more restricted regenerative ability, while no apparent activity is found in mammals. Nonetheless, the transcriptome of mammalian Müller glia cells is intriguingly similar to that of retinal progenitors (Blackshaw et al., 2004). Moreover, when the retinal progenitor genes, *Rx1*, *Notch1*, and *Hes1*, are overexpressed in proliferating postnatal rat RPCs, they generate Müller glia. Finally, in mouse, injured-activated Müller cells transplanted into normal eyes generate neurons of all the retinal layers, an effect that can be mimicked by treatment of Müller glia with Wnt and Notch (Das et al., 2006). Altogether, these data suggested that in mammals, Müller glia remain quiescent in normal conditions but can behave as retinal stem cells following specific stimuli.

The identification of retinal stem cells raised the possibility of using them in cell replacement therapy to cure inherited or age-related diseases. Many studies in this field have tested several sources of retinal stem cells (refer Lamba et al., 2008). In general, these strategies can be grouped into two distinct approaches: (i) isolate retinal stem cells or progenitors from a natural source such as the developing retina or RPE; and (ii) induce a retinal fate in embryonic stem (ES) cells. The best example of the first approach shows that postmitotic rod precursors are the most efficient source of progenitors to obtain rod photoreceptors that functionally integrate into adult retina (MacLaren et al., 2006). On the other hand, basic

studies on retinal cell fate have provided a selection of growth factors that were tested in different combinations to direct ES cells towards a retinal fate. Ikeda et al. (2005) found that treatment of mouse ES cells with dkk and leftyA, a Wnt and a nodal antagonist, respectively, together with Activin and serum results in the generation of cells with a photoreceptor phenotype when co-cultured with embryonic retinal cells. Studies on human ES cells, have shown that cocktails containing either a BMP inhibitor, a Wnt inhibitor, and IGF-1 (Lamba et al., 2006), or a combination of Wnt and nodal antagonists (Osakada et al., 2008), lead to RPCs that integrate in normal or degenerating retinas. More recently, Lamba et al. (2009) demonstrated that transplantation of retinal cells derived from human ES cells treated with their protocol restore a light response in blind $Crx^{-/-}$ mice.

Although this progress is highly significant, several steps are still necessary before these findings can be translated into a clinical treatment. To this aim, it has been proposed that human photoreceptor precursors could be obtained from ES cells extracted from embryos generated by somatic cell nuclear transfer (MacLaren and Pearson, 2007). An alternative source of retinal progenitors or RSCs could be potentially represented by induced pluripotent stem (iPS) cells. These cells, obtained from fibroblasts transformed with genes that act as key regulators of pluripotency, display most of the features of ES cells, including the ability to generate a complete mouse. A recent study defined a protocol, based on the inhibition of BMP and TGFβ, able to convert human iPS cells into neural cells (Chambers et al., 2009). If human iPS cells can be specifically directed to adopt a retinal fate, they could represent an interesting option for cell replacement therapies. In fact, it will be possible to have a large number of these cells available and they can be derived from cells of the same patient that will receive the transplantation, thus avoiding the risk of rejection. Moreover, because of their somatic origin, they will not pose the ethical concerns that have been raised about the use of human ES cells.

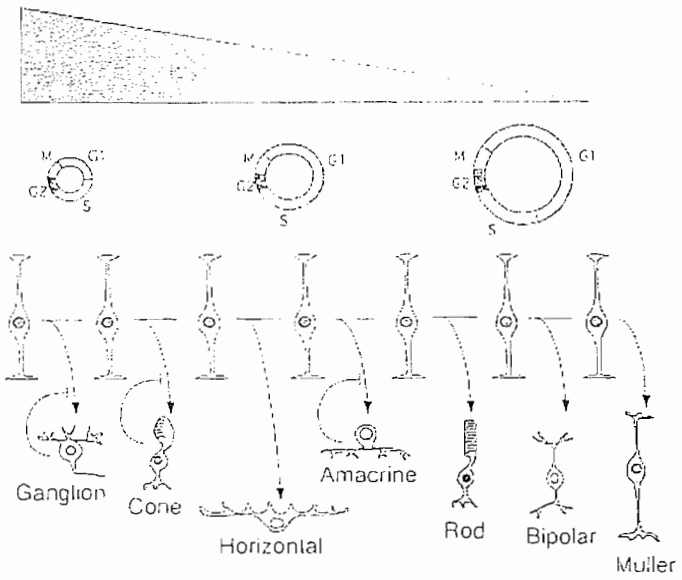

Figure 3. Model for the retina histogenic clock. A putative gradient of a growth factor (GF), such as Shh, regulates cell cycle length and the timing of activation of cell fate determining factors, thus defining the progressive change in competence of retinal progenitors. Ganglion cells, cones, and amacrine cells produce negative feedback signals that prevent the generation of the same cell type, thus contributing to the unidirectionality of the clock.

CONCLUSIONS

In vertebrates, the specification of retinal cell fate is regulated by a sequence of evolutionary conserved events that include the definition of an early retina territory, the generation, proliferation, and maintenance of multipotent retinal progenitors, and the establishment of a histogenic clock allowing the coordinated generation of all the retinal cell types.

The identification of several genes involved in each of these processes has revealed that retinal cell fate is based on coordinated interactions between transcription factors, cell cycle components, and signaling molecules. To be effective, these interactions must occur at the right time and place. In particular, the spatio-temporal expression of transcription factors is regulated not only at the transcriptional level but also through translational control, carried out by miRNAs and RNA binding proteins, as well as post-translational modifications. A possible mechanism for the histogenic clock could be based on a gradient of a growth factor, such as Shh, controlling the cell cycle length, which in turn promotes the sequential activation of transcription factors determining specific competence states and/or cell fates. Concomitantly, negative feedback signals produced by specific cell types and inhibiting the generation of the same kind of neurons, could contribute to the unidirectionality of the histogenic clock, thus driving progenitors from one competence state to the next (refer Fig. 3).

Substantial work is still required to define in detail the molecular components of the retinal clock. The knowledge gained through this work will certainly improve our understanding of inherited or age-related retinal diseases and will provide essential information

for regenerative medicine protocols aimed at directing stem cells towards specific retinal cell fates.

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UNTANGLING THE ErbB SIGNALLING NETWORK

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When epidermal growth factor and its relatives bind the ErbB family of receptors, they trigger a rich network of signalling pathways, culminating in responses ranging from cell division to death, motility to adhesion. The network is often dysregulated in cancer and lends credence to the mantra that molecular understanding yields clinical benefit: over 25,000 women with breast cancer have now been treated with trastuzumab (Herceptin®), a recombinant antibody designed to block the receptor ErbB2. Likewise, small-molecule enzyme inhibitors and monoclonal antibodies to ErbB1 are in advanced phases of clinical testing. What can this pathway teach us about translating basic science into clinical use?

MESENCHYME
Immature connective tissue that consists of cells embedded in extracellular matrix.

NEUREGULINS
EGF-like ligands whose primary receptor is ErbB3 and/or ErbB4. Four types of neuregulin are known.

STROMA
Supporting connective tissue in which a glandular or other epithelium is embedded.

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ErbBs are typical receptor tyrosine kinases that were implicated in cancer in the early 1980s when the avian erythroblastosis tumour virus was found to encode an aberrant form of the human epidermal growth factor (EGF) receptor (also known as ErbB1, HER or EGFR). Since then, the ErbB family has grown to four, and we are beginning to appreciate that the normal function of ErbBs and their ligands is to mediate cell–cell interactions in organogenesis and adulthood (reviewed in REF. 1).

In the epithelium, the basolateral location of ErbBs enables them to mediate signals between the MESENCHYME and the epithelium for cell growth[2]. The mesenchyme serves as a storehouse for many ligands including NEUREGULINS (NRGs), which bind ErbB3 and ErbB4. ErbB2 (also known as HER2) is a more potent oncoprotein than the other ErbBs, but no known ligand binds it with high affinity. It was first discovered as a rodent carcinogen-induced oncogene that encodes a variant of ErbB2 with a mutation that makes its tyrosine kinase constitutively active. ErbB2 is a shared co-receptor for several STROMAL ligands. Blocking the action of ErbB2 might thus inhibit a myriad of mitogenic pathways affecting ErbB-expressing tumour cells[3]. Although several strategies are being developed, Herceptin® — a HUMANIZED MONOCLONAL ANTIBODY to ErbB2 — has been the first to reach widespread clinical use, in particular for the treatment of metastatic breast cancer[4,5].

A layered signalling network

The components of the ErbB signalling pathway are evolutionarily ancient (BOX 1), and at first glance resemble a simple growth factor signalling pathway: ligand binding to a monomeric receptor tyrosine kinase activates the cytoplasmic catalytic function by promoting receptor dimerization and self-phosphorylation on tyrosine residues. The latter serve as docking sites for various ADAPTOR PROTEINS or enzymes, which simultaneously initiate many signalling cascades to produce a physiological outcome (FIG. 1). In higher eukaryotes, the simple linear pathway has evolved into a richly interactive, multilayered network, in which combinatorial expression and activation of components permits context-specific biological responses throughout development and adulthood.

The input layer. This comprises the ligands (EGF family of growth factors) and their receptors — the ErbBs (FIG. 1). All high-affinity ErbB ligands have an EGF-LIKE DOMAIN and three disulphide-bonded intramolecular loops. This receptor-binding domain is usually part of a large transmembrane precursor containing other structural motifs such as IMMUNOGLOBULIN-LIKE DOMAINS, heparin-binding sites and glycosylated linkers. Expression and processing of the precursor are highly regulated. For example, transformation by active Ras, or exposure to steroid hormones[6] leads to increased expres-

Box 1 | Evolution of the ErbB signalling network

Both the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster* have primordial linear versions of the ErbB signalling pathway. In higher organisms, this has evolved into a complex network, probably because an interconnected layered structure can confer selective gains in terms of adaptation, tolerance to mutations and signal diversification[91]. The main functional features of the ErbB module were defined in invertebrates. ErbB regulates the fate of diverse cell lineages in different developmental stages through short-range paracrine interactions.

C. elegans and *Drosophila* each contain a single ErbB homologue; however, the only EGF-like ligand of *C. elegans*, called Lin-3, is replaced by four ligands in *Drosophila*. Vulva development is a well-characterized function of the Lin-3 signalling pathway: the six vulva precursor cells (VPCs) respond to an inductive signal from a gonadal anchor cell, which is thought to secrete Lin-3. Lin-3 binds the juxtaposed receptor on one of the VPCs and instructs it to undergo several cell cycles and develop concomitantly a more differentiated phenotype. The Lin-3 pathway functions in other inductive morphogenic events; loss-of-function mutations in the receptor result not only in a vulvaless phenotype, but also in sterility, abnormal male tail development and death[92].

The *Drosophila* EGF receptor (DER) is used repeatedly in several stages of development, including oogenesis, embryogenesis, and wing and eye development. Likewise, differentiation of the DER-expressing tendon cell is regulated by the myotube-derived NRG-like ligand, Vein[93]. Gurken, a homologue of transforming growth factor-α (TGF-α), functions primarily in the oocyte. Activation of another ligand, Spitz, which is anchored to the cell surface, requires proteolytic cleavage[94]. By contrast, Argos, a secreted DER ligand, is unique in that it negatively acts on receptor signalling[95].

sion of several ErbB ligands, and cleavage of ligand precursors by a METALLOPROTEINASE can be stimulated by activation of other receptors, such as G-protein-coupled receptors[7] (FIG. 2).

An important issue relates to the multiplicity and possible redundancy of ErbB ligands. This issue is particularly relevant to the many NRGs and their splice variants. Studies in cultured cells and initial attempts to address this issue in animals suggest that ErbB ligands have non-overlapping functions. For example, ligands such as EGF and NRG4, which bind to ErbB1 and ErbB4, respectively, have narrow specificity, whereas others such as epiregulin, NRG1β and betacellulin bind to two distinct primary receptors[5]. Overexpression of ErbB2, which biases heterodimer formation, can broaden ligand specificity (FIG. 1, dotted lines), and ligands that are better at recruiting this co-receptor can reduce the binding of less effective ligands. In addition, splice variants of NRGs and various ligand–receptor complexes also differ in their ability to recruit a partner receptor[8–11], which affects their potency and kinetics of signalling.

The four ErbBs share an overall structure of two cysteine-rich regions in their extracellular region, and a kinase domain flanked by a carboxy-terminal tail with tyrosine autophosphorylation sites. With few exceptions (for example, haematopoietic cells), ErbB proteins are expressed in cells of MESODERMAL and ECTODERMAL origins.

Examination of the intracellular and extracellular domains of the ErbBs provides a satisfying explanation as to why a horizontal network of interactions is crucial to the ErbB signalling pathway. ErbB3 is devoid of intrinsic kinase activity[12], whereas ErbB2 seems to have no direct ligand[13]. Therefore, in isolation neither ErbB2 nor ErbB3 can support linear signalling (FIG. 5). Most inter-receptor interactions are mediated by ligands, and ErbB2-containing heterodimers are formed preferentially[14,15]. Nevertheless, overexpression of a specific receptor can bias dimer formation, especially in the case of ErbB2, whose homodimers can spontaneously form in ErbB2-overexpressing cells. Many cancers of epithelial origin have an amplification of the ErbB2 gene, which pushes the equilibrium towards ErbB2 homodimer and heterodimer formation. By contrast, ErbB4, whose expression pattern is relatively limited, has several isoforms that differ in their juxtamembrane and carboxyl termini, resulting in differences in the recruitment of phosphatidylinositol-3-OH kinase (PI(3)K)[16], which activates cell-survival pathways.

Signal-processing layers. The specificity and potency of intracellular signals are determined by positive and negative effectors of ErbB proteins, as well as by the identity of the ligand, oligomer composition and specific structural determinants of the receptors. The main determinant, however, is the vast array of phosphotyrosine-binding proteins that associate with the tail of each ErbB molecule after engagement into dimeric complexes (FIG. 1). Which sites are autophosphorylated, and hence which signalling proteins are engaged, are determined by the identity of the ligand as well as by the heterodimer partner[17]. The Ras- and Shc-activated mitogen-activated protein kinase (MAPK) pathway is an invariable target of all ErbB ligands, and the PI(3)K-activated Akt kinase and *p70S6K/p85S6K pathway* are downstream of most active ErbB dimers. The potency and kinetics of PI(3)K activation differ, however, probably because PI(3)K couples directly with ErbB3 and ErbB4, but indirectly with ErbB1 and ErbB2 (REF. 18).

Simultaneous activation of linear cascades, such as the MAPK pathway, the stress-activated protein kinase cascade, protein kinase C (PKC) and the Akt pathway translates in the nucleus into distinct transcriptional programmes. These involve not only the proto-oncogenes *fos*, *jun* and *myc*, but also a family of zinc-finger-containing transcription factors that includes Sp1 and Egr1, as well as Ets family members such as GA-binding protein (GABP)[19]. Despite sharing some pathways, each receptor is coupled with a distinct set of signalling proteins. For example, unlike ErbB1, the kinase-defective ErbB3 cannot interact with the adaptor protein and ubiquitin ligase c-Cbl, the adaptor protein Grb2, the second-messenger-generating enzyme phospholipase Cγ or the Ras-specific GTPase-activating protein (GAP)[20], but it can associate with the adaptors Shc and Grb7 (FIG. 1). In addition to combinatorial interactions, an important determinant of signalling outcome is variation in the kinetics of specific pathways. The principal process that turns off signalling by the ErbB network is ligand-mediated receptor endocytosis, and the kinetics of this process also depend heavily on receptor composition (BOX 3).

The output layer. The output of the ErbB network ranges from cell division and migration (both associated with tumorigenesis) to adhesion, differentiation and

HUMANIZED MONOCLONAL ANTIBODY
An antibody, usually from a rodent, engineered to contain mainly human sequences. This process reduces the immune response to the antibody in humans.

ADAPTOR PROTEINS
Proteins that augment cellular responses by co-ordinating other proteins in a complex. They usually contain several protein–protein interaction domains.

EGF-LIKE DOMAIN
A motif with ~50 amino acids, including six cysteine residues and a mainly β-sheet structure, found in all ErbB-binding growth factors and in extracellular matrix proteins.

IMMUNOGLOBULIN-LIKE DOMAIN
A protein domain composed of two β-pleated sheets held together by a disulphide bond.

METALLOPROTEINASES
Proteinases that have a metal ion at their active sites.

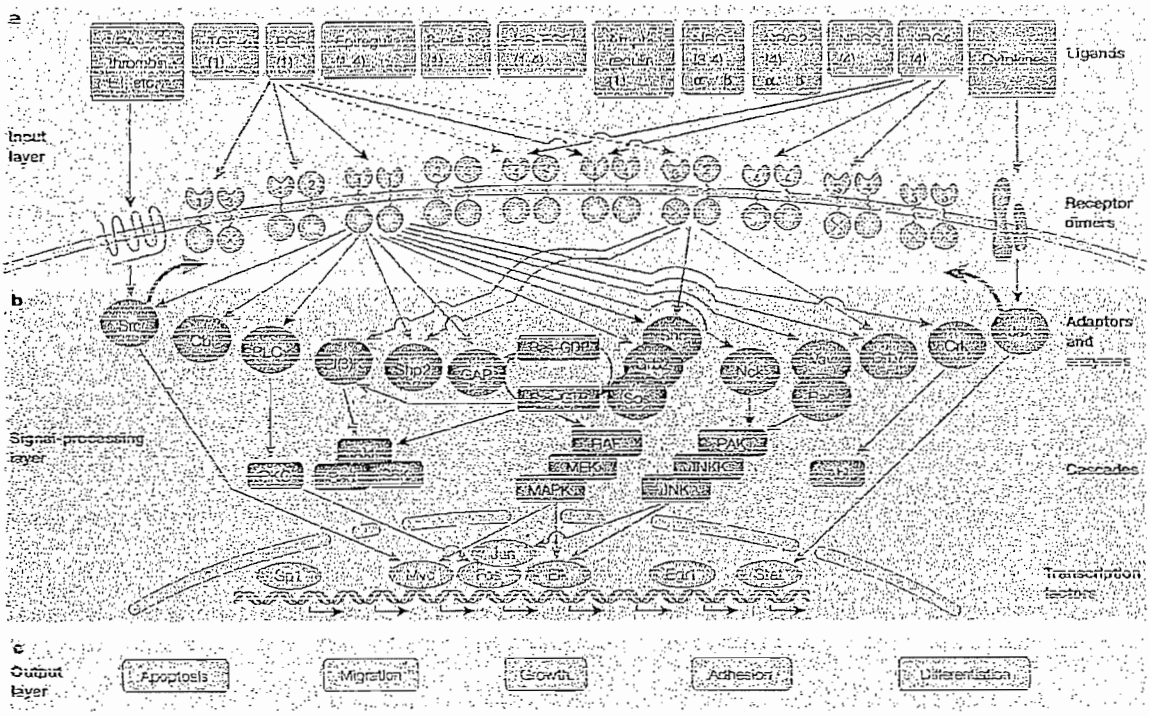


Figure 1 | **The ErbB signalling network. a** | Ligands and the ten dimeric receptor combinations comprise the input layer. Numbers in each ligand block indicate the respective high-affinity ErbB receptors[3]. For simplicity, specificities of receptor binding are shown only for epidermal growth factor (EGF) and neuregulin 4 (NRG4). ErbB2 binds no ligand with high affinity, and ErbB3 homodimers are catalytically inactive (crossed kinase domains). *Trans*-regulation by G-protein-coupled receptors (such as those for lysophosphatidic acid (LPA), thrombin and endothelin (ET)), and cytokine receptors is shown by wide arrows. **b** | Signaling to the adaptor/enzyme layer is shown only for two receptor dimers: the weakly mitogenic ErbB1 homodimer, and the relatively potent ErbB2–ErbB3 heterodimer. Only some of the pathways and transcription factors are represented in this layer. **c** | How they are translated to specific types of output is poorly understood at present. (Abl, a proto-oncogenic tyrosine kinase whose targets are poorly understood; Akt, a serine/threonine kinase that phosphorylates the anti-apoptotic protein Bad and the ribosomal S6 kinase (S6K); GAP, GTPase activating protein; HB-EGF, heparin-binding EGF; Jak, janus kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ; Shp2, Src homology domain-2-containing protein tyrosine phosphatase 2; Stat, signal transducer and activator of transcription; RAF–MEK–MAPK and PAK–JNKK–JNK, two cascades of serine/threonine kinases that regulate the activity of a number of transcription factors.)

MESODERM
The middle germ layer of the developing embryo. It gives rise to the musculoskeletal, vascular and urinogenital systems, and to connective tissue (including that of the dermis).

ECTODERM
The outermost germ layer of the developing embryo. It gives rise to the epidermis and the nerves.

AKT PATHWAY
Akt (or protein kinase B) is a serine/threonine protein kinase activated by the phosphatidylinositol-3-OH kinase pathway that activates survival responses.

apoptosis (FIG. 1). Output depends on cellular context, as well as the specific ligand and ErbB dimer. This has been best shown in terms of mitogenic and transforming responses: homodimeric receptor combinations are less mitogenic and transforming than the corresponding heterodimeric combinations, and ErbB2-containing heterodimers are the most potent complexes[21–23] (FIG. 3).

Perhaps the best example of the ability of the ErbB module to tune mitogenic signalling is provided by the ErbB2–ErbB3 heterodimer: although neither ErbB2 nor ErbB3 alone can be activated by ligand, the heterodimer is the most transforming[24,25] and mitogenic[21] receptor complex. The ErbB2–ErbB3 heterodimer also increases cell motility on stimulation with a ligand[26]; but the other NRG receptor, ErbB4, which exists in several isoforms, has been associated with processes varying from cellular chemotaxis[27] to proliferation and differentiation[28].

A network of networks?

The ErbB network might integrate not only its own inputs but also heterologous signals, including hormones, neurotransmitters, lymphokines and stress inducers[29] (FIG. 1). Many of these *trans*-regulatory interactions are mediated by protein kinases that directly phosphorylate ErbBs, thereby affecting their kinase activity or endocytic transport[29]. The most extensively studied mechanism involves activation of G-protein-coupled receptors (GPCRs) by agonists such as lysophosphatidic acid (LPA), carbachol (which specifically activates muscarinic acetylcholine receptors) or thrombin (FIG. 2).

Experiments done with mutants and inhibitors of ErbBs imply that the mitogenic activity of some GPCR agonists requires transactivation of ErbB proteins. These agents increase tyrosine phosphorylation of ErbB1 and ErbB2, either by increasing their intrinsic

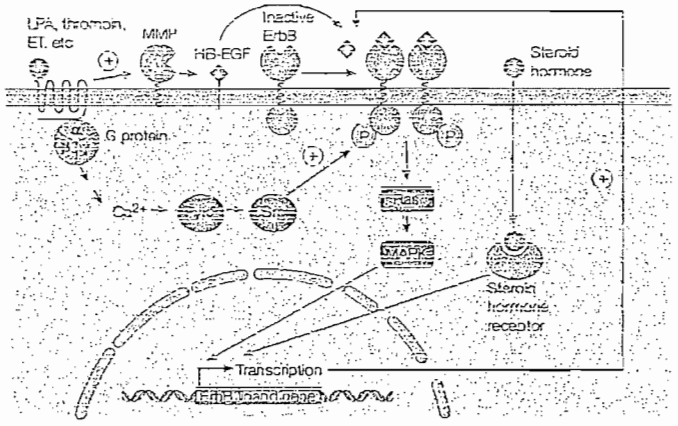


Figure 2 | **Crosstalk between the ErbB network and other signalling pathways.** G-protein-coupled receptors (GPCRs) such as those for lysophosphatidic acid (LPA), thrombin and endothelin (ET) can have positive effects on ErbB signalling through two mechanisms. First, through a poorly defined mechanism, they can activate matrix metalloproteinases (MMPs), which cleave membrane-tethered ErbB ligands (such as heparin-binding EGF-like factor, HB-EGF), thereby freeing them to bind to ErbBs. Second, GPCRs indirectly activate Src (perhaps via Pyk2), which phosphorylates the intracellular domains of ErbBs on tyrosine residues. Steroid hormones can have a positive effect on ErbB signalling by activating the transcription of genes encoding ErbB ligands. Finally, ErbB activation can activate a positive feedback loop through the Ras–MAPK (mitogen-activated protein kinase) pathway, which also activates transcription of ErbB ligand genes.

kinase activity[30] or by inhibiting an associated phosphatase activity. Signalling events downstream of ErbB1 are subsequently triggered, and this might account for the mitogenic potential of the heterologous agonists. Apparently, a cascade of tyrosine kinases links GPCRs such as the LPA receptor or the β adrenergic receptor to ErbB1 and subsequently to MAPK. The cascade culminates in the stimulation of Src family kinases[31], which are recruited by either the calcium-regulated tyrosine kinase Pyk2 (REF. 32) or a GPCR-coupled kinase and an adaptor protein (*for example, arrestin*[33]). Another kinase that phosphorylates ErbB1 is the cytokine-regulated tyrosine kinase Jak2: on stimulation of adipocytes by growth hormone, Jak2 phosphorylates ErbB1, thus allowing MAPK activation even by a kinase-defective mutant of ErbB1 (REFS 34,35).

Yet another cytokine, interleukin-6, elevates tyrosine phosphorylation of ErbB2 by increasing its intrinsic catalytic activity[36]. By contrast, factors that activate PKC, such as certain growth factors and hormones (for example, PDGF, LPA and EGF by itself), increase threonine and serine phosphorylation of ErbB1 and ErbB2, which decrease tyrosine phosphorylation and ligand binding affinity through a mechanism involving accelerated recycling of internalized receptors (BOX 2). These interconnections to other signalling modules help to integrate and coordinate cellular responses to extracellular stimuli.

Integrating developmental cues

The ErbB network is a key developmental signalling pathway throughout evolution. Its functions in worm and fly development are now well understood (BOX 1), but recent research using knockout and transgenic mice is beginning to clarify the functions of individual ErbBs and specific ligands in mammalian development.

ErbB1 and its ligands. Inactivation of ErbB1 impairs epithelial development in many organs, including those involved in tooth growth and eye opening[37–39]. Likewise, transgenic and *in vitro* studies implicate ErbB1 in promoting proliferation and differentiation of the epithelial component of skin, lung, pancreas and the gastrointestinal tract. These processes are probably regulated by growth factors from the local mesenchyme. Mice lacking expression of transforming growth factor-α (TGF-α) have abnormal skin, hair and eye development[40,41] but, in contrast with ErbB1 deficient mice, which undergo massive apoptosis in cortical and thalamic

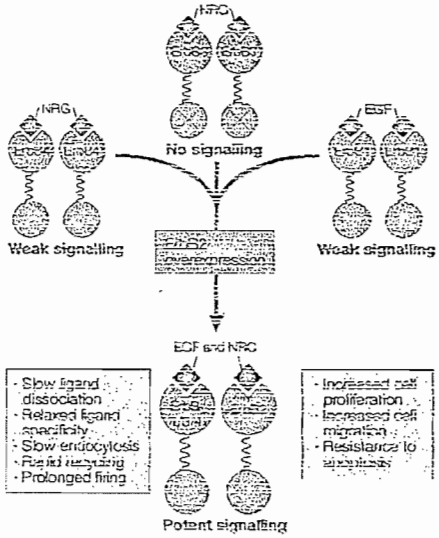


Figure 3 | **Signalling by ErbB homodimers in comparison with ErbB2-containing heterodimers.** Receptors are shown as two lobes connected by a transmembrane stretch. Binding of a ligand (EGF-like or NRG) to the extracellular lobe of ErbB1, ErbB3 (note inactive kinase, marked by a cross) or ErbB4 induces homodimer formation. When ErbB2 is overexpressed, heterodimers form preferentially. Unlike homodimers, which are either inactive (ErbB3 homodimers) or signal only weakly, ErbB2-containing heterodimers have attributes that prolong and enhance downstream signalling (green box) and their outputs (yellow box). Apparently, homodimers of ErbB2 are weaker signalling complexes than heterodimers containing ErbB2. (EGF, epidermal growth factor; NRG, neuregulin.)

STRESS-ACTIVATED PROTEIN KINASES
Member of the mitogen-activated protein kinase (MAPK) family that respond to stress. They include the Jun amino-terminal kinases (JNKs) and the p38 MAPKs.

UBIQUITIN LIGASES
Enzymes that catalyse the last stage of ubiquitylation, in which the small protein ubiquitin is transferred from a ubiquitin-conjugating enzyme (UBC or E2) to its target protein. They are also known as E3 enzymes.

GAPS
Proteins that inactivate small GTP-binding proteins, such as Ras family members, by increasing their rate of GTP hydrolysis.

Box 2 | Turning off the ErbB response

On ligand binding, ErbB1 molecules cluster over clathrin-coated regions of the plasma membrane, which invaginate to form endocytic vesicles. These mature to early and late endosomes, while gradually decreasing their internal pH and accumulating hydrolytic enzymes that lead to receptor degradation. Importantly, the other three ErbB proteins are endocytosis impaired and are more often recycled back to the cell surface[21,96]. Sorting to degradation is determined by the composition of the dimer: ErbB1 homodimers are targeted primarily to the lysosome; ErbB3 molecules are constitutively recycled[97]; and hetero-dimerization with ErbB2 decreases the rate of endocytosis and increases recycling of its partners[95,96]. Receptor internalization is determined by cytoplasmic motifs[100], but sorting in the early endosome seems to depend on the differential dissociation of ligand–ErbB complexes at mildly acidic pH. Complex dissociation leads to recycling, whereas continuous activation of tyrosine phosphorylation in the endosome leads to recruitment of c-Cbl, a ubiquitin ligase that preferentially binds to ErbB1 homodimers[101] and directs them to lysosomal degradation by tagging with polyubiquitin tracts[102].

brain regions[58], mice homozygous for a disrupted TGF-α gene show no brain abnormalities. So, the limited penetrance of TGF-α mutations and the confinement of the phenotype to the skin and eye suggest that each ErbB ligand has a distinct functional role and tissue specificity, analogous to the different roles played by each of the *Drosophila* EGF receptor ligands in insect development (BOX 2).

Neuregulins and their receptors. Like ErbB1 and its ligands involved in mesenchyme–epithelium interactions, the NRGs and their receptors are involved in the interaction between nerves and their target cells (for example, muscle, GLIA and SCHWANN CELLS), and are essential for cardiac and neural development. Mice defective in ErbB4, ErbB2 and NRG-1 die at embryonic day 10.5 from similar heart defects[1]. Endocardium-derived

Table 1 | **Expression of ErbBs and their ligands in cancer**

Molecule	Nature of dysregulation	Type of cancer	Notes	References
Ligands				
TGF-α	Overexpression	Prostate	Expressed by stroma in early, androgen-dependent prostate cancer and by tumours in advanced, androgen-independent cancer	52
	Overexpression	Pancreatic	Correlates with tumour size and decreased patient survival; may be due to overexpression of Ki-Ras, which also drives expression of HB-EGF and NRG1	108
	Overexpression	Lung, ovary, colon	Correlates with poor prognosis when co-expressed with ErbB1	51
NRG1	Overexpression	Mammary adeno-carcinomas	Necessary, but not sufficient for tumorigenesis in animal models	109
Receptors				
ErbB1	Overexpression	Head and neck, breast, bladder, prostate, kidney, non-small-cell lung cancer	Significant indicator for recurrence in operable breast tumours; associated with shorter disease-free and overall survival in advanced breast cancer; may serve as a prognostic marker for bladder, prostate, and non-small-cell lung cancers	110,111
	Overexpression	Glioma	Amplification occurs in 40% of gliomas; overexpression correlates with higher grade and reduced survival	35
	Mutation	Glioma, lung, ovary, breast	Deletion of part of the extracellular domain yields a constitutively active receptor	54
ErbB2	Overexpression	Breast, lung, pancreas, colon, oesophagus, endometrium, cervix	Overexpressed owing to gene amplification in 15–30% of invasive invasive ductal breast cancers. Overexpression correlates with tumour size, spread of the tumour to lymph nodes, high grade, high percentage of S-phase cells, aneuploidy and lack of steroid hormone receptors	56
ErbB3	Expression	Breast, colon, gastric, prostate, other carcinomas	Co-expression of ErbB2 with ErbB1 or ErbB3 in breast cancer improves predicting power	64,65
	Overexpression	Oral squamous cell cancer	Overexpression correlates with lymph node involvement and patient survival	112
ErbB4	Reduced expression	Breast, prostate	Correlates with a differentiated phenotype	66
	Expression	Childhood medullo-blastoma	Co-expression with ErbB2 has a prognostic value	67

(TGF-α, transforming growth factor-α; NRG1, neuregulin-1; HB-EGF, heparin-binding epidermal growth factor.)

GLIA
Supporting cells of the nervous system, including oligodendrocytes and astrocytes in the central nervous system, and Schwann cells in the peripheral nervous system. Glia surround neurons, providing mechanical and physical support, and electrical insulation between neurons.

SCHWANN CELLS
Cells that produce myelin and ensheath axons in the peripheral nervous system.

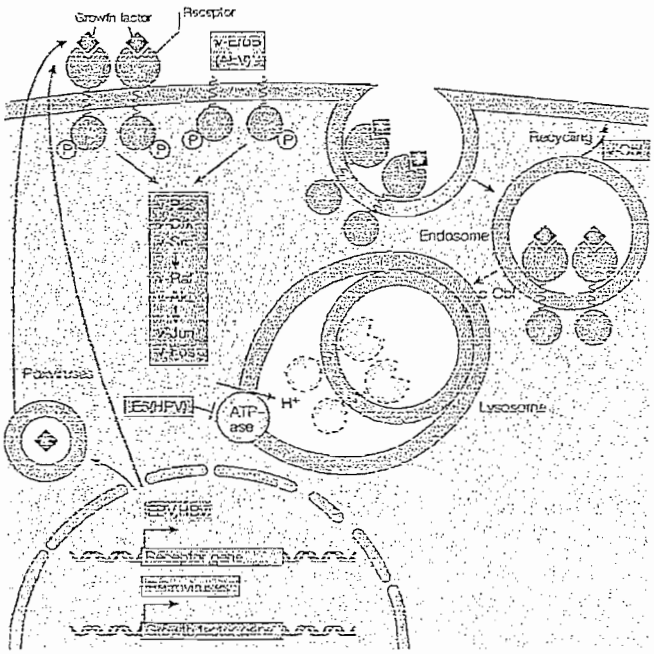
NRG1 stimulates an ErbB2–ErbB4 heterodimer on adjacent myocytes to initiate formation of the TRABECULAE. Surprisingly, the immunoglobulin domain and the cytoplasmic part of NRG1 — regions that are not involved in receptor binding — are essential for proper heart development[42,43]. ErbB3-deficient mice survive to embryonic day 13.5 and suffer from defective cardiac formation[44,45]. The alternative NRG-promoted heterodimer, ErbB2–ErbB3, is involved in different morphogenic events: mice lacking ErbB2, ErbB3 or NRG1 have a severely underdeveloped SYMPATHETIC GANGLION chain. This is probably caused by defective migration of neural progenitors from the NEURAL CREST[44].

The Schwann cell lineage is also controlled by the ErbB2–ErbB3 heterodimer. *In vitro* studies showed that NRG1 biases differentiation of neural crest progenitors towards a glial fate, and ErbB3-deficient mice showed partial lack of Schwann cells along peripheral and sensory neurons[45,46]. The ability of NRGs to control transcription of several ion channels underlies involvement of ErbBs in the neuromuscular junction[47]. NRGs elevate transcription of all subunits of the postsynaptic nicotinic acetylcholine receptor, but a nerve-derived splice variant seems to bias replacement of the γ-subunit with the ε-chain, which increases single-channel conductance. A similar subunit switch might occur at central synapses; NRG1β can markedly increase expression of the NR2C subunit of the *N*-methyl-D-aspartate receptor in slices of cerebellum[48].

The cancer connection

The potent cell proliferation signals generated by the ErbB network are used by cancer cells to fix oncogenic mutations by CLONAL EXPANSION. In addition, many types of oncogenic viruses exploit the ErbB network by manipulating its components (BOX 3). Human cancers use several mechanisms to activate the network at different layers. In many different cancer cell types, the ErbB pathway becomes hyperactivated by a range of mechanisms, including overproduction of ligands, overproduction of receptors, or constitutive activation of receptors (TABLE 1). It is extremely useful to know whether a

Box 3 | How do viruses harness the ErbB network?



Several transforming and non-transforming viruses constitutively elevate ErbB signalling by expressing an active component or by interfering with signalling shut-off. The hepatitis B virus (HBV), which is associated with hepatocellular carcinoma, upregulates transcription from the ErbB1 promoter[103]. Likewise, expression is deregulated by LMP1, a protein encoded by the Epstein–Barr virus (EBV), which is associated with several malignancies, including nasopharyngeal carcinoma[104]. Most members of the largest group of DNA viruses, poxviruses, encode EGF-like ligands, whose expression at sites of infection significantly increases pathogenicity[105]. RNA tumour viruses present the most divergent strategy to harness ErbB signalling: the avian erythroblastosis virus (AEV) encodes a truncated form of ErbB1 lacking most of the ectodomain and carrying many intracellular mutations. The oncoprotein v-ErbB forms ligand-independent covalent dimers at the cell surface[106]. Active mutants of various ErbB target proteins, including small GTP-binding proteins (v-Ras), adaptors (v-Crk), protein kinases (v-Src, v-Akt, v-Raf) and transcription factors (v-Jun, v-Fos), are encoded by oncogenes of different strains of retroviruses. In addition, the mouse Cas NS-1 retrovirus, which induces pre-B cell lymphomas and myeloid leukaemia, encodes a dominant active form of c-Cbl, a ubiquitin ligase that targets ErbB proteins to lysosomal degradation[107]. This interferes with receptor ubiquitylation and degradation, similar to the effect of E5, a product of the human papilloma virus (HPV) that inhibits ErbB1 degradation through inhibition of an endosomal proton-ATPase[107]. Both E5 and v-Cbl increase the rate of receptor recycling back to the cell surface.

TRABECULAE
Finger-like projections of cardiac muscle cells that form ridges in the ventricular wall.

SYMPATHETIC GANGLIA
Clusters of sympathetic neurons in which a glandular or other epithelium is embedded.

NEURAL CREST
A group of embryonic cells that separate from the embryonic neural plate and migrate, giving rise to the spinal and autonomic ganglia, peripheral glia, chromaffin cells, melanocytes and some haematopoietic cells.

CLONAL EXPANSION
Growth of a population of cells from a single precursor cell.

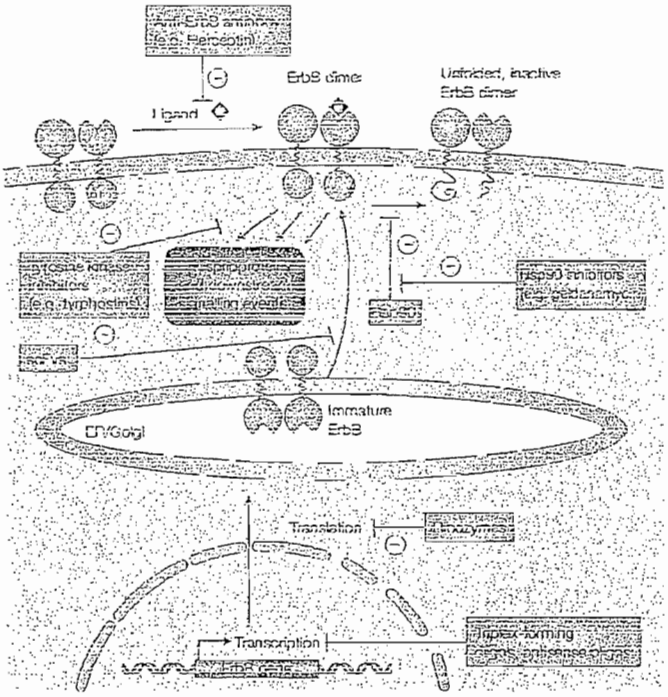


Figure 4 | **Therapeutic strategies for blocking the ErbB signalling network.** Anti-ErbB antibodies (such as Herceptin®, which binds ErbB2) block ligand binding and stimulate receptor internalization. Tyrosine kinase inhibitors such as tyrphostins block downstream signalling of the receptor–ligand complex, and Hsp90 inhibitors (for example, geldanamycin) prevent stabilization of ErbBs at the membrane. The active conformation of ErbB2 is maintained through interactions with a chaperone (Hsp90), and therefore chaperone antagonists inactivate the oncoprotein. It might also be possible to prevent ErbBs from reaching the cell surface, by blocking their transcription with triplex-forming oligonucleotides, their translation with antisense oligonucleotides or ribozymes, or their trafficking to the cell surface with intracellular single-chain Fv fragments of antibodies (scFvs). (ER, endoplasmic reticulum.)

CARCINOMA
A malignant tumour of epithelial origin.

PROGNOSIS
The likely outcome or course of a disease.

ANDROGEN-DEPENDENT PROSTATE CANCER
An early form of prostate cancer that is responsive to androgens and anti-androgen therapy.

AUTOCRINE
Activation of cellular receptors by ligands produced by the same cell.

GENE AMPLIFICATION
A differential increase in a specific portion of the genome. Amplification is associated with neoplastic transformation and acquisition of drug resistance.

particular tumour has an overactive ErbB pathway because of mutation, overexpression or amplification of a component of the ErbB pathway, as it can tell us what the patient's chance of survival is and with what drug they should be treated (FIG. 4).

Ligands. The relationship between ErbB ligand expression and tumorigenicity is complex: growth factors can be induced secondarily by a primary oncogene; either the stroma or the tumour can act as a ligand source; or the ligand can be expressed but unprocessed or sequestered in an inactive form[48].

Of all the ErbB ligands, the relevance of TGF-α to human cancer is best characterized. TGF-α and ErbB1 are co-expressed in several types of CARCINOMAS[36], and expression of TGF-α, particularly in lung, ovary and colon tumours co-expressing ErbB1, correlates with poor PROGNOSIS (reviewed in REF. 51). In prostate cancer, the pattern of expression of TGF-α seems to change as the disease progresses[52]. In early, ANDROGEN-DEPENDENT PROSTATE CANCER, TGF-α is expressed primarily in the tumour stroma, which suggests paracrine signalling. In advanced, androgen-independent disease, TGF-α is expressed by the tumour cells themselves, indicating AUTOCRINE signalling. Less information is available on other ligands (TABLE 1).

ErbB1. Both overexpression and structural alterations of ErbB1 are frequent in human malignancies. However, *in vitro* studies suggest that overexpression of the normal receptor leads to transformation only in the presence of a ligand. Accordingly, expression of EGF-like ligands often accompanies ErbB1 overexpression in primary tumours. Overexpression of ErbB1 is a very frequent genetic alteration in brain tumours; amplification of the gene occurs in 40% of gliomas[53]. Overexpression is associated with higher grade, higher proliferation and reduced survival. In a significant proportion of tumours, GENE AMPLIFICATION is accompanied by rearrangements. The most common mutation (type III) deletes part of the extracellular domain[35], yielding a constitutively active receptor. Recent studies identified an identical alteration in carcinomas of the lung, ovary and breast, suggesting broader implications to human cancer[54].

ErbB2. Several types of cancers overexpress ErbB2 (reviewed in REF. 36). The association of ErbB2 expression with cancer is best studied in breast cancer, where protein is overexpressed owing to gene amplification in 15–30% of invasive DUCTAL BREAST CANCERS[55]. Overexpression correlates with tumour size, spread of the tumour to lymph nodes, high grade, high percentage of S-phase cells, ANEUPLOIDY and lack of steroid hormone receptors, implying that ErbB2 confers a strong proliferative advantage to tumour cells[56,57]. Paradoxically, a higher degree of ErbB2 overexpression is reported in early forms of breast cancer relative to more advanced invasive carcinomas, suggesting that alterations in ErbB2 alone are insufficient for breast tumour progression from a relatively benign to a more malignant phenotype[58].

The identification of ErbB2 amplifications by FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH; FIG. 5) has now been approved by the US Food and Drug Administration to pinpoint patients at high risk for recurrence and disease-related death with node-negative invasive breast cancer[56,58]. Efforts are also being made to correlate ErbB2 status with predictive value — in other words, do patients with ErbB2 amplifications benefit from particular types of therapy? Again FISH technology can identify patients who might benefit from more aggressive therapy[59]. Several studies have shown that ErbB2 overexpression is associated with resistance to anti-oestrogen therapy[60]. Most ErbB2-overexpressing tumours do not express the oestrogen and progesterone receptors, indicating inverse relationships between the steroid hormone axis and the ErbB network.

Clinically, this crosstalk might be critical: patients treated with an anti-oestrogen drug were found to have a worse outcome if their tumours overexpressed ErbB2

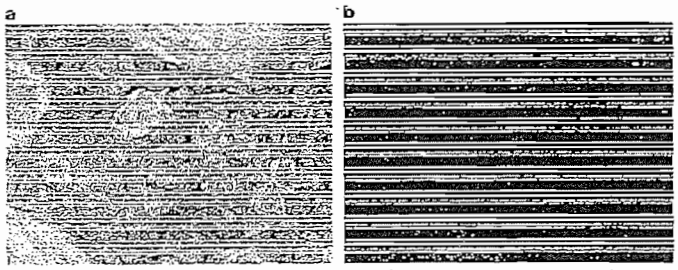


Figure 5 | **Molecular diagnosis of breast cancer. a** | Immunohistochemistry and **b** | fluorescence *in situ* hybridization (FISH) analysis of ErbB2 in human breast cancer. Immunohistochemistry was performed using HercepTest and FISH using a PathVysion ErbB2 DNA probe kit. The ErbB2 gene is seen as red fluorescence and the chromosome-17 centromeric α-satellite probe as green fluorescence. (Image courtesy of D. Eberhard, E. Huntzicker and R. Wright, Genentech, Inc.)

DUCTAL BREAST CANCER
Cancer arising from the lining of the milk ducts, as opposed to the lobules of the breast (lobular breast cancer).

ANEUPLOIDY
An abnormal number of chromosomes caused by their inaccurate segregation during cell division.

FLUORESCENCE *IN SITU* HYBRIDIZATION
Visualizing a genetic marker on a chromosome by using a fluorescently labelled polynucleotide probe that hybridizes to a gene on a chromosome during metaphase.

FARNESYLTRANSFERASE INHIBITORS
Inhibitors that block the activity of Ras by preventing the addition of a farnesyl group that targets it to the plasma membrane.

TYROSTINS
A type of tyrosine kinase inhibitor.

(REF. 61). On the one hand, *in vitro* studies indicate that overexpression of ErbB2 or NRG confers resistance to anti-oestrogens and renders cancer cells independent of oestrogen[62]. On the other hand, oestrogen suppresses transcription from the ErbB2 promoter, and specifically inhibits growth of ErbB2 overexpressing mammary cells[63]. Taken together, the molecular and clinical observations imply that the steroid and ErbB pathways are alternative, but functionally linked pathways that enhance cell proliferation (FIG. 2).

Neuregulin receptors. The catalytically inactive member of the ErbB family, ErbB3, is expressed in several cancers, but there is no evidence for gene amplification and overexpression is limited. However, a large recent study found that co-expression of ErbB2 with ErbB1 or ErbB3 in oral squamous-cell carcinoma was significant and it critically improved the predicting power[64], consistent with the non-autonomous role of ErbB3. Similarly, analysis of prostate cancer suggests the existence of a paracrine loop involving NRG1 and the ErbB2–ErbB3 heterodimer[65]. Some studies observed lower expression of ErbB4 in breast and prostate tumours relative to normal tissues, and an association with a relatively differentiated histological phenotype[66]. By contrast with epithelial tumours, childhood medulloblastomas often express ErbB4, whose co-expression with ErbB2 has a prognostic value[67], in line with the importance of receptor heterodimerization.

The network as a target for cancer therapy

The central role of the ErbB network in the development of solid tumours, its availability to extracellular manipulation, and detailed understanding of the underlying biochemistry have made the ErbB network an attractive target for pharmacological intervention (FIG. 4). Most efforts have concentrated on ErbB2 and ErbB1 owing to their increased expression in certain tumour cells relative to normal cells.

Immunological strategies. One approach — a humanized antibody to ErbB2 called Herceptin® — has been approved for clinical use, both alone and in combination with chemotherapeutic agents. In addition to downregulating surface ErbB2, Herceptin induces the cyclin-dependent kinase inhibitor $p27^{kip1}$ and the Rb-related protein p130, which reduce the number of cells in S phase[68]. The recruitment and activation of immune effector cells to the ErbB2-overexpressing tumour might also contribute to Herceptin's mechanism of action[69].

Alternative approaches to the use of naked monoclonal antibodies to ErbBs include making antibodies toxic to cancer cells by linking them to radionuclides, toxins or prodrugs. Active immunization with portions of ErbB2 is another promising approach[70]. Monoclonal antibodies directed to a mutant form of ErbB1 (EGFRvIII) found in gliomas and carcinomas inhibit brain tumours in a manner dependent on the Fc receptor[71]. Comparison of two tumour-inhibitory monoclonal antibodies to ErbB1 revealed that only one depends on immune mechanisms; the other acts primarily by altering receptor functions. The chimeric version of this antibody, C225, competes with ligand binding to ErbB1 and arrests cultured cells at G1 because of an elevation in $p27^{kip1}$ (REF. 72). This therapeutic antibody is now in late stage clinical testing in patients with colorectal or head and neck cancers.

Low molecular weight inhibitors. The discovery of naturally occurring compounds capable of inhibiting the ErbB network (for example, herbimycin, genistein and emodin) led to the synthesis of analogues specific to the nucleotide-binding sites of ErbB proteins or their putative chaperones, the 90-kDa heat-shock proteins (Hsp90)[73]. The chaperone might escort ErbB proteins from the endoplasmic reticulum to the plasma membrane, where it might stabilize the active conformation of the kinase. The crystal structures of related kinases were used to enhance selectivity of synthetic tyrosine kinase inhibitors to ErbBs[74].

Both reversible and irreversible inhibitors[74] capable of discriminating between ErbBs and other kinases have been developed. When applied *in vitro* and in animal models, the compounds variably inhibited cell growth with some specificity for ErbB1- and ErbB2-expressing cells. At least five of these compounds are now being tested in human clinical studies. Because some studies indicated that Ras and Src are essential for transformation by ErbB proteins, FARNESYL TRANSFERASE INHIBITORS, Src-specific TYROSTINS, MAPK inhibitors and Akt inhibitors might also be therapeutically effective in containing activated ErbB pathways[75].

Gene therapy. Strategies aimed at blocking transcription, translation or maturation of ErbB transcripts or proteins are candidates for gene therapy. Early studies have shown that the adenovirus type 5 early region 1A (E1A) gene product can block ErbB2 overexpression and suppress the tumorigenic potential of ErbB2-overexpressing ovarian cancer cells[76]. This method is now being tested in a phase I trial with ovarian cancer patients. Intracellular single chain antibodies (scFvs) directed to either ErbB1 or ErbB2 can effectively inhibit

receptor transfer from the endoplasmic reticulum to the plasma membrane, and thereby reduce signalling[77].

A human protocol for the treatment of ErbB2-positive ovarian cancer with scFvs has been developed following demonstration of selectivity and phenotypic effects *in vitro*[78]. Triplex-forming oligonucleotides that bind to a purine-rich sequence in the ErbB2 promoter are potent and specific inhibitors of ErbB2 transcription in an *in vitro* assay[79]. Antisense oligonucleotides[80], various dominant-negative mutants of ErbBs[81] and specific ribozymes[82] show specificity and efficacy in blocking receptor expression in cultured cells, and therefore might also prove useful as therapeutic lead compounds.

Perspectives

Successful treatments have been or are being developed to target aberrant ErbB receptor signalling in cancer; however, the potential for exploiting this pathway is still in its infancy. Antagonizing ErbB signalling might be a useful strategy for treating proliferative diseases other than cancer. One such opportunity might be coronary atherosclerosis. The migration of vascular smooth muscle cells in the arterial intima contributes to this cardiovascular disorder, particularly restenosis. Activation of the thrombin receptor is required for smooth muscle cell migration and proliferation, and activation of this G-protein-coupled receptor depends on transactivation by ErbB1 in response to heparin-binding EGF. Blockade of ErbB1 activation might therefore aid in the treatment of this disorder[83].

Another opportunity for intervention by targeted ErbB therapy might be psoriasis[84]. In normal skin, ErbB1 expression is restricted to the basal layer whereas in psoriatic skin, ErbB1 and one of its ligands, amphiregulin, are highly expressed throughout the entire epidermal layer[85]. Inhibition of ErbB1 activation might help control the spread or recurrence of psoriatic lesions.

In contrast to inhibiting ErbB signalling, potential also exists for activating the pathway in clinically meaningful ways. For example, ErbB ligands might promote wound healing[86]. ErbB signalling is also involved in fetal lung development, and appropriate activation of these pathways might benefit premature infants[87]. Neuregulins, which are also known as glial growth factors, are potent mitogens for Schwann cells[88]. Activation of Schwann cells with NRG might help resolve peripheral nerve injuries or neuropathies[89].

In summary, the ErbB field has made significant strides since Stanley Cohen's initial observation that EGF induces precocious eyelid opening in neonatal mice[90]. Although many of the individual molecules involved in ErbB signalling have been characterized, a full understanding of how the network functions in homeostasis — or malfunctions in a number of diseases — requires further definition. Regardless, the interface between basic and translational science has been established, and exploiting the ErbB pathway will probably yield other meaningful advances in the very near future.

Links

DATABASE LINKS ErbB1 | NRGs | ErbB3 | EGF | epiregulin | NRG1β | betacellulin | PI(3)K | Shc | p70S6K | PKC | Akt | Grb7 | *fos* | *jun* | *myc* | zinc finger | Sp1 | Egr1 | GABP | Grb2 | phospholipase Cγ | Src | Pyk2 | arrestin | Jak2 | interleukin-6 | TGF-α | *Drosophila* EGF receptor | NRG1 | Herceptin | $p27^{Kip1}$ | Rb | p130 | C225 | Hsp90 | amphiregulin | Vein | Gurken | Spitz | Argos

FURTHER INFORMATION The tumour gene database

ENCYCLOPEDIA OF LIFE SCIENCES *C. elegans* vulval induction | *Drosophila* embryo: dorsal–ventral specification

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Review

Neuregulins: Versatile growth and differentiation factors in nervous system development and human disease

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ABSTRACT

The neuregulins are a family of growth and differentiation factors with a wide range of functions in the nervous system. The power and diversity of the neuregulin signaling system comes in part from a large number of alternatively-spliced forms of the NRG1 gene that can produce both soluble and membrane-bound forms. The soluble forms of neuregulin are unique from other factors in that they have a structurally distinct heparin-binding domain that targets and potentiates its actions. In addition, a finely tuned, bidirectional mechanism regulates when and where neuregulin is released from neurons in response to neurotrophic factors produced by both neuronal targets and supporting glial cells. Together, this produces a balanced intercellular signaling system that can be localized to distinct regions for both normal development and maintenance of the mature nervous system. Recent evidence suggests that neuregulin signaling plays important roles in many neurological disorders including multiple sclerosis, traumatic brain and spinal cord injury, peripheral neuropathy, and schizophrenia. Here, we review the basic biology of neuregulins and relate this to research suggesting their involvement with and potential therapeutic uses for neurological disorders.

Contents

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1. Introduction and overview

Nowhere is the correct spatial and functional organization of multiple cell types more critical and complex than in the nervous system. During nervous system development, this precise complexity is achieved through ongoing cell–cell interactions that are mediated in part by specific growth and differentiation factors. One such factor called neuregulin-1 (NRG1) is essential for normal nervous system development, as well as maintaining normal function in the mature nervous system (Falls, 2003). The neuregulins are a large family of growth and differentiation factors with important functions in heart and nervous system development and in a number of malignancies (Gilmour et al., 2002; Hansen and Linthicum, 2004; Pinkas-Kramarski et al., 1994; Raabe et al., 2004; Zhao et al., 1998). Since its discovery over 10 years ago, NRG1 has been found to participate at all levels of nervous system development from the end of peripheral nerves at the neuromuscular junction, to the spinal cord, brainstem and neocortex.

NRG1 is uniquely qualified to carry out these diverse roles in development through both its pattern of expression together with its extensive repertoire of alternatively spliced forms that serve to target NRG1 to specific cellular regions at specific stages of development. It is therefore not surprising that NRG1 has become a central focus both for the pathogenesis and treatments of a wide variety of human diseases. Here, we will review the structural and functional aspects of NRG1 that enable it to carry out its important roles in nervous system development, working our way from the peripheral to the central nervous system. For both peripheral nervous system (PNS) and central nervous system (CNS) regions, shown in Fig. 1, we will discuss what is known about NRG1 signaling and the potential impact of that signaling in human disease.

2. Alternative splicing determines NRG1 localization and function

At least 4 distinct genes make up the family of neuregulin signaling factors (Carraway et al., 1997; Chang et al., 1997; Fischbach and Rosen, 1997; Harari et al., 1999; Ishiguro et al., 1998; Zhang et al., 1997). Most research interests have focused on the NRG1 gene, in part because this was the first neuregulin gene discovered, and also because of its known importance in normal development. Therefore, this review will continue that focus.

Within the NRG1 gene, at least 16 different structurally distinct gene products are encoded through a combination of

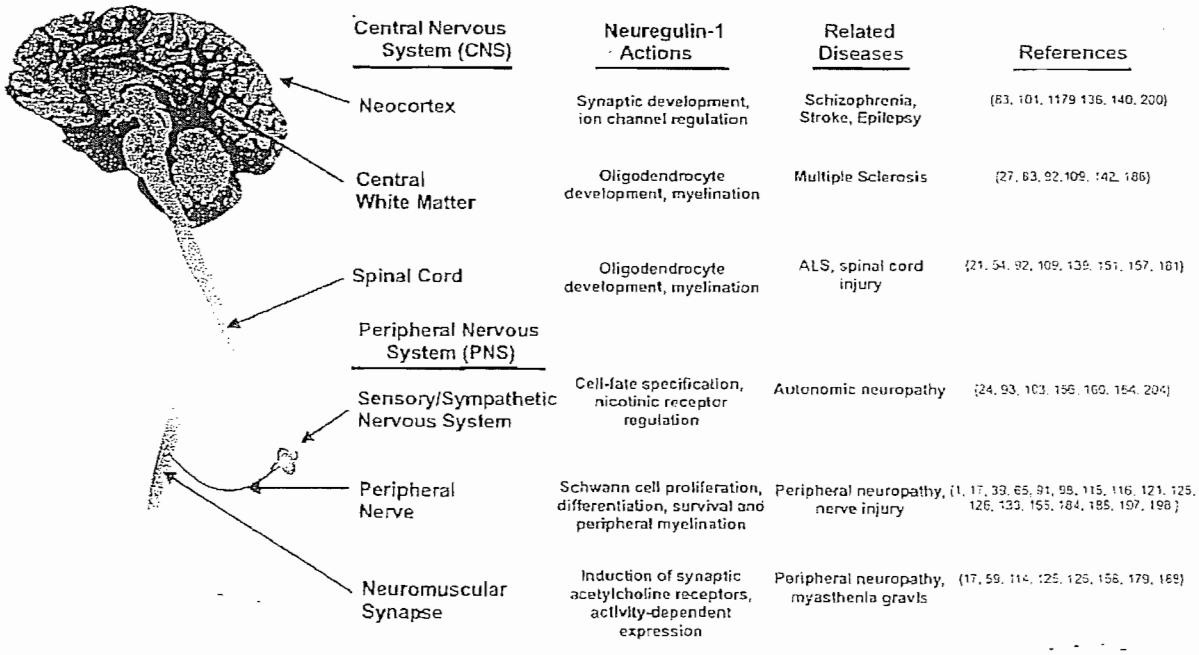

Fig. 1 – Sites of NRG1 action throughout the nervous system. NRG1 is produced in neurons of both the CNS and PNS. Shown here are known sites of NRG1 activity and the diseases that relate to those areas that could be impacted by NRG1 actions.

multiple promoter usage and alternative-splicing (Falls, 2003; Steinthorsdottir et al., 2004). In addition, an extensive structural analysis of the *NRG1* gene has recently predicted six novel alternative 5′-exons for the *NRG1* gene, that could yield at least 3 additional classes of NRG1 proteins (Steinthorsdottir et al., 2004). Historically, many *NRG1* alternatively spliced forms were discovered independently based on their biological activities and were given unique names before it was realized that they were all derived from the same gene. These include neu differentiation factor (NDF), heregulin, acetylcholine receptor inducing activity (ARIA), and glial growth factor (GGF). While NDF and heregulin were purified based on their growth and differentiation activities in breast epithelial cells, ARIA and GGF were purified based on their roles in neuromuscular and Schwann cell development, respectively (Corfas et al., 1993; Falls et al., 1993; Holmes et al., 1992; Marchionni et al., 1993; Wen et al., 1992, 1994).

Currently, these forms are loosely organized into three types based on significant structural and functional differences in their NH_2-terminal regions (Fig. 2). These proteins all share a unique epidermal growth factor (EGF)-like domain needed to activate its receptors. Type I forms have an extracellular heparin-binding, immunoglobulin-like domain (HBD); type II NRGs also have this heparin-binding domain plus a kringle domain, and type III NRGs have a cysteine-rich domain (CRD) on their NH_2-terminus. This latter form has also been referred to in the literature as sensory and motor neuron-derived factor (SMDF) (Ho et al., 1995). As shown in Fig. 2, most NRG1 forms are first expressed as transmembrane precursors often called "proNRG" that undergo a regulated cleavage, however, efficient release of soluble NRG1 is achieved only by the Type I/II forms (Wang et al., 2001). The inefficient release of type III isoforms is likely the result of the highly hydrophobic CRD domain that remains tethered in the membrane even after proteolytic processing. Type III forms

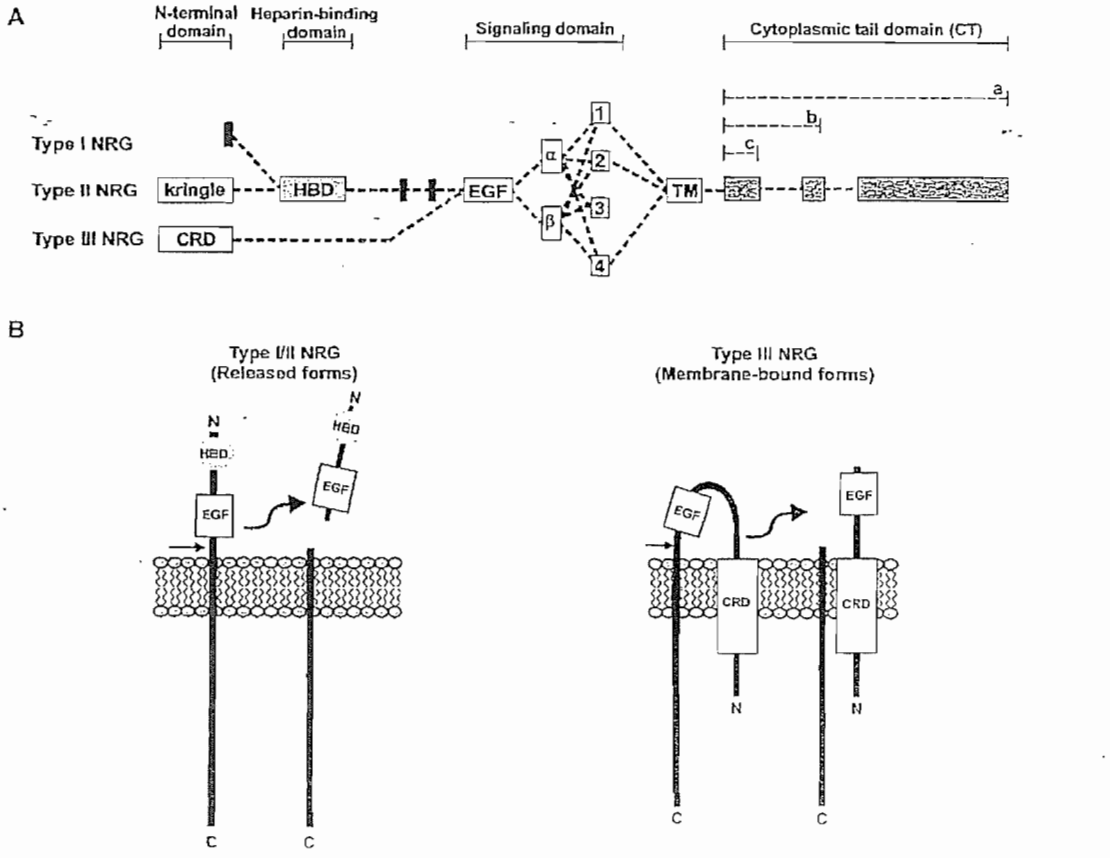

Fig. 2 – Alternative splicing of the NRG1 gene yields distinct isoforms. (A) The NRG1 gene produces at least 15 different proteins through alternative splicing through the use of multiple promoters. There are three major types of NRG1 proteins (I, II, III) distinguished by the N-terminal domain. All forms of NRG1 share a common EGF-like domain that is necessary and sufficient for signaling. Most forms of NRG1 are synthesized as transmembrane (TM) precursors with one of three possible cytoplasmic tails (CT), a, b, and c. (B) The Type I and II forms of NRG1 contain a heparin-binding domain (HBD) near their N-terminus, which functions to target NRG1 activity through interactions with HSPGs. After proteolytic cleavage and release of the ectodomain, these forms of NRG1 signal to nearby cells in a paracrine manner. The Type III forms of NRG1 have a cysteine-rich domain (CRD) near their N-terminus, which loops back into the plasma membrane to function as a transmembrane anchor. Following proteolytic cleavage, this form of NRG1 signals to adjacent cells in a juxtacrine manner.

are therefore thought to work through direct cell–cell or juxtacrine interactions from closely appositioned cells (Cabedo et al., 2002; Falls, 2003; Schroering and Carey, 1998; Wang et al., 2001). There is also evidence that membrane bound forms of NRG1 may be bi-directional signaling molecules, mediated through proteolytic cleavage and translocation of the intracellular domain of NRG1 to the nucleus (Bao et al., 2003).

Once released, localization of heparin-binding Type I/II forms are restricted through interactions with cell-surface and extracellular matrix heparan-sulfate proteoglycans (HSPGs) (Li and Loeb, 2001; Li et al., 2004a,b; Loeb, 2003; Loeb and Fischbach, 1995; Loeb et al., 1999; Meier et al., 1998). Negatively charged sulfate groups located on various positions of the HSPGs encode an enormous degree of structural diversity that is highly coordinated during development through the expression of synthetic enzymes (Nogami et al., 2004). A hierarchy of importance for each sulfate group mediating NRG1 binding has been demonstrated in vitro, and suggests the regulation of HSPG sulfation may be an important biological mechanism regulating the localization of NRG1 with HSPGs along central and peripheral axons and at neuromuscular synapses (Pankonin et al., 2005). HSPG interactions also potentiate NRG1 signaling through concentrating NRG1 at the synapse, and provide a sustained signal critical for up-regulating acetylcholine receptor genes (Li and Loeb, 2001) (Fig. 3).

Both distinct and overlapping functions of Type I/II and III forms of NRG1 proteins have been elucidated in mice with targeted deletions of specific NRG1 isoforms or their receptors. These studies demonstrate the critical importance of both forms for nervous system development and will be discussed below in the context of each anatomic region (Corfas et al., 1995; Dong et al., 1995; Falls, 2003; Falls et al., 1990; Gassmann et al., 1995; Jo et al., 1995; Lee et al., 1995; Morrissey et al., 1995a,b; Trachtenberg and Thompson, 1996; Wen et al., 1994).

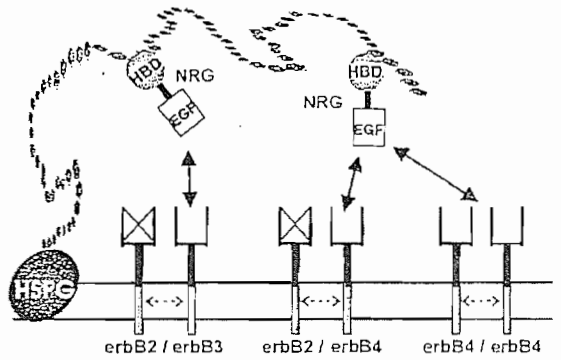

Fig. 3 – NRG1 signaling through erbB receptors is mediated by HSPG interactions. Once released from the neuron, NRG1 interacts with HSPGs in the extracellular matrix through its heparan-binding domain (HBD). This serves to target NRG1 to specific sites of action. NRG1 signals by binding and activating members of the erbB family of receptor tyrosine kinases. NRG1 can bind to either erbB3 or erbB4. ErbB2 can form heterodimers with either erbB3 or erbB4 to transduce NRG signals, but cannot bind NRG1 directly. ErbB3 has a non-functional kinase domain and must form a heterodimer with either erbB2 or erbB4 to transduce NRG1 signals.

3. Regulation of NRG1 signaling between cells through reciprocal neurotrophic factor interactions

The most common feature of all known NRG1 forms is an EGF-like domain that is necessary and sufficient for binding to and activating receptors belonging to the EGF family of receptor tyrosine kinases erbB2, erbB3, and erbB4 (see Fig. 3). These receptors are also referred to as Neu/HER2 (erbB2), HER3 (erbB3), and HER4 (erbB4). NRG1 does not bind the prototype EGF receptor (erbB1). NRG1 binds to either erbB3 or erbB4 after which it can produce erbB3/erbB2 or erbB4/erbB2 heterodimers or erbB4 homo-dimers all capable of generating active signaling complexes (Lee et al., 1995; Pinkas-Kramarski et al., 1996a,b; Riese et al., 1995). This complex receptor signaling system has been extensively studied and reviewed elsewhere (Riese and Stern, 1998). While a number of other soluble ligands also bind to erbB4 including HB-EGF and amphiregulin, NRG1 and NRG2 are the only known ligands for erbB3 (Hobbs et al., 2002; Schroering and Carey, 1998). Receptor dimerization leads to activation of an intracellular kinase domain present on erbB2 and erbB4 that phosphorylates specific tyrosine residues within the cytoplasmic tail of the receptor. This, in turn, leads to the activation of signaling pathways that include the mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI-3K) pathways (Bagossi et al., 2005; Ferguson, 2004; Gerecke et al., 2001; Holbro and Hynes, 2004; Loeb and Fischbach, 1995). This complex signaling network provides an enormous potential for diversification of biological effects mediated by NRG1.

The NRG1-erbB ligand–receptor signaling system is perfectly positioned for important signaling interactions in the normal PNS. NRG1 is highly expressed in spinal motor neurons and sensory ganglia neurons shortly after their birth and is efficiently transported down their axons to reach their synaptic targets (Loeb et al., 1999). ErbB receptors are highly expressed in those same targets that include muscle, skin, and Schwann cells that support and myelinate those axons (Calaora et al., 2001; Gerecke et al., 2001; Moscoso et al., 1995; Negro et al., 2004; Pinkas-Kramarski et al., 1997; Zhu et al., 1995). The erbB receptors are also expressed in the CNS in oligodendrocytes, where NRG1 from a variety of neuronal populations promotes their development (Cannella et al., 1999; Canoll et al., 1999; Park et al., 2001a,b; Vartanian et al., 1997, 1999). Thus, based on the expression of NRG1 in neurons and NRG1's receptors in cells that contact those neurons in both the peripheral and central nervous systems, NRG1 is poised to be an important communicator both for neuron–neuron and neuron–glial interactions during development and in the mature nervous system.

The simple apposition of NRG1-expressing neurons with erbB receptor-expressing cells does not guarantee the type of dynamic, two-way communication needed for development and maintenance of the nervous system. Recent work in our laboratory has uncovered a higher level of reciprocal communication between neuronal NRG1 and target-derived neurotrophic factors. Neurotrophic factors were initially identified

based on their ability to support the survival of peripheral and central neurons both during development and after injury (Houenou et al., 1996; Novak et al., 2000; Oppenheim et al., 2000; Risling et al., 1993; Yan et al., 1992, 1993). The first neurotrophic factor identified was nerve growth factor (NGF) followed by its other family members brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Other target-derived neurotrophic factors include glial cell line-derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF), and cardiotrophin-1 (CT-1). Each of these has been shown to promote the survival of a variety of neuronal subtypes both in vitro and in vivo, however, it is becoming clear that their functions go well beyond neuronal survival. For example, BDNF, NT-3, and GDNF stimulate the expression of NRG1 in motor neurons both in vitro and in vivo (Loeb and Fischbach, 1997; Loeb et al., 2002). Furthermore, applied locally, NGF, BDNF, NT-3, and GDNF induce the rapid release of NRG1 from neurons and their axons (Esper and Loeb, 2004). Therefore, these, and potentially other neurotrophic factors that are expressed by glial cells and targets of innervation, have the potential to modulate the local release of NRG1 in a positive-feedback loop (Fig. 4). This notion has been substantiated at neuromuscular synapses in vivo where, both BDNF and GDNF were able to "rescue" NRG1 expression at neuromuscular synapses after activity blockade with curare (Loeb, 2003; Loeb et al., 2002).

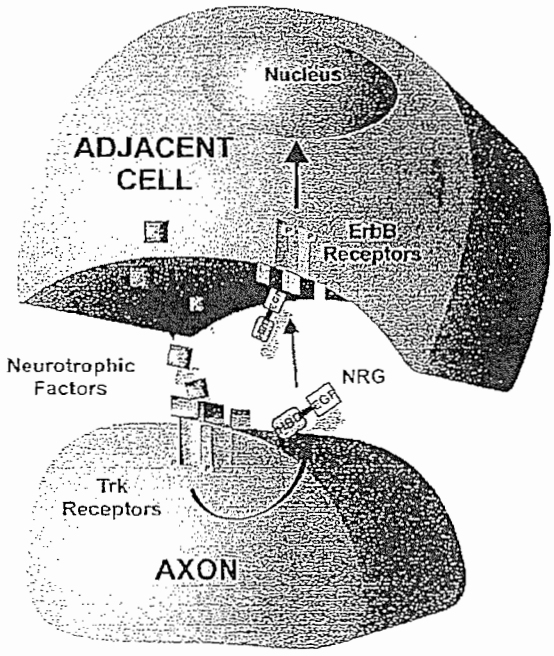

Fig. 4 – NRG1 and neurotrophic factors can be part of a bidirectional signaling system. Glial and muscle cells in both the CNS and PNS secrete neurotrophic factors that bind and activate their receptors on adjacent axonal membranes. In response, axons rapidly release NRG1 which can bind to and activate erbB receptors on those same glia and muscle cells. In this way, cells that contact those axons can control the amount of NRG1 stimulation they receive.

4. NRG1 is critical for PNS development

The PNS consists of both myelinated and unmyelinated motor, sensory, and autonomic nerve fibers that enable effective communication between the CNS and the periphery. Mounting evidence suggests that NRG1 is an important mediator for the development and maintenance of the PNS and is highly expressed in each of these neuronal subtypes (Falls, 2003; Fischbach and Rosen, 1997; Jessen and Mirsky, 1999; Mirsky et al., 2002). Indeed, NRG1 is transported down motor axons as they first emerge from the neural tube and later becomes highly concentrated in the synaptic basal lamina of neuromuscular junctions (NMJs) (Goodearl et al., 1995; Loeb et al., 1999; Sanes and Lichtman, 1999). There it has been proposed to have an important role in promoting both the expression and insertion of synaptic acetylcholine receptors (AChRs) into the muscle membrane (Fischbach and Rosen, 1997; Sandrock et al., 1997). Recent evidence suggests that NRG1 works synergistically with an AChR clustering proteoglycan called agrin to maintain the extremely high densities of AChRs needed for efficient synaptic transmission (Li et al., 2004a,b; Meier et al., 1998). This may occur through direct NRG1-agrin binding between the heparin-binding domain of NRG1 and agrin's glycosaminoglycan side chains that serves to concentrate NRG1 at NMJs. As synapses mature, both the density of HSPGs and heparin-binding forms of NRG1 increase so as to provide sustained support for those synapses (Loeb, 2003; Loeb et al., 1999). This mechanism is complemented by an activity-dependent mechanism that controls NRG1 release presynaptically in response to postsynaptic neurotrophic factors so that more active synapses release more NRG1 and thus are functionally stronger (Esper and Loeb, 2004; Loeb et al., 2002). It should be noted however, that the relative contributions NRG1 and agrin make to the formation of synapses at the NMJ is still controversial. Escher et al. recently demonstrated that mouse NMJs still form normally in the absence of erbB2 and erbB4, raising the possibility that the effects of NRG1 signaling to muscle may be mediated indirectly through other mechanisms such as Schwann cell activation (Escher et al., 2005).

Other than at the earliest times of axonal outgrowth, neuronal axons are always surrounded and supported by Schwann cells in the peripheral nerve that receive critical signaling from NRG1. Genetic studies in mice have shown that disruption of NRG1 signaling, either by knocking out all NRG1 gene isoforms, the type III (CRD) isoform, or the genes encoding the NRG1 receptors erbB2 or erbB3 leads to an almost complete loss of Schwann cells followed thereafter by death of the sensory and motor neurons that they support (Adlkofer and Lai, 2000; Garratt et al., 2000; Meyer and Birchmeier, 1995; Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999). The reason for this likely stems from NRG1's roles in promoting both Schwann cell proliferation and survival (Ciutat et al., 1996; Grinspan et al., 1996; Trachtenberg and Thompson, 1996; Winseck et al., 2002).

While NRG1 serves a critical role during development of the peripheral nerve, additional roles for NRG1 signaling for the mature PNS have been elucidated (Baek and Kim, 1998; Dong et al., 1995; Grinspan et al., 1996; Jessen and Mirsky, 2002; Levi

et al., 1995; Meyer and Birchmeier, 1995; Mirsky et al., 1996; Syroid et al., 1996; Topilko et al., 1996). As the peripheral nerve matures, each Schwann cell must make a commitment to either myelinate an adjacent axon, or remain "non-myelinating". Over- and under-expression studies of type III (CRD) isoforms in mice suggest that NRG1 promotes myelin sheath thickness (Kerber et al., 2003; Michailov et al., 2004). Paradoxically, treating neuron/Schwann cell co-cultures with GGF-2, a type II isoform of NRG1, resulted in significant demyelination (Zanazzi et al., 2001). In addition, transgenic mice induced to over-express this same isoform developed demyelination, resting tremors, gait abnormalities, decreased hind limb strength, and paralysis by approximately 7 months of age (Huijbregts et al., 2003). These seemingly conflicting results suggest a delicate balance for NRG1 signaling in the peripheral nerve where different isoforms have specific effects on developing and adult myelinating Schwann cells. NRG1 also appears to have continued roles for non-myelinating Schwann cells, since mice where erbB signaling is blocked develop a progressive loss of non-myelinating Schwann cells producing a sensory neuropathy with subsequent loss of DRG neurons (Chen et al., 2003). However, this phenotype is not necessarily a NRG1-specific effect, since the approach taken to generate mice expressing a dominant negative erbB4 in Schwann cells could also have blocked other erbB ligands as well. Extensive studies have been done with knockout mice for several NRG1 isoforms and reviewed in detail (Falls, 2003).

Just as described above for the NMJ, there is some evidence to suggest a feedback loop between Schwann cell-derived neurotrophic factors and NRG1 produced by neuronal axons in peripheral nerve development. Supporting this are in vitro studies that demonstrate the rapid, localized release of NRG1 from motor and sensory axons in response to Schwann cell-produced neurotrophic factors (Esper and Loeb, 2004). This model shown in Fig. 4 is complemented by studies showing that BDNF promotes myelination both in vivo and in vitro (Chan et al., 2001; Cosgaya et al., 2002). Similarly, GDNF, which strongly stimulates NRG1 release from neurons, can cause non-myelinating Schwann cells to begin myelinating in adults (Hoke et al., 2003). Whether either of these effects are due directly to BDNF and GDNF on the Schwann cells or to an indirect effect by promoting NRG1 release from the neurons that then act on the Schwann cells remains to be seen, however, both groups of factors will likely need to be considered when devising therapeutic interventions for peripheral nerve diseases.

4.1. *NRG1 in peripheral neuropathies*

Given the critical nature of NRG1 signaling in PNS development and maintenance, it seems likely that NRG1 signaling will be important both for the understanding and treatment of human peripheral neuropathies. Peripheral neuropathy is a common and often painful condition that effects myelinated and non-myelinated axons. It can result from a large variety of toxic, infectious, metabolic, genetic, and traumatic causes (Di Trapani et al., 1986; Facer et al., 2000; Martin et al., 2003; McArthur, 1996; McCarron et al., 1999; Nations and Barohn, 2002; Schifitto et al., 2001; Steiner et al., 1988; Takahashi et al., 1995; Valensi et al., 1997; Winer et al., 1992). Unlike in the CNS, damaged peripheral nerves have the ability to regenerate axons that in many ways recapitulate early developmental events. Studies on Schwann cells in injured nerves demonstrate a multitude of changes in gene expression that lead to their proliferation and promotion of local axonal re-growth (Carroll et al., 1997; Eckersley, 2002; Fan and Gelman, 1992; Pellegrino et al., 1986; Scherer, 1997; Stoll et al., 1989). Abrogation of this natural repair process may contribute to chronic peripheral neuropathies, including that seen in diabetes (Boulton and Malik, 1998; Yasuda et al., 2003).

Because of the reciprocal relationship described above, both NRG1 and neurotrophic factors have the potential to serve as therapeutic agents in peripheral neuropathies (Apfel, 2001; Apfel and Kessler, 1995; Dobrowsky et al., 2004; ter Laak et al., 2000). In response to nerve damage and loss of axonal contact, Schwann cells undergo numerous changes including production of NRG1 that has been suggested to promote Schwann cell proliferation in an autocrine manner (Carroll et al., 1997; Oka et al., 2000). NRG1 mRNA levels have also been found to be dramatically increased in DRG sensory neurons following axotomy (Li et al., 1997), and administration of exogenous NRG1 blocked the injury-induced apoptosis of terminal Schwann cells at the NMJ (Trachtenberg and Thompson, 1996). Neurotrophic factor expression is also significantly changed after axotomy or crush injury causing levels of BDNF, NGF, and CNTF to increase (Cho et al., 1998; Funakoshi et al., 1993; Sebert and Shooter, 1993; Vischer, 1997; Widenfalk et al., 2001).

A number of animal studies have demonstrated considerable benefit for peripheral neuropathies with either NRG1 or neurotrophic factor treatment. The addition of exogenous NRG1 was shown to promote nerve regeneration through its effects on Schwann cells in damaged nerves (Bennett et al., 1998; Munson and McMahon, 1997; Munson et al., 1997; Verge et al., 1996). In a toxin-induced neuropathy model, the application of NRG1 provided significant protection (ter Laak et al., 2000). Similarly, neurotrophic factors including NGF, BDNF, NT-3, GDNF, CNTF, or LIF can promote the regeneration of sensory and motor neurons following axotomy (Boyd and Gordon, 2003; Munson et al., 1997; Oudega and Hagg, 1999; Sayer et al., 2002; Schnell et al., 1994; Sendtner et al., 1990; Zhang et al., 2004). These promising results in animals have led to human clinical trials using NGF to treat diabetic neuropathies. While there was some significant improvement in some patient outcomes in early trials, these studies had to be discontinued due to painful side effects (Apfel, 1999a,b, 2001, 2002; Apfel et al., 1994; Apfel and Kessler, 1995, 1996; Apfel et al., 1998, 2000; Pittenger and Vinik, 2003). On the other hand, human trials using NRG1 in peripheral neuropathy have yet to be reported. Given NRG1's numerous functions in many tissues other than peripheral nerve, a major obstacle is efficiently delivering NRG1 to the desired target without causing unwanted side effects elsewhere. Clearly, a better understanding of the relationship between NRG1 and neurotrophic factor signaling as well as the development of improved delivery methods for these important growth and differentiation factors to sites where they are needed will be required to develop safe and effective treatments for peripheral neuropathies.

5. NRG1 in the CNS

Neuregulin-1 signaling is also critical for the normal development and spatial distribution of several cell types in the CNS. For example, NRG1 promotes the proliferation, migration, and survival of neural precursors (Lai and Feng, 2004; Rio et al., 1997). Cortical interneurons depend on NRG1 for proper migration within the telencephalon and outward toward the cortex (Flames et al., 2004). Cultured hippocampal neurons increase neurite outgrowth, area, length, and branching when exposed to NRG1 (Gerecke et al., 2004). Studies with NRG1 mutants have also demonstrated the importance of NRG1-erbB signaling for the generation of radial glia and their transformation into astrocytes in the cerebral cortex (Schmid et al., 2003).

Unfortunately NRG1 knockout mice die at embryonic day 10.5 from severe cardiac defects, making it difficult to utilize genetic models to study later aspects of CNS development such as oligodendrocyte generation and lineage development. However, several in vitro and in vivo studies have supported the notion that NRG1 is required for early fate determination, proliferation of precursors, as well as subsequent differentiation of oligodendrocytes (Falls, 2003; Marchionni et al., 1999; Park et al., 2001a,b). For example, spinal cord explant cultures from NRG1 knockout mice fail to generate oligodendrocytes unless exogenous NRG1 is added, suggesting an important role for NRG1 in oligodendrocyte lineage specification (Vartanian et al., 1999). Interestingly, explants from dorsal chick spinal cord, which normally lack oligodendrocyte precursors, generate immature oligodendrocytes when treated with sonic hedgehog and NRG1 (Sussman et al., 2000). Subsequent to oligodendrocyte birth, NRG1 may also play a role in promoting the transition to mature myelinating oligodendrocytes as well. For example, erbB2 signaling is required for the development of terminally differentiated oligodendrocytes both in vitro and in vivo. In this study, transgenic mice expressing a dominant negative erbB2 receptor exhibited hypomyelination, a decrease in mature oligodendrocytes, and an increase in the number of progenitor cells (Kim et al., 2003). While this could be due to impaired NRG1 signaling, the hypomyelination seen could have resulted from the disruption of other signaling pathways known to activate erbB2.

The pleiotrophic effects of NRG1 on oligodendrocyte development are determined by developmental stage, culture conditions, and other co-factors in the myelination process. Recent studies suggest a context dependent switch of NRG1 induced proliferation to differentiation of oligodendrocytes is required for cells to respond dynamically to NRG1 during different stages of development. The switch may in part be determined by the expression of different integrins on the oligodendrocyte cell surface. Myelinating oligodendrocytes express alpha-6 integrins that interact with axonal laminins and activate a switch in survival signaling from one NRG1 signaling pathway to another (Colognato et al., 2002, 2004).

While developmental studies have been essential for understanding many functions of NRG1 in the nervous system, much work is still needed to determine the role of NRG1 in the adult CNS. NRG1 has been shown to be expressed in mature brain (Law et al., 2004). NRG1 mRNA levels and protein were localized to human prefrontal cortex, hippocampal formation, cerebellum, and substantia nigra. NRG1 in mature brain may play a role in post-injury regeneration, since the mRNA of several NRG1 isoforms were up-regulated in axotomized motor neurons (Kerber et al., 2003).

NRG1's erbB2, erbB3 and erbB4 receptors are also expressed in several cell types of the adult CNS, including oligodendrocytes (Deadwyler et al., 2000), astrocytes (Cannella et al., 1999), and microglial cells (Cannella et al., 1999). Interestingly, the erbB4 receptor is enriched in the postsynaptic density (PSD) and associates with PSD-95, possibly suggesting a role for NRG1 in CNS synaptic plasticity (Garcia et al., 2000; Huang et al., 2000). Consistently, a number of in vitro models have suggested that NRG1 signaling modulates the expression and function of several neurotransmitter receptors in the CNS, just as it does in the PNS. For example, NRG1 increased the expression of the NR2C subunit of the NMDA receptor by over 100-fold in cerebellar slices (Ozaki et al., 1997). Another study demonstrated that bath perfusion of NRG1 significantly reduced whole-cell NMDA receptor currents in acutely isolated and cultured prefrontal cortex (PFC) and pyramidal neurons (Gu et al., 2005). NRG1 has also been shown to reduce the expression of the GABA(A) receptor α subunit, and induce the expression of the GABA(A) β2 subunits in hippocampal slices and cultured rat cerebellar granule neurons (Rieff et al., 1999; Xie et al., 2004). Given its peripheral role in regulating AChRs, it is not surprising that NRG1 also promoted the up regulation of the α-5, α-7, β-4, δ and ε subunits of neuronal acetylcholine receptors (Buonanno and Fischbach, 2001; Liu et al., 2001; Sapru et al., 1998; Yang et al., 1998).

In summary, given both the pattern of NRG1 expression in the developing and adult CNS, and its observed activities on oligodendrocyte development and synaptic neurotransmitter receptors, it is well positioned to have important roles in both diseases of white matter and grey matter. At present time, most disease-related research on NRG1 has focused on diseases of myelin such as multiple sclerosis (MS), and schizophrenia. A role for NRG1 in MS makes perfect sense given its roles in early oligodendrocyte development. A relationship between NRG1 and schizophrenia is not so obvious, however, the recent linkages to NRG1 in a number of schizophrenic families has led to increased interest in this psychiatric disorder (Corfas et al., 2004). A number of other studies suggest a protective role of NRG1 after traumatic spinal cord and brain injuries, as well as ischemic stroke (Kerber et al., 2003; Oudega and Hagg, 1999; Shyu et al., 2004). Each of these conditions will be briefly reviewed with emphasis on possible mechanisms of NRG1 involvement, followed by a discussion on future possibilities of using NRG1 as a therapeutic agent.

5.1. NRG1 *in multiple sclerosis*

Multiple sclerosis (MS) is a chronic and debilitating disease of the CNS characterized by multifocal demyelination and axonal loss (Franklin, 2002; John et al., 2002). Although most of the therapeutic strategies in MS have focused on reducing the associated inflammatory process, the etiology and pathogenesis of the disease are still unclear. No matter what the initial cause, increasing the number of functional

oligodendrocytes at the lesion site would be beneficial. Theoretically, this could be achieved by increasing the survival of mature myelinating oligodendrocytes, or promoting the proliferation and migration of pre-myelinating oligodendrocyte precursor cells (OPCs). In fact, it has been suggested that expression of NRG1 is reduced in active MS lesions, raising the possibility that a deficiency of NRG1 contributes to the paucity of remyelination seen in MS (Viehover et al., 2001). Regardless, NRG1's importance for oligodendrocyte survival, proliferation and differentiation, make it an attractive candidate for further study as a therapeutic agent in MS. The therapeutic use of growth factor supplementation in human MS patients is still in its infancy, but several animal studies suggest that NRG1 can promote remyelination in models of MS. Systemic delivery of NRG1 to mice with experimental autoimmune encephalitis (EAE), a mouse model of MS, delayed signs of the disease, decreased the severity, and resulted in significant reductions in relapse rate (Cannella et al., 1998; Marchionni et al., 1999). In addition, NRG1 treated groups displayed more remyelination in CNS lesions than controls. Although the application of exogenous NRG1 had a therapeutic effect on these EAE mice, the mechanism behind the observed functional improvement is not clear. In contrast to this EAE model, another study that directly infused NRG1 into areas of toxicity-induced demyelination did not alter remyelination in the rat CNS (Penderis et al., 2003). This raises the question of whether the beneficial effects of systemically administered NRG1 may have been indirect, through modulation of the immune system. Further studies are needed to determine the precise mode of action of NRG1 on these cells in vivo to maximize their therapeutic potential.

5.2. NRG1 in schizophrenia

Schizophrenia remains one of the more mysterious and elusive diseases of the nervous system. An estimated 1% of the world's population is afflicted with the severe behavioral, emotional, and cognitive impairments characteristic of the disease. Currently, it is considered a syndrome with no neuropathological hallmarks, causative agents, or sharply defined diagnostic criteria. Strong familial characteristics of schizophrenia suggest a genetic component, and several genome wide scans have identified a number of susceptibility genes, including NRG1 (Li et al., 2004a,b; Stefansson et al., 2003). NRG1 gene polymorphisms have been identified based on their higher association in family members with schizophrenia than within families from normal populations. Such association studies, however, only demonstrate that a given genetic locus correlates with the schizophrenia trait, and do not necessarily implicate a direct causal effect of the gene or its protein products. One approach has been to pursue genes identified by genome wide scans that have biological roles that overlap with current ideas of how schizophrenia develops. NRG1's observed effects on nervous system development and regulation of neurotransmitter receptors makes it an attractive candidate gene to examine further in this disease (Corfas et al., 2004).

Parallels exist between NRG1's effects on the expression of the NMDA receptors and reduced levels of NMDA receptor subunits in several regions of brain tissue from people with schizophrenia (Akbarian et al., 1996; Carlsson and Carlsson, 1990). Consistently, mice with reduced NRG1 expression demonstrated a 16% decrease in functional NMDA receptors compared with wild type litter mates (Stefansson et al., 2002). In addition, mice expressing lower levels of the NR1 subunit of the NMDA receptor exhibited behavioral features thought to be similar to schizophrenic symptoms (Mohn et al., 1999). Some have argued that disrupting the expression of NRG1's erbB4 receptor in mice produced behavioral characteristics consistent with those observed in patients with schizophrenia, including a lower level of spontaneous motor activity and reduced grip strength compared to wild type mice (Golub et al., 2004).

The exact role for NRG1 in the etiology or treatment of schizophrenia is not clear. Future studies will need to develop a better understanding of the effects of increasing local NRG1 expression in the CNS, or modulating its downstream signaling pathways that promote increased NMDA receptor expression. If improper NRG1 signaling turns out to be an etiological mechanism for schizophrenia, therapies attempting to restore this system may prove more effective in correcting the disease process.

5.3. NRG1 in traumatic spinal cord and brain injuries

Given the roles for NRG1 and neurotrophic factors in development of the nervous system, it is likely that these factors also play a role in the response to traumatic injuries. In addition to the direct effects of the injury, additional damage to the spinal cord can occur post-impact from inflammation, excitotoxicity, and actions of immune system cells. With the advent of functional genomic methods, a large number of changes in gene transcription and protein production have been observed in spinal cord injury that include the levels of NRG1, neurotrophic factors, and their respective receptors (Brown et al., 2004; Hayashi et al., 2000; Lindholm et al., 2002; Murakami et al., 2002; Nakamura and Bregman, 2001; Tokita et al., 2001). Similar increases in NRG1 expression occur in the brain following traumatic injury (Kerber et al., 2003), and after focal ischemic stroke (Parker et al., 2002). These changes may be part of an attempt at repair and regeneration. Whereas the PNS is capable of robust self-repair, CNS neurons appear to have limited regenerative capacity in vivo. This may be due to either intrinsic features of these cells, the presence of extracellular inhibitory factors, or the lack of appropriate growth factors in the damaged CNS.

Application of exogenous neurotrophic factors, most notably NGF and NT-3, can stimulate limited axon regeneration in damaged rat spinal cords (Oudega and Hagg, 1999). The purpose of adding these neurotrophic factors was to promote survival and regeneration of neurons. This approach has lead to some axonal re-growth, but failed to stimulate functional recovery. Although each of these factors is capable of differentially promoting robust axon outgrowth in vitro, they may be singly insufficient to promote regeneration in the complex damaged spinal cord. An alternative approach may be to promote axonal regeneration indirectly, by stimulating glial cells with exogenous NRG1 protein. NRG1 promotes oligodendrocyte precursor proliferation during development,

and has been shown to cause mature oligodendrocytes to dedifferentiate and proliferate (Canoll et al., 1996, 1999). There are currently no reports of using exogenous NRG1 in animal models of traumatic CNS injury, but NRG1 has reduced damage due to experimental brain ischemia in rats (Parker et al., 2002; Shyu et al., 2004; Xu et al., 2004).

Recent evidence also suggests that NRG1 may aid in the regeneration of damaged spinal cord. Traumatized CNS neurons can regrow axons if they are placed in a growth factor-rich environment that among other things, facilitates both NRG1 and neurotrophic factor signaling (Bray et al., 1991; Richardson et al., 1980; Risling et al., 1993; Tobias et al., 2003). Creating such an environment at the site of CNS injury may be the key to developing effective methods to regenerate damaged spinal cords or brain tissue. One way this can be achieved is by transplanting olfactory ensheathing cells (OECs) into damaged spinal cords (Barnett and Riddell, 2004; Bunge and Pearse, 2003; Moreno-Flores et al., 2002; Raisman, 2001; Resnick et al., 2003). Initial evidence suggests these glial cells may be able to promote functional recovery, however, the exact way this occurs is not clear (Boruch et al., 2001; Thompson et al., 2000; Williams et al., 2004). OECs secrete a variety of neurotrophic factors including NGF and BDNF that stimulate neurons to regrow axons both in vitro and in vivo, and the OECs may be able to myelinate them in vivo (Barnett et al., 2000; Kato et al., 2000; Smith et al., 2001). Conversely, the OECs both produce and respond to a NRG1, possibly through an autocrine mechanism (Woodhall et al., 2003). The exact roles for the OEC-derived growth factors in spinal cord regeneration have yet to be determined, but transplanting these cells into the traumatized spinal cord appears to create an environment permissive to natural repair (Garcia-Alias et al., 2004; Lakatos et al., 2003). Consistently, transplanted OECs genetically modified to over-express GDNF, yielded significant functional recovery in injured rats (Cao et al., 2004). Given that glia-derived GDNF induces the rapid release of NRG1 from axons, it is possible that the observed neuronal recovery in these animal spinal cord injury models could be due to GDNF-stimulated release of endogenous NRG1.

6. Conclusions

In this review, we have discussed basic mechanisms of NRG1 activity in the development of both the PNS and CNS, and how NRG1 may be involved in various disease processes. Understanding basic mechanisms of NRG1 expression, interaction with neurotrophic factors, and targeting through HSPG interactions will also be critical in determining NRG1's role in disease. Continued research into NRG1 actions in normal and diseased nervous system tissues will provide important new insights as well as suggest new therapeutic options.

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FUTURE PERSPECTIVE IN PERIPHERAL NERVE RECONSTRUCTION

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Nerve injuries induce severe disability and suffering for patients. Profound alterations in nerve trunks, neurons, and the central nervous system are induced rapidly after injury. This includes activation of intracellular signal transduction mechanisms aiming at the transfer of the cells into a regenerative state through the induction of the appropriate gene programs. The understanding of the neurobiological mechanisms that occur after injury can be used to design modern strategies for reconstruction after nerve injuries. Signal transduction mechanisms for instance may be targets for pharmacological intervention to stimulate nerve regeneration. Nerve injuries, particularly where there is a defect between the severed nerve trunks like in brachial plexus lesions, remain a challenge for the surgeon. Reconstruction of nerve injuries with a defect requires utilization of graft material, which can be of various designs. Application of autologous nerve grafts and use of nerve transfers are the most common clinical solutions to overcome problems with nerve defects. In this chapter we discuss the future perspective of nerve reconstruction with focus on signal transduction mechanisms and new avenues to bridge nerve defects using nanomodified graft surfaces.

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I. Introduction

In spite of extensive research on nerve regeneration, with focus on both clarifications of the delicate molecular and cellular mechanisms, as well as direct clinically applied projects, results after nerve injury and repair are generally still insufficient. The outcome is particularly troublesome when larger nerve defects have to be reconstructed. A variety of factors influence the results after nerve injury and repair and reconstruction (Dahlin, 2008a,b,c, 2009). A poor result may often be observed when a nerve injury occurs in an adult, when a mixed nerve is injured proximally, such as the median and ulnar nerves, or when a nerve trunk or a spinal nerve root is severely lacerated, such as in the brachial plexus. Hence, such nerve injuries cause frustration not only for the patient, but also for the surgeon that has to deal with poor outcome. In contrast, injuries in young children, injuries to a pure motor nerve, such as the posterior interosseous nerve, and injuries where there is a short distance to the target, may sometimes have a favorable outcome, particularly in recovery of motor function. The present issue of *The International Review of Neurobiology* focuses on repair and reconstruction of nerve injuries today as well as future possibilities. Here we focus on future perspectives of nerve repair and in particular the possibility of targeting signal transduction to improve regeneration and secondly the possibility to use nanomodified surfaces for nerve reconstruction.

II. Intracellular Signaling

The reactions of neurons and nonneuronal cells after nerve injury are very complex processes that consist of temporally and spatially orchestrated mechanisms aimed at cellular repair. After an injury in larger and mature organisms, the axons have to grow over long distances, usually along basal lamina tubes. Sometimes the basal lamina tubes have to be recreated. An important question is if these pathways are optimal and if the Schwann cells along the paths are not optimally receptive for the outgrowing axons. A possible strategy to explore for improvements would be to try to mimic some of the developmental mechanisms of axonal growth. In such conditions there is a growth of axons over a limited distance with very receptive cells in a perfect environment with appropriate tropic and trophic signals to guide the axons to their target. Finally, the brain is very well adaptive to the new signals received from the periphery in young subjects and during development. Intensive research on mechanisms of nerve regeneration is necessary to clarify the events induced in neurons, Schwann cells, and other cells, like endothelial and inflammatory cells, in the regeneration process (Dahlin, 2008b).

It can be anticipated that stimulation or inhibition of these events with proper timing and positioning can promote both pathfinding and axonal outgrowth. In analogy with a symphony orchestra, a large number of molecular instruments are played in the cells, each with their own specific and optimal function, but in contrast to an orchestra, in the cellular context, the conductor—the surgeon—may have very limited influence on the cellular orchestra in a mature subject. New findings have been presented on the subcellular mechanisms, particularly when it comes to intracellular signaling—signal transduction, which are initiated rapidly after a cellular injury and then continuous during the entire regeneration process through autocrine and paracrine cellular signaling. Such injury-induced signal transduction will be one of the topics discussed in the present review.

III. Development of Nerve Repair and Reconstruction

Although nerve repair and reconstruction is problematic, some success has yet been achieved with the aim to improve outcome for nerve repair following injury. This progress was initiated by researchers in the beginning of the last century, such as by Ramon y Cajal (1928), who described, in meticulous studies, the biology after nerve injuries. With focus on clinical nerve repair and reconstruction, Bunnell (1944) presented an impressive description of the problem of nerve injury and repair. He described factors that influenced the outcome, techniques for how to repair nerves after injury, and also results of repair in individual patients. Other important contributions to the understanding of nerve injuries have been made by Sunderland, Seddon, Moberg, Narakas, Gilbert, Birch and many others. During the last 50 years, new strategies for nerve repair and reconstruction have evolved (Lundborg, 2000). Nerve graft techniques have been introduced by the pioneering work of particularly Millesi *et al.* (1972), and such procedures are now routine in the clinic. Thus, extensive reconstruction of brachial plexus lesions is possible by the use of nerve grafts performed at specific centers in the world, where also various nerve transfers are utilized to improve function after particularly nerve root avulsions. Recently, nerve transfers were described, which were made more distally in the arm and hand, and this technique is now frequently used (Brown and Mackinnon, 2008). For shorter nerve defects, various conduits have been, or will be, introduced in the clinic based on extensive experimental research in our laboratories (Dahlin *et al.*, 2007; Lundborg *et al.*, 2004; Nilsson *et al.*, 2005a; Scherman *et al.*, 2001, 2004). Specific rehabilitation strategies with sensory re-education are routine in the clinic. Finally, better tools to treat pain and allodynia in the injured patients are also available.

However, in spite of these improvements the outcome of nerve repair is still poor, particularly when sensory functions are considered.

IV. Nerve Reconstruction: Technique and Alternatives

The clinical routine to bridge defects between injured nerve trunks is the use of autologous nerve grafts. Many problems remain after such a procedure, such as the likely discrepancy in caliber between the graft and repaired nerve. In addition, there is probably a limit of how long a graft can be, which will still permit the growth of axons to reinnervate the target. Furthermore, there may also be a lack of availability of graft material. Alternatives to nerve grafts have been experimentally developed, but few of them are clinically applied. For short gaps, and in specific circumstances as an alternative to nerve repair, various conduits are available (Lundborg *et al.*, 2004; Weber *et al.*, 2000). Other alternatives are the simple technique by the use of longitudinal sutures to bridge short defects, which is developed in our laboratories (Scherman *et al.*, 2001). Acellular nerve grafts have been developed experimentally and are becoming more popular in the clinic. The presumption is that acellular nerve grafts are less prone to be attacked by the immune system than cell-containing nerve grafts (Hudson *et al.*, 2004; Kvist *et al.*, 2008; Sondell *et al.*, 1997). Making grafts acellular may for this reason even allow xenografting making the problem of shortage of graft material and the sacrifice of healthy donor nerve void. Recently, acellular nerve allografts, additionally treated with chondrotinase A (Krekoski *et al.*, 2001), have been used to bridge short defects in digital nerves (Karabekmez *et al.*, 2009). By making nerve grafts acellular with different techniques (Hudson *et al.*, 2004; Krekoski *et al.*, 2001; Sondell *et al.*, 1999), three-dimensional structures are obtained which still contain growth-stimulating substances like laminin. To improve the regeneration process through such acellular nerve grafts and other matrices used for bridging (e.g., tendon autografts), Schwann cells from the recipient, cultured or acutely dissociated from the injured nerve segment (Brandt *et al.*, 2005; Nilsson *et al.*, 2005a), have been added to such structures. Although axonal outgrowth can be improved initially in such Schwann cell enriched structures (Nishiura *et al.*, 2004), long-term functional recovery may be disappointing (Arino *et al.*, 2008), although a "blow-through" effect in experimental studies has been suggested (Keune *et al.*, 2006). However, further clinical studies are required to elucidate long-term outcomes. In the future, we may expect that stem cells from the recipient may be used. These may have advantageous influence on axonal outgrowth through different structures, where the stem cells can get the same characteristics and function as Schwann cells after differentiation (see Terenghi *et al.*, Chapter 21, this issue).

V. Signal Transduction in Peripheral Nerve Regeneration

Some decades ago the research on effects of different growth factors showed promising results with hopes to apply these factors to improve peripheral nerve regeneration. However, the use of neurotrophic factors to improve regeneration in the peripheral nervous system has not come to clinical use, either since the mechanisms of nerve regeneration are much more complex than first anticipated or because the treatment has drawbacks, like the induction of allodynia by NGF treatment.

Harvey *et al.* (2006) cites the neurobiologist Larry Benowitz, who described the regenerative response in neurons in the central nervous system with the analogy of the "break and the gas pedal." Growth-inhibitory substrates are the breaks, whereas the growth and trophic factors that provide growth enhancing signals is the accelerator or the gas pedal. To continue the analogy of a moving vehicle, it is not enough to take the foot from the break or to push harder on the accelerator if the handbrake is still engaged. In addition, we also have to add the "steering wheel" that directs the extension of the growth cones with their filopodia. Finally, a vehicle also has a clutch, which may represent so-far unrecognized signaling pathways, or specific switches in the cell machinery that have to be engaged for an optimal and directed growth (Harvey *et al.*, 2006).

A. The Injury Signal and the Cell Body Reaction

Cells respond to signals in their environment by translating them into intracellular messengers, which through their actions induce the appropriate stimuli-specific response. The neural response to an axonal injury is not simply localized to the site of the damage, but profound changes also occur in the cell body, sometimes long distances away from the injury site. These changes are collectively known as the cell body reaction, and involves alterations in transcription, translation, and posttranslational processes. How these changes are induced and orchestrated, both spatially and temporally, and how the information of the injury is conveyed from the injury site to the cell body still remains an enigma, but this matter is the focus of intense research (Ambron and Walters, 1996; Ambron *et al.*, 1995, 1996; Befort *et al.*, 2003; Boeshore *et al.*, 2004; Brindle and Montminy, 1992; Bussmann and Sofroniew, 1999; Chen and Strickland, 2003; Chen *et al.*, 1996; Costigan *et al.*, 2002).

Microarray analysis of injured neurons has revealed injury-induced regulation of hundreds of genes (Curtis *et al.*, 1998; Drysdale *et al.*, 1996; Gunstream *et al.*, 1995; Gupta *et al.*, 1996), including those encoding neurotrophin receptors, transcription factors and cytoskeletal components. It is hypothesized that such transcriptional changes come about following injury as a response to both negative and positive signals, that is, lack of signals from target tissue and injury-induced

signals from the damaged axon, respectively. The cell body reaction can, for instance, be initiated by the disruption of retrograde trophic support from the target tissue. There is ample experimental support for such negative control of the cell body response in injured sensory neurons, and it has been shown that pharmacological inhibition of retrograde axonal transport mimics several aspects of the cell body reactions associated with axotomy (Hai *et al.*, 1989, 1999; Hibi *et al.*, 1993). Also, injury-induced downregulation of the neurotransmitters substance P and neuropeptide Y in sensory neurons can be mitigated by distal application of nerve growth factor (NGF) or acidic fibroblast growth factor (aFGF), respectively, to the nerve (Ji *et al.*, 1996; Kallunki *et al.*, 1996). However, the cell body may also be triggered by positive signaling, that is, retrograde transport of proteins from the site of injury. The mollusk *Aplysia californica* has been extensively used as a model system to illustrate such positive regulatory mechanisms (Ambron *et al.*, 1995; Costigan *et al.*, 2002; Karin, 1995; Leah *et al.*, 1991).

Currently, there is no established treatment other than surgery for peripheral nerve injuries. However, the molecular mechanisms that regulate the neuronal injury response could in the future be used as the basis for developing new clinical therapies. Ultimately, the goal would be to modify how a peripheral axonal lesion activates the intrinsic growth capacity of the injured neuron, which in turn would be aimed to promote the speed and accuracy of regeneration.

The intrinsic growth capacity of peripheral neurons has been suggested to be mediated through the actions of cyclic adenosine monophosphate (cAMP) (Liang *et al.*, 1996; Lindwall and Kanje, 2005; Lindwall *et al.*, 2004), which ultimately regulates organization of the cytoskeleton (Snider *et al.*, 2002). Also, among others, two transcription factors that have been demonstrated to be rapidly induced by peripheral nerve injury are c-Jun (Raivich *et al.*, 2004) and activating transcription factor 3 (ATF3) (Lindwall *et al.*, 2004) (Fig. 1). c-Jun is the fundamental component of the activating protein 1 (AP-1) complex (Karin, 1995), and is one of the targets of the stress activated c-Jun *N*-terminal kinase (JNK), which catalyzes its phosphorylation (Neumann *et al.*, 2002; Nilsson *et al.*, 2005b; Perlson *et al.*, 2004a). Conditional knockout of c-Jun (Raivich *et al.*, 2004) as well as pharmacological inhibition of JNK (Lindwall *et al.*, 2004) has been demonstrated to inhibit nerve regeneration. Thus, the JNK family of kinases is required for successful regeneration of peripheral sensory neurons (Middlemas *et al.*, 2003; Perlson *et al.*, 2004b; Qiu *et al.*, 2002a), and as such represents a target for future clinical therapies. ATF3 is a member of the ATF/CREB transcription factor family (Qiu *et al.*, 2002b; Raivich *et al.*, 2004), and is rapidly induced by a variety of signals, including agents that induce the JNK signaling pathway (Seijffers *et al.*, 2006, 2007; Snider *et al.*, 2002; Sung *et al.*, 2001; Tanabe *et al.*, 2003). Inhibition of JNK reduces ATF3 protein levels (Lindwall *et al.*, 2004), which in turn hamper regeneration. Importantly, ectopic expression of ATF3 can actually promote neurite outgrowth of peripheral neurons, possibly through an increase in the intrinsic

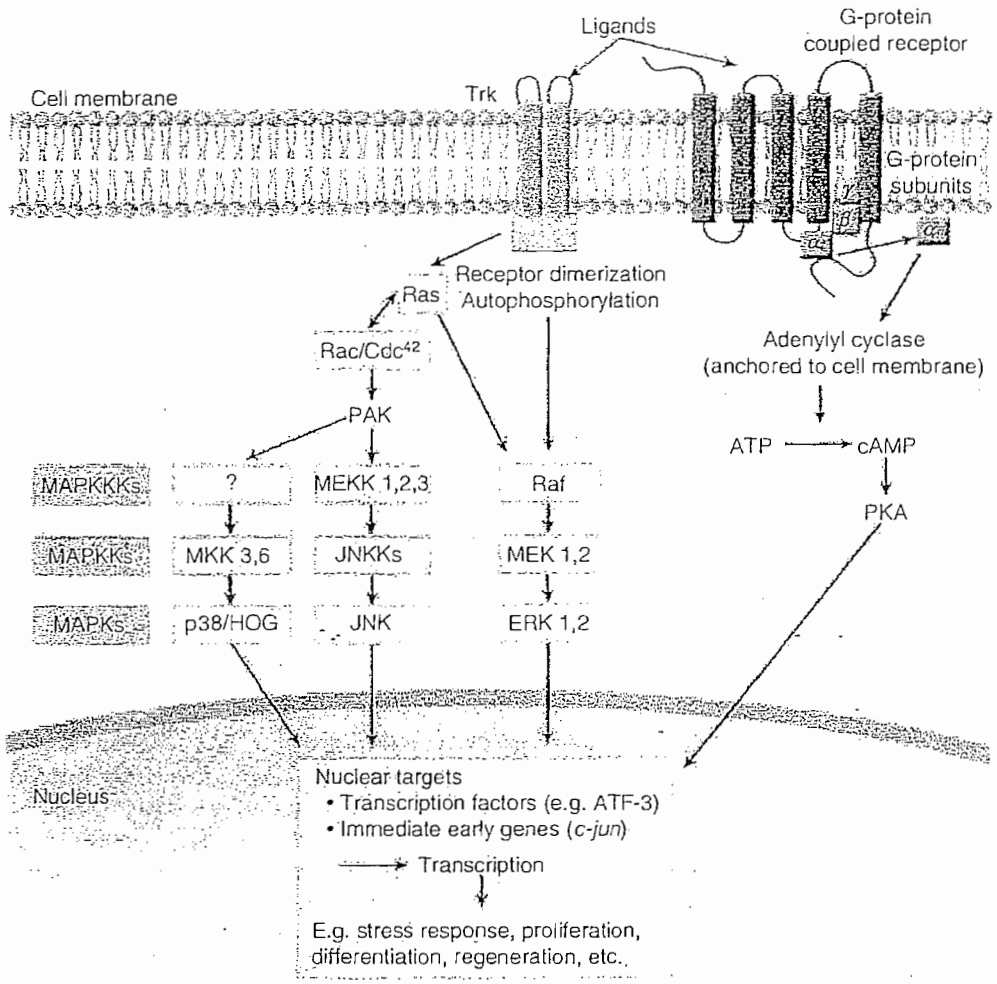

FIG. 1. Signal transduction steps in neurons and nonneuronal cells that occur after nerve injury. The schematic drawing shows the various steps that are needed to activate cells in response to a trauma. Different intracellular steps include phosphorylation steps by the MAPK modules. (Reproduced by kind permission of Elsevier.)

growth state of the neurons (Wong and Oblinger, 1991; Woolf *et al.*, 1990). The presence of these factors over time does probably influence the efficiency of axonal outgrowth; knowledge of outmost importance when considering timing of nerve repair (see below). Thus, if the levels of injury induced molecules, such as JNK, c-Jun, and ATF3, can be selectively modified following a peripheral nerve injury, augmentation of neuritogenesis can be obtained. Such treatment strategies for severe nerve injuries must, however, await a better understanding of the intrinsic molecular mechanisms initiating, and underlying, the regeneration process.

B. Extrinsic Properties Required for Axon Growth and Target Finding

The intrinsic growth capacity of peripheral nerve regeneration has to be combined with a proper environment to encourage axonal growth. Normally, peripheral axons are ensheathed and myelinated by Schwann cells. These cells also provide a basal lamina surrounding bundles of axons. Following an injury, Schwann cells de-differentiate and aid in the clearing of damaged debris, while during regeneration they act as guides for sprouting axons. During the regenerative process they upregulate several genes; the protein products of which may be involved in the guidance of axonal sprouts by Schwann cell-axon attachment (Martini *et al.*, 1994). For instance, the previously mentioned extracellular matrix (ECM) molecule laminin, which is produced by Schwann cells, plays a significant role during regeneration. Laminin receptors, such as integrins, are expressed on the growing axons, which supports regeneration. On the other hand, in mouse knockout models of laminin, axonal regeneration is significantly impaired (Zhang and Ambron, 2000). Thus, regeneration depends on a complex interplay and signals between several cell types within the nerve.

During regeneration the axonal sprouts grow down the distal nerve segment and, if successful, reinnervate their correct targets. Axonal outgrowth is, however, slow in humans, and occur at a rate of around 1 mm per day. Success of regeneration can only be judged following reinnervation of the target tissue, a process which, depending on where the damage was done, may take weeks or months after the initial insult, although the regeneration process can be followed by advancement of the Tinel sign. Unfortunately, at the time of reinnervation the window of successful regeneration may already have passed. Axons must also make correct discriminatory choices in order to reinnervate the correct target tissue, and during this process they are often misrouted. In order to develop therapeutic strategies to improve both rate and accuracy of target reinnervation we need to clarify the molecular events that influence the intrinsic growth capacity as well as axonal discrimination of the extrinsic cues, both substrate bound and diffusible, that is encountered by the axon along the regenerative pathway (Fig. 2). However, it should be stressed that these signal transduction mechanisms occur not only in neurons and its axons but also in all types of cells in a temporal and spatial resolution.

VI. Nanotechnology and Nerve Regeneration

The complex treatment of peripheral nerve injuries but also of the injured spinal cord, which involves a variety of strategies, has been emphasized by many authors (see, e.g., Garbossa *et al.*, 2006). To enhance axonal regeneration with a possible application in spinal cord repair a new generation of tissue compatible

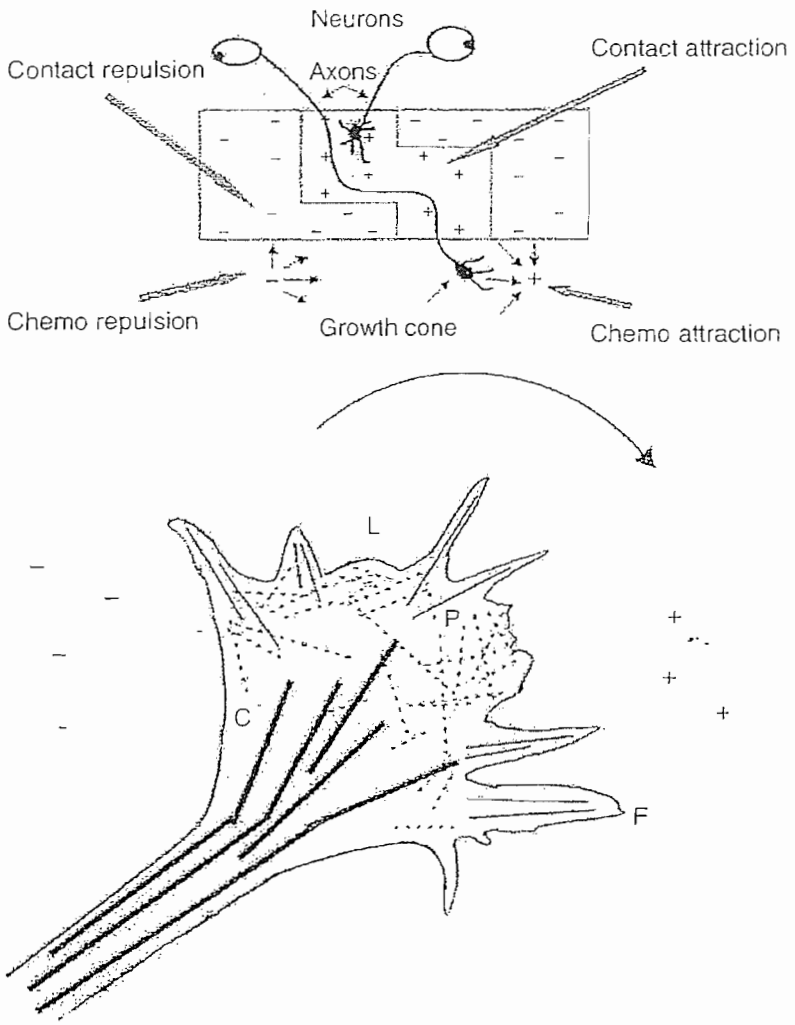

FIG. 2. Schematic drawing showing four different mechanisms that direct axonal outgrowth after nerve injury involving both attraction and repulsion of the growth cone (upper drawing). The lower drawing show details of a growth cone formed on the tip of each of numerous sprouts, which originate from the proximal end of the transected axon. C = domain where microtubules are located. P = domain where F-actin monomers and actin filaments are found. F = finger-like filopodia that palpates the surroundings directing growth. L = veil-like lamellipodia. (Reproduced by kind permission of American Society for Surgery of the Hand.)

matrices are currently developed using recent developments in nanotechnology. Nanostructures can be a suitable environment for outgrowing axons in different situations; not only to create bridges for nerve defects but also for other reasons with focus on brain machine interface (BMI) issues. Such reasons are directionality of axonal growth and sorting of nerve fibers (Johansson *et al.*, 2006; Prinz *et al.*, 2008). Nanotechnology and nerve regeneration is a future exciting field which

also will be covered in the present chapter. Development of biologically compatible scaffolds that can serve as permissive substrates for growth of neurons, migration of Schwann cells, influence differentiation, and minimize scar and inhibitory environment is a challenge where specific criteria have been outlined for application in the central nervous system (Ellis-Behnke *et al.*, 2007). In the CNS, the four important P's of regeneration as a framework has been stressed (Ellis-Behnke *et al.*, 2007): *Preservation* of neurons (no cell death); Growth-*permissive* environment; *Promotion* of growth through the permissive environment of preserved neurons, axons and their sprouts and growth cones, and, finally, utilized and improved *plasticity* after reconnection. Nanotechnology may offer solutions to several of these criteria. In combination with genomic and proteomic revolution this nanomic one will help to understand pathophysiological events and to improve results as has been suggested for the visual system (Harvey *et al.*, 2006).

A. Nanostructures for Neurite Regeneration

For the development of a new generation of artificial scaffold implants, which are tissue compatible, which smoothly integrate with the host, and which also enhance axonal regeneration, the influence of the implant surface, that is, the topography and chemophysical properties on which the cells/neurites will grow, is of paramount importance. Such tissue engineering principles can also be adopted for the study of cellular behavior associated with regenerating nerve tissue *in vitro*.

Nanotechnology has provided us with new tools that allow the design of structures with dimensions of only a few nanometers that may interact with cells and subcellular processes on a suitable cellular scale (Yim *et al.*, 2005). Extensive research on cell reactions to nanostructures *in vitro*, as well as on cell and cell extensions—neurites—has been performed during the last decade. It is conceivable that nanostructured implant surfaces can be tuned to interact smoothly with the tissue on the implant site and evoke less of an immune response than would nonstructured surfaces. Furthermore, such surfaces can be modified for the organization of the attached cells and thereby tissue formation, resulting in enhanced regeneration. We are presently pursuing these ideas for the repair and reconstruction of peripheral nerves.

B. Neurites and Topography: From Micro to Nano

During nerve regeneration the outgrowth of axons is influenced by a variety of factors, local or distant, and by the cues in the surrounding (Fig. 2). Almost a hundred years ago, R. G. Harrison reported that cells and neurites grown on threads from a spider's web followed the fibers (Harrison, 1911). In 1945,

the American developmental neurobiologist Paul Weiss named this behavior "contact guidance" (Weiss, 1945), but it was not until the early 1970s that biologists seriously tested the idea of contact guidance again, starting with growing cells on grooved substrata and on spheres (Maroudas, 1972; Rovensky *et al.*, 1971). Since then, the cellular responses to topographical cues of many different kinds have been tested, including curved surfaces, single steps, angled planes, pillars, pits, pores, cylinders, spheres, and last, but not least, the most studied structure, parallel grooves and ridges (Flemming *et al.*, 1999). The explosion of research on such artificial topographical cues was mainly due to the rapid development of techniques in the computer industry. Hence, structures with micrometer, and in the last 10–15 years even submicrometer, sized objects are possible to produce and have become available for biomedical research.

Although the exact cell reaction to a specific topography may not easily be predicted, since it is cell type dependent, a great pool of structures and cell types have been tested. Today, it is clear that structures as small as 5–10 nm can change the morphology of some cells, that is, macrophages (Wojciak-Stothard *et al.*, 1996) and that axons may follow grooves and fibers with widths of around 100 nm (Johansson *et al.*, 2006). The latter is perhaps not too surprising considering the fasciculation (minifasciculation) that occurs once a pioneering axon (that in mice may be as thin as 100 nm) has found a path during embryogenesis or regeneration. A simple, although elegant, model for neurite guidance on fibers with different diameters has been presented (Smeal *et al.*, 2005), and shows an enhanced neurite alignment along thin fibers as compared with thicker ones. The basic idea for this guidance phenomenon appears to be the stiffness of the cytoskeleton of the extending neurites. These extensions can simply not curve around a fiber with too small a curvature radius and therefore extend along the fiber in an aligned manner.

For tissue engineering applications in general, and nerve grafts in particular, ordered outgrowth/morphology is often requested. Even though 100 years have passed, the old finding of Harrison is still applicable, not only in the micrometer domain, but also in the nanometer range: parallel structures of grooves with ridges and fibers will orient cells and cell extensions, for example, axons along the structures. Such guided axons often display a simplified growth cone and a higher outgrowth rate as compared to a similar smooth or irregular surface (Corey *et al.*, 2007), a desirable feature in clinical applications. We have found excellent axonal guidance on substrates with rows of 2.5 μm long, vertically standing nanowires separated by 400 nm. Axons from dorsal root ganglia (mouse) were found to be unable to cross between, or climb the nanowires when the distance between two standing wires was small enough (sufficient with 400 nm separation but not with 1 μm) (Prinz *et al.*, 2008). Again, the explanation of this behavior is probably due to the rigidity of the cytoskeleton, a model that may explain several contact guidance phenomena.

On nonordered substrates, such as porous silicon, which has a sponge-like appearance, attachment and proliferation have been shown to be dependent on pore size *in vitro* (Bayliss *et al.*, 1999a,b, 2000; Sapelkin *et al.*, 2006). In this way, we have demonstrated ordered axonal outgrowth from mouse dorsal root ganglia on porous stripes in otherwise smooth silicon (Johansson *et al.*, 2005, 2008). Such porous silicon has also been shown to induce less encapsulation than smooth silicon *in vivo*, indicating a more biocompatible structure (Rosengren *et al.*, 2002). On random meshes of polymer fibers produced by electrospinning (see below), axonal outgrowth is hampered as compared to aligned fibers of the same material, probably due to irregular contact guidance cues (Corey *et al.*, 2007; Wang *et al.*, 2008). For clinical applications, such as nerve grafts where fast Schwann cell migration and axonal regeneration is crucial, ordered linear structures are obviously of essence.

Some of the intracellular molecular components of the guiding system have been identified (Nobes and Hall, 1999; Patel and Van Vactor, 2002), although still many pieces in the puzzle are missing. All cell reactions to the topographies described above depend on highly coordinated assembly and disassembly of the cytoskeleton and in particular microfilaments. The intracellular signaling pathways arising from the extracellular cues, and leading to the rearrangement of the cytoskeleton, involve signal transduction described previously.

For migrating cells the small GTPases, Rac, Cdc42, Ras, and Rho have been shown to be important for organizing the cytoskeleton during migration. Rac is essential for the protrusion of lamellipodia and thereby forward movement, Cdc42 is necessary for maintaining cell polarity, while Ras regulates focal adhesions and associated actin fibers (Nobes and Hall, 1999). The last one, Rho, has been reported as necessary for cell adhesion during movement and thereby contact guidance (Nobes and Hall, 1999; Rajnicek *et al.*, 2008). The guidance of axons thus depends on the same molecules, Cdc42 and Rac, that mediate growth cone attraction and elongation, while Rho mediates repulsion and growth cone collapse (Patel and Van Vactor, 2002). The alternating activation GTPases of by the external cues via the membrane receptors can thus guide the axon in a stop and go fashion. These mechanisms are examples of the intracellular signal transduction pathways.

C. Why Nanostructures?

So, if the results of an experiment performed 100 years ago on spider silk gave the same result as the most advanced structures today, why is there still an interest in nanotechnology in tissue engineering? Artificial nanopatterns can be controlled with respect to size, chemical composition, and physical properties. From an engineering point of view, the spatial resolution is extremely high using

nanotechnology enabling influence and guidance on single axons (Figs. 3 and 4). This may be very important for high-resolution neural interfaces (BMI) that may support axons bypassing injuries, controlling artificial limbs or restore other functions including hearing and vision (Donoghue, 2002). From a more biological/clinical point of view, the ECM is composed of fibers and fibrils

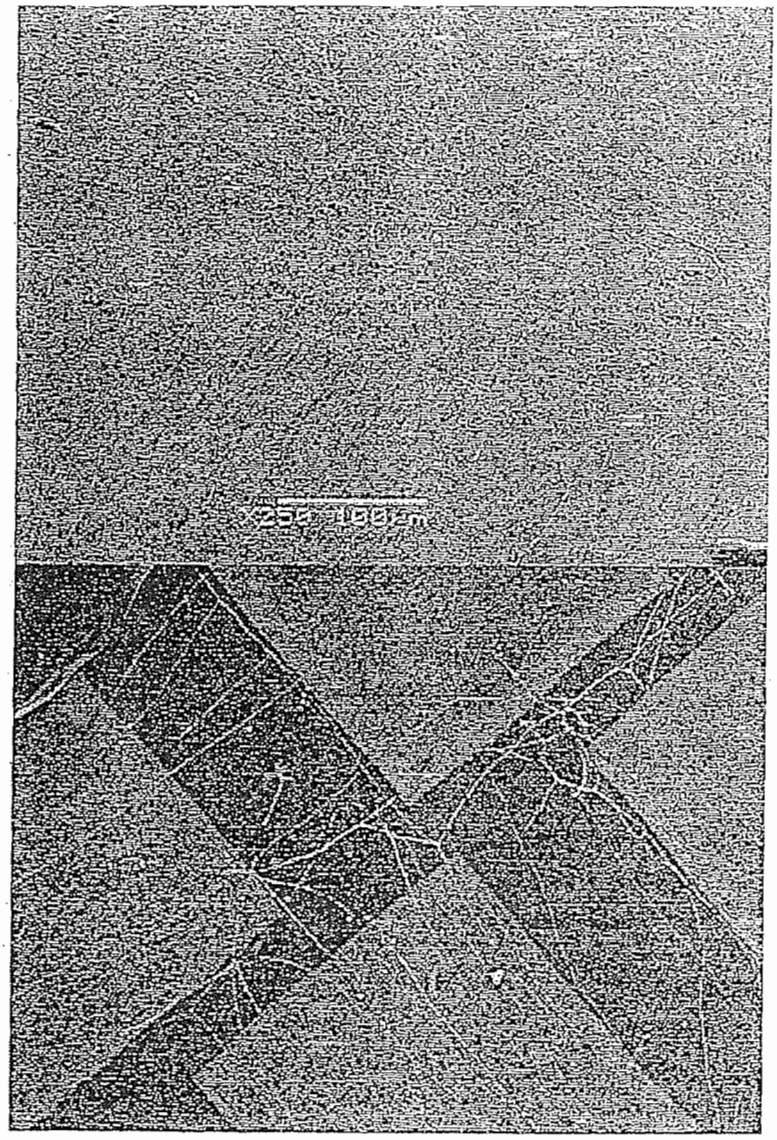

FIG. 3. Upper and lower: Axons grown *in vitro* are highly sensitive for topographies such as grooves and ridges. Here, DRG axons grown on grooves and ridges as small as 100 nm wide, display contact guidance and follow the patterned areas (squares with orthogonal grooves/ridges in an otherwise plane polymer surface produced with Nanoimprint Lithography). (Scanning electron microscope images.)

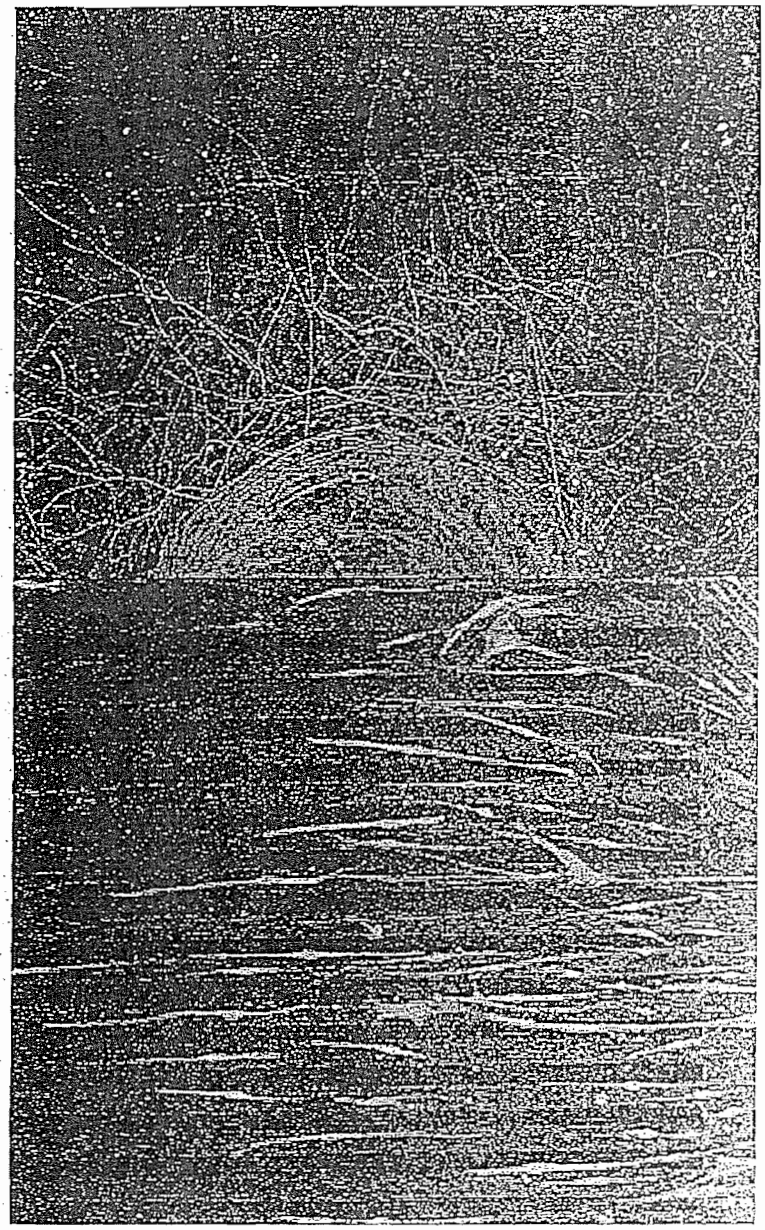

FIG. 4. *Top*: A DRG mounted in Matrigel sends out axons in a random fashion on a flat polymer surface. Once entering squares with grooves and ridges, most axons are guided along the imprinted topography. The top row holds horizontal grooves, while the lower row holds vertical grooves. Note that the thicker axons appears to be less guided than the thinner on those nanometer sized grooves. (Flourescence microscope image.) *Bottom*: On stripes (light green) of porous silicon, pore sizes of 500 nm, in an otherwise flat substrate (black), both axons and Schwann cells from an explanted DRG *in vitro*, prefer to grow and elongate on the porous stripes rather than on the flat areas. This behavior could be utilized for guidance of such cells and extensions in many different applications. (Flourescence microscope image.)

ranging from nanometers to micrometers. Hence, the addition of nanostructures for tissue engineered implants can mimic the ECM structure—the natural environment of cells and growing axons *in vivo* (Ma *et al.*, 2005).

In many other bio-nano applications, such as quantum dots, the effect of quantum physics is taken advantage of. These quantum effects and the potential for such nanostructures are so far not included in tissue engineering, although the extreme area to volume ratio can be employed for built-in drug delivery systems. The substance that should be delivered can either be adsorbed on the artificial substrate, and then the area-to-volume ratio is critical for how much substance an implant can hold. For biodegradable implants (usually polymers), where substances can be incorporated in the implant material, the delivery rate depends on the area rather than the volume when the substrate is degraded. Nanostructured/porous substrates will therefore represent faster delivery systems than bulk substrates of the same volume. The possibility to include substances which promotes survival of neurons in nanostructured nerve implants should be explored.

D. From Cell Reactions to Nanostructures *In Vitro* to Nerve Regeneration Applications

Most *in vitro* studies on nanostructures have been made on flat (rather 2.5-D than true 3-D), hard substrates such as silicon, glass, and plastics. The reason is the limitation of the patterning techniques inherited from the computer industry, that is, photolithography and electron beam lithography that usually must be used at some stage in processing a structured surface. "Soft lithography" is an overall description of many techniques where rubber molds from templates, created by the techniques mentioned above, can produce new topographical or chemical patterns. The use of such techniques can transit such structured surfaces onto irregular shapes to some degree, but only within certain limits. Although very different from an *in vivo* situation, such flat test structures have supplied us with most of the basic knowledge concerning cell adhesion, migration, and alignment etc.

From the clinical perspective, a technique called electrospinning may be better suited for nerve repair. In short, this technique is based on a polymer that is pushed out of a thin syringe. At the syringe tip, the polymer is surface charged and forms a jet stream toward an electrically grounded target, where the polymer is collected when the solvent evaporates. The thickness of such polymer fibers can be tuned from some nanometers to micrometers and the fiber alignment can be manipulated by rapid movement of the target. In this way, fabrics of

nanometer polymer fibers resembling the ECM have been produced (Kumbar *et al.*, 2008; Ma *et al.*, 2005; Murugan and Ramakrishna, 2006) and aligned such fibers guide extending neurites and migrating Schwann cells (Kim *et al.*, 2008; Schnell *et al.*, 2007; Yang *et al.*, 2005). The layers of ECM-like, biodegradable polymers, for example, poly-L-lactic acid (PLLA), can be added onto many macroscopic surfaces as a way to enhance biofunctionality, or work on its own as an artificial ECM scaffold. Besides the obvious resemblance of the ECM structure, the polymer itself can be blended with axon promoting factors, such as laminin (Koh *et al.*, 2008). By using highly aligned structures, contact guidance may also help to enhance axonal outgrowth and nerve regeneration *in vitro* and *in vivo* (Kim *et al.*, 2008; Wang *et al.*, 2008). The use of biodegradable polymers, such as PLLA with the opportunity to blend in other substances, together with the porous structure of the fabric that enables diffusion of nutrition and oxygen prior to vascularization, fulfills many clinical requirements of a nerve graft.

The use of a tissue-engineered nerve graft that performs as good as, or even better, than a standard autologous graft may minimize costs and trauma after nerve injuries. Nanostructures assist in mimicking actual tissue, enable designs on a subcellular level, and may thus be used in future nerve grafts.

VII. Clinical Development: Future Perspectives

To improve nerve regeneration and the outcome after various injuries there is a requirement for the exploration of new research avenues. Such avenues can be signal transduction and nanotechnology as discussed above. There are several other aspects which require attention from the clinical perspective. One is the problem of comparing new with conventional repair and reconstruction techniques. Another is the timing of repair and reconstruction. A third is the problem of neuronal cell death which may be a target for pharmacological intervention. Finally, focus is now also directed towards brain plasticity and the patient's ability to utilize coping strategies to adjust to the impaired function.

In the short perspective, in clinical studies, and particularly multicenter studies, we can investigate the effectiveness of different nerve reconstruction techniques, such as the new alternatives to nerve grafts. However, in more extensive nerve injuries, like in brachial plexus injuries, there are difficulties to evaluate used repair and reconstruction techniques since no lesion is similar to the other, that is, there are differences in the individual extent of injury and thereby the need for different reconstruction procedures. Thus, it is difficult to collect an appropriate number of patients with similar injuries, where such injuries are reconstructed with well-defined techniques. Previous findings have revealed an impaired functional recovery if nerve reconstruction of the brachial plexus lesion

is done 6 months or later following the injury. The timing for nerve reconstruction has been emphasized based on neurobiological alterations in neurons and Schwann cells (Saito and Dahlin, 2008). Interestingly, Kay and co-workers (Jivan *et al.*, 2009) have recently presented data indicating that functional outcome after brachial plexus lesions involving C5-C6 is better if reconstruction is done within 2 weeks after injury. Such notion is supported by the neurobiological data indicating that cellular alterations in both neurons and Schwann cells are time dependent. In Schwann cells, signal transduction mechanisms are rapidly initiated, even within 30 min, which are important for the proliferation of the Schwann cells after a nerve injury (Martensson *et al.*, 2007) and thereby the outgrowth of axons. Schwann cells can also modify the growth environment in the distal nerve segment after injury (Danielsen *et al.*, 1995). Transcription factors, upregulated rapidly in Schwann cells and neurons, subside over time with a subsequent impaired activation of Schwann cell in the distal nerve segment and decreased axonal outgrowth (Saito and Dahlin, 2008). Similarly, a rapid upregulation of the transcription factor ATF3 in neurons is also deteriorated over time. The diminution of that ATF3 response in neurons seems to correlate to impaired nerve regeneration (Saito and Dahlin, 2008). Interestingly, the decline of ATF3-containing neurons is more rapid in motor neurons than in sensory neurons (Kataoka *et al.*, 2007; Saito and Dahlin, 2008). However, the cell death of neurons is more pronounced in sensory neurons than in motor neurons (Hart *et al.*, 2004; McKay Hart *et al.*, 2002). Neuronal cell death can also be diminished if nerve trunks are repaired early after injury (Ma *et al.*, 2003). To prevent or decrease neuronal cell death, particularly among motor neurons, a pharmacological intervention can be considered. Experimental data indicate that early treatment, perhaps within the first 24 h after injury, with *N*-acetylcystein (Hart *et al.*, 2002, 2004) can reduce the number of neurons that go through programmed cell death, apoptosis. However, such treatment has to be tested clinically, preferably in multicenter studies, utilizing the specific protocols for evaluation of function after nerve injury and repair.

A problem after repair and reconstruction of proximal nerve injuries is the extended time before reinnervation of the target can be expected. The Schwann cell response to injury deteriorates over time leading to impaired axonal regeneration after proximal nerve injuries. In this respect, the described nerve transfers in the hand and distal forearm (Brown and Mackinnon, 2008) is an alternative since the surgeon can transfer the nerve injury from a proximal to a distal one. The growing axons from the transferred nerve are thus allowed to grow into an environment that is still permissive in the originally injured distal nerve segment. Nerve transfers can also be applied for a distal nerve segment when there is a lack of a proximal nerve trunk as a source of axon—end-to-side nerve repair (see Bontioti and Dahlin; Chapter 12, this issue). In addition, there are also a large number of other clinically potentially exciting additional treatments as an adjunct

to conventional nerve repair and nerve grafting techniques, which can improve nerve regeneration covered in the present issue of *International Review of Neurobiology*.

Following nerve reconstruction the surgeon also has to plan and initiate the rehabilitation phase and focus on the central nervous system, where extensive reorganization in the cerebral cortex and other levels occur after injury. Early, before reinnervation (phase I), new concepts for rehabilitation have to be considered followed by novel rehabilitation techniques when reinnervation of the hand and arm has occurred (phase II). Several new strategies have been introduced in recent years including the use of EMLA® (local anesthetics) cream application to the forearm leading to improved sensibility in the hand after repaired median and ulnar nerve injuries (Lundborg *et al.*, 2007; Rosen *et al.*, 2006). Thus, brain plasticity is a central issue in nerve reconstruction. Furthermore, individual care of the patients is crucial to direct them along their inborn strategies to cope with such an injury (Cederlund *et al.*, 2008), strategies which they may not immediately be aware of.

Taken together, although conventional nerve reconstruction techniques are used frequently in clinical practice, the outcome is generally still insufficient. Thus, new treatment strategies have to be introduced based on new avenues of research. The utilization of knowledge of intracellular signal transduction mechanisms, and the use of nanotechnologies are exciting perspectives in nerve reconstruction in the future.

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NEUROTROPHINS AND THEIR RECEPTORS: A CONVERGENCE POINT FOR MANY SIGNALLING PATHWAYS

Moses V. Chao

The neurotrophins are a family of proteins that are essential for the development of the vertebrate nervous system. Each neurotrophin can signal through two different types of cell surface receptor — the Trk receptor tyrosine kinases and the p75 neurotrophin receptor. Given the wide range of activities that are now associated with neurotrophins, it is probable that additional regulatory events and signalling systems are involved. Here, I review recent findings that neurotrophins, in addition to promoting survival and differentiation, exert various effects through surprising interactions with other receptors and ion channels.

LONG-TERM POTENTIATION (LTP). An enduring increase in the amplitude of excitatory postsynaptic potentials as a result of high-frequency (tetanic) stimulation of afferent pathways. It is measured both as the amplitude of excitatory postsynaptic potentials and as the magnitude of the postsynaptic-cell population spike. LTP is most often studied in the hippocampus and is often considered to be the cellular basis of learning and memory in vertebrates.

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The era of growth factor research began fifty years ago with the discovery of nerve growth factor (NGF). Since then, the momentum to study the NGF — or neurotrophin — family has never abated because of their continuous capacity to provide new insights into neural function; the influence of neurotrophins spans from developmental neurobiology to neurodegenerative and psychiatric disorders. In addition to their classic effects on neuronal cell survival, neurotrophins can also regulate axonal and dendritic growth and guidance, synaptic structure and connections, neurotransmitter release, LONG-TERM POTENTIATION (LTP) and synaptic plasticity[1–3].

The surprising discovery that neurotrophins and their receptors do not exist in *Drosophila melanogaster* or *Caenorhabditis elegans* reinforced the idea that these proteins are not absolutely necessary for the development of neuronal circuits *per se*, but are involved in 'higher-order' activities. For example, neurotrophins and their receptors influence many aspects of neuronal activity that result in the generation of new synaptic connections, which can be long lasting[3]. Alterations in neurotrophin levels have profound effects on a wide variety of phenomena, including myelination, regeneration, pain, aggression, depression and substance abuse.

The actions of neurotrophins depend on two different transmembrane-receptor signalling systems[4] — the Trk receptor tyrosine kinases and the p75 neurotrophin receptor[5,6]. Despite considerable progress in understanding the roles of these receptors, additional mechanisms are needed to explain the many cellular and synaptic interactions that occur between neurons. An emerging view is that neurotrophin receptors act as sensors for various extracellular and intracellular inputs, and several new mechanisms have recently been put forward. Here, I will consider several ways in which Trk and p75 receptors might account for the unique effects of neurotrophins on behaviour and higher-order activities.

The levels of neurotrophins are important

It is well established that the overall levels of neurotrophins determine the balance between cell survival and APOPTOSIS during development. Neural activity has profound effects on the levels of neurotrophins. Indeed, the idea that neurotrophins are crucial for synaptic plasticity came from observations that they are synthesized and released in an activity-dependent manner[7–9]. NGF and brain-derived neurotrophic factor (BDNF) messenger RNAs (mRNAs) are highly regulated by electrical stimulation and epileptic activity[10], and BDNF in particular is rapidly released by neuronal activity during periods of activity-dependent synaptic remodelling[11–13].

Studies of mice that express reduced levels of neurotrophins have shown surprising effects on adult brain function and behaviour. Mice that completely

Box 1 | Haploinsufficiency of neurotrophins

NGF$^{+/-}$ mice
- Decreased cholinergic innervation of the hippocampus[15]
- Deficiency in memory acquisition and retention[15]
- Loss of neurons of the peripheral nervous system[121]

BDNF$^{+/-}$ mice
- Hyperphagia, obesity[16–18]
- Impairment of long-term potentiation[19,20,122]
- Elevated striatal dopamine levels[123]
- Loss of mechanosensitivity[124]
- Loss of neurons of the peripheral nervous system[125,126]

NT3$^{+/-}$ mice
- Deficient amygdala kindling activity[127]
- Cardiovascular defects[128]
- Reduced mechanoreceptors[129]
- Loss of neurons of the peripheral nervous system[130]

lack neurotrophins die during the first few weeks following birth. Heterozygous mice in which neurotrophin levels are reduced by half are viable but, strikingly, they show other unanticipated deficits (BOX 1). For example, lowering the level of NGF leads to several deficits in memory acquisition and retention[15]. In the absence of normal levels of BDNF, mice show enhanced aggressiveness, hyperactivity and hyperphagia[16–18]. Intracerebroventricular infusion of BDNF or neurotrophin 4 (NT4) reverses the hyperphagic phenotype[17]. In *BDNF*$^{+/-}$ heterozygous mice, 5-HT (5-hydroxytryptamine, serotonin)-mediated neuronal function is abnormal in the forebrain, cortex, hippocampus and hypothalamus, and administration of the selective 5-HT-reuptake inhibitor fluoxetine reduces the aggressive behaviour, hyperphagia and hyperlocomotor activity[18]. A conditional deletion of BDNF in the brains of postnatal mice also leads to hyperphagia and hyperactivity, as well as to higher levels of anxiety as measured by a LIGHT/DARK EXPLORATION TEST[18]. Therefore, the feeding phenotype and the other behavioural abnormalities are mediated by the action of BDNF in the central nervous system (CNS), not in the periphery. Abnormal behaviours, indicative of impulse-control disorders, are also elicited by partial deletion of BDNF.

Lack of BDNF also causes deficits in memory tasks; for example, *BDNF*$^{+/-}$ mice show impairments in spatial memory. This is consistent with defects in LTP that are found in the hippocampus. Interestingly, *BDNF*$^{-/-}$ and *BDNF*$^{+/-}$ mice show the same deficits in LTP[19,20], indicating that not only the availability of BDNF, but also its levels, can profoundly alter plasticity.

Neurotrophins and their receptors

The neurotrophins are initially synthesized as precursors or pro-neurotrophins, which are cleaved to produce the mature proteins[22]. Pro-neurotrophins are cleaved intracellularly by FURIN or pro-convertases at a highly conserved dibasic amino-acid cleavage site to release carboxy-terminal mature proteins. The mature proteins, which are about 12 kDa in size, form stable, non-covalent dimers, and are normally expressed at very low levels during development. The amino-terminal half (or pro-domain) of the pro-neurotrophin is believed to be important for the proper folding and intracellular sorting of neurotrophins.

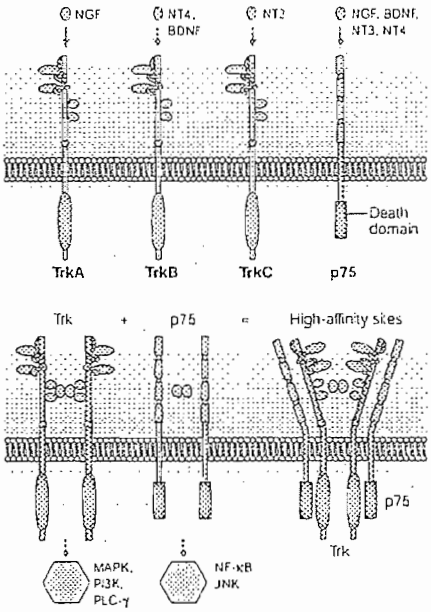


Figure 1 | **Models of Trk and p75 receptor activation.** Neurotrophin binding results in dimerization of each receptor. Neurotrophins bind selectively to specific Trk receptors, whereas all neurotrophins bind to p75. Trk receptors contain extracellular immunoglobulin G (IgG) domains for ligand binding and a catalytic tyrosine kinase sequence in the intracellular domain. Each receptor activates several signal transduction pathways[5,34,35]. The extracellular portion of p75 contains four cysteine-rich repeats, and the intracellular part contains a death domain. Neurotrophin binding to the p75 receptor mediates survival, cell migration and myelination[135] through several signalling pathways[36]. Interactions between Trk and p75 receptors can lead to changes in the binding affinity for neurotrophins[37]. BDNF, brain-derived neurotrophic factor; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; NT, neurotrophin; PI3K, phosphatidylinositol 3-kinase; PLC-γ, phospholipase Cγ.

Receptors encode specificity and responsiveness. Different neurotrophins show binding specificity for particular receptors — NGF binds preferentially to tyrosine receptor kinase A (TrkA); BDNF and NT4 to TrkB; and neurotrophin 3 (NT3) to TrkC (FIG. 1). These interactions have generally been considered to be of high affinity. However, in reality, the binding of NGF to TrkA, and of BDNF to TrkB is of low affinity[23–25], but it can be regulated by receptor dimerization, structural modifications

APOPTOSIS
The process of programmed cell death, characterized by distinctive morphological changes in the nucleus and cytoplasm, chromatin cleavage at regularly spaced sites, and the endonucleolytic cleavage of genomic DNA.

LIGHT/DARK EXPLORATION TEST
This test depends on the natural tendency of rodents to explore the environment in the absence of a threat and to retreat to an enclosed area when fearful. The animals are placed in an apparatus that has a dark and an illuminated compartment. Reduced exploration of the bright compartment and a reduced number of transitions between compartments are commonly interpreted as measures of anxiety.

FURIN
An endopeptidase with specificity for the consensus sequence Arg-X-Lys/Arg-Arg.

KINDLING
An experimental model of epilepsy in which an increased susceptibility to seizures arises after daily focal stimulation of specific brain areas (for example, the amygdala) — stimulation that does not reach the threshold to elicit a seizure by itself.

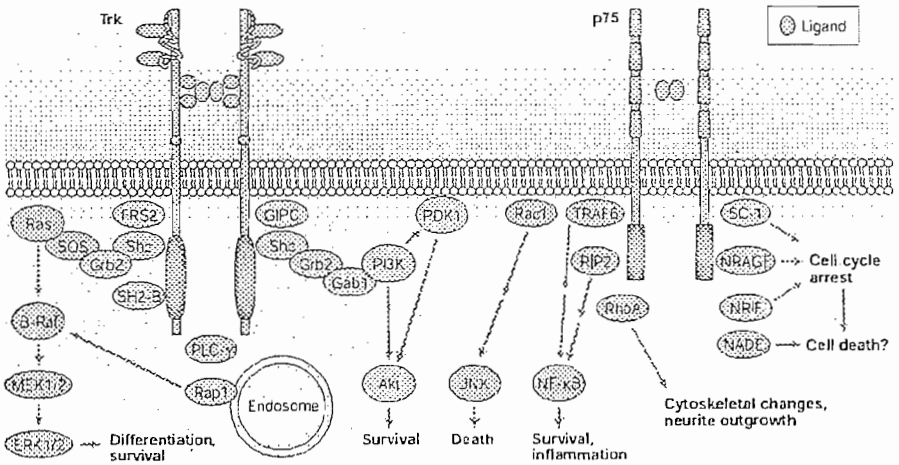


Figure 2 | **Neurotrophin receptor signalling.** Trk receptors mediate differentiation and survival signalling through extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLC-γ) pathways[3,5]. Trk family members recruit and increase the phosphorylation of PLC-γ and Src homologous and collagen-like adaptor protein (Shc), which leads to activation of PI3K and ERK. Rap1 exerts its actions from an endosomal location[136]. The p75 receptor predominantly signals to activate NF-κB and Jun N-terminal kinase (JNK), and modulates RhoA activity. These responses are mediated through adaptor proteins that bind to the cytoplasmic domain of p75, including neurotrophin-receptor interacting factor (NRIF), neurotrophin-associated cell death executor (NADE), neurotrophin-receptor-interacting MAGE homologue (NRAGE), Schwann cell 1 (SC1) and receptor-interacting protein 2 (RIP2)[35,35], which can exert effects on apoptosis, survival, neurite elongation and growth arrest. Akt, protein kinase B; FRS2, fibroblast growth factor receptor substrate 2; Gab1, Grb2-associated binder-1; Grb2, growth factor receptor-bound protein 2; GIPC, GAIP interacting protein, C terminus; MEK, mitogen-activated protein kinase (MAPK)/ERK kinase; PDK1, phosphoinostide-dependent kinase 1; SH2B, Src homology 2-B; SOS, Son of Sevenless; TRAF6, tumour necrosis factor receptor-associated factor 6.

or association with the p75 receptor[26,27]. The p75 receptor can bind to each neurotrophin, and also acts as a co-receptor for Trk receptors (FIG. 1). Expression of p75 can increase the affinity of TrkA for NGF and can enhance its specificity for cognate neurotrophins[28–30]. As a result, increased ligand selectivity can be conferred on the Trk receptors by the p75 receptor.

The ability of Trk and p75 receptors to present different binding sites and affinities to particular neurotrophins determines both their responsiveness and specificity. The ratio of receptors is important in dictating the numbers of surviving cells, and interactions between p75 and Trk receptors provide greater discrimination between different neurotrophins. A similar mechanism is also observed in other ligand–receptor systems, such as the glial-derived neurotrophic factor (GDNF)–Ret receptor[31], in which preferential interactions between GDNF ligands and the Ret receptor are facilitated by expression of GDNF family receptor subunits (GFRα). Not surprisingly, Ret receptors use signalling pathways similar to those used by Trks.

The effects of neurotrophins on axon guidance can also be modulated by the intracellular location of the neurotrophin–receptor complex. During development, neurotrophins are produced and released from the target cells and become internalized into vesicles, which are then transported to the cell body. The biological effects of neurotrophins require that signals be conveyed over long distances, from the nerve terminal to the cell body[32]. Both Trk and p75 receptors undergo retrograde and anterograde transport. Several proteins are associated with the Trk and p75 receptors during transport, and signalling persists after internalization[33]. The proper distribution of these proteins in the growth cone could result from movement of the neurotrophin receptors.

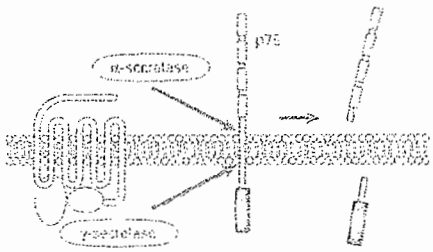
These receptor functions do not necessarily provide an explanation for the numerous phenotypes that are shown by mice that are deficient in neurotrophins. In addition to forming complexes, Trk and p75 receptors show independent signalling properties[5,34–36], and downstream signal transduction pathways significantly contribute to individual physiological responses. Neurotrophins bind as dimers to p75- and Trk-family members. Trk receptor dimerization leads to *trans*-autophosphorylation and to the activation of intracelluiar signalling cascades. The Src homologous and collagen-like (Shc) adaptor protein links the activated Trk receptor to two separate intracellular signalling pathways (FIG. 2). Neuronal survival requires Shc binding to the Trk receptor, which results in increases in phosphatidylinositol 3-kinase (PI3K) and Akt (protein kinase B) activities (FIG. 2). Phosphorylation of Shc by Trk also leads to increases in the activity of Ras and the extracellular signal-regulated kinase (ERK). These events in turn influence transcriptional events, such as the induction of the cyclic AMP-response element binding (CREB) transcription factor. CREB has effects

Box 2 | Processing of the p75 receptor

The p75 receptor undergoes cleavage by metalloproteinases[131] — such as α-secretase — to produce an ectodomain piece and a fragment containing the transmembrane and cytoplasmic domains (see figure). Unexpectedly, this membrane-spanning region is further cleaved by a presenilin-dependent γ-secretase (REF. 132; T.-W. Kim, unpublished observations). The generation of proteins by regulated intramembrane proteolysis is a universal mechanism[133] that acts on several proteins, including the amyloid precursor protein, Notch and the ErbB4 receptor tyrosine kinase. Whether the proteolytic enzymes involved in these events are regulated is not known. However, the generation of a p75 intracellular domain implies that neurotrophins might use regulated intramembrane proteolysis to transmit an intracellular signal. Analogous to Notch, the p75 intracellular domain might function in the nucleus as a transcriptional modifier. The intracellular domain might be involved in activation or repression of neurotrophin-related genes. Several p75 adaptor proteins, such as neurotrophin-receptor interacting factor (NRIF), tumour necrosis factor receptor-associated factors (TRAFs), receptor-interacting protein 2 (RIP2) and Schwann cell 1 (SC1) (FIG. 2), are candidates for nuclear translocation.

As p75 is expressed after nerve damage, in inflammatory conditions such as multiple sclerosis and in neuronal populations that degenerate in Alzheimer's disease, it is tempting to speculate that the γ-secretase cleavage of p75 reflects an early event in the pathogenesis of neurodegenerative diseases that are characterized by a chronic inflammation reaction.

Together with the preponderance of proneurotrophins in Alzheimer's disease-affected tissues[134], proteolytic cleavage of neurotrophins and their receptors represent an intriguing regulatory mechanism for neuronal survival and regeneration during injury and ageing.



on the cell cycle, neurite outgrowth and synaptic plasticity[37]. The small G protein Rap1 accounts for the ability of neurotrophins to signal through ERK for sustained periods[38]. In addition, phospholipase Cγ (PLC-γ) binds to activated Trk receptors and initiates an intracellular signalling cascade, resulting in the release of inositol phosphates and activation of protein kinase C (PKC).

Through a different set of adaptor proteins (FIG. 2), p75 produces increases in Jun N-terminal kinase (JNK), NF-κB and ceramide[66]. One established function of p75 is to promote cell death[15]. This might provide a means for the refinement of correct target innervation during development and eliminate cells during periods of developmental cell death[39]. Apoptosis by p75 is also manifested after seizure or inflammation[40,41]. Injury to the spinal cord leads to oligodendrocyte death that is p75-dependent — a phenomenon that has also been observed in culture[42]. This apoptotic function is accompanied by an increase of Rac and JNK activities (FIG. 2), which are essential for NGF-dependent death[43]. Another function of p75 might be to mediate a non-apoptotic or survival response[44,45], similar to the behaviour of other tumour necrosis factor receptors.

Surprisingly, pro-neurotrophins are more selective ligands for the p75 receptor than mature forms[46], and are more effective at inducing p75-dependent apoptosis[42,47]. This indicates that the biological actions of neurotrophins can be regulated by proteolytic cleavage, with pro-forms preferentially activating p75 to mediate apoptosis and mature forms selectively activating Trk receptors to promote survival. Like the neurotrophins, the p75 receptor can also undergo cleavage (BOX 2).

Many of the components of the pathways that mediate neurotrophin signalling, such as ERK, Akt, PLC, PKC, Ras, JNK and NF-κB (FIG. 2), are not unique to neurotrophins. Each signalling component is used in many different contexts and by other growth factors and cytokines. This complicates the problem of ascribing specific mechanisms to a particular response[47]. Clearly, the effects of neurotrophins depend on various factors — their levels, their affinity of binding to transmembrane receptors, and the duration and intensity of downstream signalling cascades that are stimulated after receptor activation. From these considerations alone, it is still not evident how changes in behaviour and neuronal activity can be explained simply by a 50% reduction in levels of neurotrophins or their signalling components.

Neurotrophin-mediated plasticity

Many observations have indicated that neurotrophins influence both the frequency and amplitude of synaptic currents. Neurotrophins such as BDNF and NT3 produce rapid increases in synaptic strength in nerve–muscle synapses, as well as increases in excitatory post-synaptic currents in hippocampal neurons[48–50]. BDNF and NT3 also induce rapid and long-lasting enhancement of synaptic strength through LTP in hippocampal slices. These effects are not due to the nonspecific effects of using large amounts of proteins in electrophysiological recordings *in vitro*, as mice deficient in BDNF or NT4 show a notable impairment of LTP in hippocampal slices[51,52]. Similarly, the effects on LTP are not due to a developmental or structural alteration created by gene targeting, as normal LTP can be rescued by addition of exogenous BDNF[50,53].

Despite considerable evidence for the effects of neurotrophins on synaptic strength[52–54], there are few molecular and signalling mechanisms that could explain these effects. The use of protein kinase inhibitors has indicated that intracellular protein phosphorylation is important, as well as phosphatidylinositol lipids and inositol-1,4,5-triphosphate ($Ins(1,4,5)P_3$) receptors[55]. A CONDITIONAL MUTATION of the *TrkB* gene results in deficits in memory acquisition and consolidation in several hippocampus-dependent learning tasks[56]. These studies provide convincing evidence that signalling by TrkB receptors is directly responsible for promoting hippocampal LTP[57]. Mutagenesis of the Shc and PLC-γ binding sites in the *TrkB* gene shows that downstream activation of CREB and calcium/calmodulin-dependent kinase II is responsible for the ability of TrkB to modulate LTP. Although Trk receptors are implicated in many forms of neuronal plasticity, there are reports that p75 signalling might also exert some effects on behaviour. Analysis of mice deficient in the full-length p75 receptor

CONDITIONAL MUTATION: A mutation that can be selectively targeted to specific organs (or cell types within an organ) or induced at a specific developmental stage.

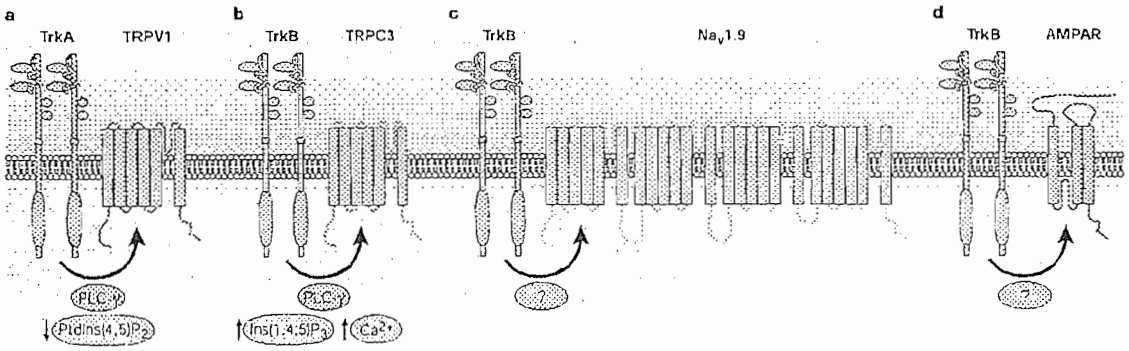


Figure 3 | **Examples of ion channel interactions with Trk neurotrophin receptors.** Several examples of interactions between Trk receptors and ion channels are known. **a** | TrkA with the transient receptor potential (TRP) channel TRPV1 (or VR1)[9]. **b** | TrkB with TRPC3 (REF. 65). **c** | TrkB with $Na_v1.9$ (REF. 77). **d** | α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) activity can be modified by brain-derived neurotrophic factor (BDNF) binding and activation of TrkB receptors[77]. TrkA-mediated PLC-γ activation decreases levels of cellular PIP2, which leads to the opening of TRPV1 channels. In the case of TrkB and TRPC3, TrkB-mediated PLC-γ activity leads to the opening of TRPC3 through inositol-1,4,5-triphosphate ($Ins(1,4,5)P_3$) generation and store-operated calcium release. These associations have been confirmed by co-immunoprecipitation experiments. $PtdIns(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate.

has revealed slight impairments in several learning tasks[58]. Another p75-mutant mouse that lacks both the full-length and the short isoform[59] shows a more severe phenotype and could provide additional insight into the role of p75 in higher-order functions.

However, full explanation of the ability of neurotophins to regulate synaptic plasticity in the adult brain requires a better understanding of how neurotrophin-receptor signalling is linked to ion channel function. Recent analysis of the role of ephrins at synapses has shown that EphB2 receptors can regulate postsynaptic function through an interaction with NMDA (*N*-methyl-D-aspartate) receptors[60–63]. These studies highlight the possibility that other receptor tyrosine kinase systems might regulate ion channel function.

The study of transient receptor potential (TRP) ion channels has provided new insights into this question. The TRP superfamily includes more than twenty cation channels, some of which have been shown to be sensitive to cold and hot temperatures, and to pheromones[64]. TRPC3 is a non-voltage-gated, store-operated cation channel that is highly expressed in brain regions where TrkB receptors are found. Treatment of pontine neurons with BDNF resulted in a delayed inward current after 30 s (REF. 65). This response was specific to BDNF, as other ligands — such as fibroblast growth factor and insulin-like growth factor — did not elicit an increase in cation current. The BDNF-induced current depended on activation of TrkB and PLC (FIG. 3). The biological consequences of interactions between TrkB and TRPC3 have not been fully defined, but the increase in cation flux implies a unique neurotrophin-specific function. The abundant expression of TRPC3 during neonatal development indicates that it might have a role in neurotrophin-dependent plasticity.

Neurotrophins and pain

Another TRP family member that has intimate ties with the Trk receptor is the TRPV1 (VR1) channel or capsaicin receptor, a non-selective cation channel that is activated by heat, noxious vanilloid compounds such as capsaicin, and extracellular protons[66]. Previously, NGF was shown to potentiate the responses of nociceptive sensory neurons to capsaicin[67]. This indicated that crosstalk between capsaicin and NGF occurred within sensory neurons. The idea that TRPV1 channels are necessary for NGF-induced thermal hypersensitivity was also underscored by observations of mice lacking TRPV1. In contrast to NGF-injected normal mice, which showed a marked decrease in paw withdrawal latency in response to a thermal stimulus, injection of NGF into TRPV1-deficient mice did not produce any sensitization[68].

Strikingly, NGF produced an approximately 30-fold increase in proton-evoked currents in *Xenopus* oocytes that co-expressed TrkA and TRPV1. Diminution of phosphatidylinositol-4,5-bisphosphate levels through antibody sequestration or PLC-mediated hydrolysis mimicked the potentiating effects of NGF at the cellular level (FIG. 3). Moreover, recruitment of PLC-γ to TrkA was essential for NGF-mediated potentiation of channel activity (FIG. 3). Co-immunoprecipitation studies indicated that TRPV1 associates with TrkA and PLC-γ to form a complex. As an interaction was also observed between TrkB and TRPC3 (REF. 65), it is likely that common sequences are required for these interactions.

Neurotrophins have been shown to produce acute pain as a side effect in clinical trials for neuropathy and neurodegeneration[69,70]. NGF is present at high levels after inflammation and promotes nociceptor sensitization. These responses might reflect the same process as potentiation of thermal sensitivity by TRPV1 or related heat-activated ion channels. In NGF-responsive

nociceptive sensory neurons, TrkA and TRPV1 are frequently co-expressed. In other neuronal populations, similar mechanisms might account for the pronounced pain that is observed when high levels of neurotrophins are administered in animal models or in human clinical trials.

Other ion channels

Increasing numbers of interactions between Trk receptors and ion channels are being discovered. Increased tyrosine phosphorylation of NMDA and voltage-gated potassium channels occurs as a result of treatment with BDNF[71,72]. In the hippocampus, Trk receptors are expressed both pre- and postsynaptically, and both pre- and postsynaptic mechanisms have been proposed to account for changes in synaptic activity[50,73–76]. Electrophysiological measurements show that BDNF can actually suppress $K_v3.3$ currents[72] and block postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor-mediated currents[77]. The catalytic activity of TrkB receptors is required for the decrease in AMPA receptor activity, implying that there might be a close association between TrkB and AMPA receptors (FIG. 3). Alternatively, the exo- and endocytosis of AMPA receptors[78], which determine activity-dependent changes of synaptic efficacy, could be influenced by BDNF signalling.

One reason for suspecting a direct interaction between Trk receptors and ion channels comes from recent studies in which the sodium channel $Na_v1.9$ was activated by BDNF[79]. The effects of BDNF on hippocampal neurons of the CA1 region are remarkable for the rapidity of their response — an inward sodium current was detected within milliseconds of BDNF treatment. Curiously, the BDNF-mediated increase was blocked by K-252a, a Trk-specific inhibitor[80]. This requirement for receptor tyrosine kinase activity is difficult to reconcile with the time course of $Na_v1.9$ activation, as phosphorylation takes a considerably longer time than the patterns of activity that are stimulated by BDNF. For example, the earliest tyrosine phosphorylation events require nearly a minute of neurotrophin treatment[81]. Also, it takes up to a minute of exposure to NGF to elicit a change in sodium channel mRNA expression[82], a far longer time interval than is required for BDNF to activate the $Na_v1.9$ channel.

Although the exact mechanisms for receptor–ion channel interactions are unknown, the considerations outlined in the previous paragraph indicate that the TrkB BDNF receptor might exist in a complex with the $Na_v1.9$ channel (FIG. 3). Conformational changes in the receptor or the channel might account for the ability of sodium channels to be rapidly influenced by the binding of BDNF to TrkB. Previous studies have indicated that conformational changes in the TrkA receptor might account for changes in its NGF-binding properties[25]. In addition, TrkA receptor dimerization and activation might simply result from a point mutation in the extracellular domain of the receptor[83]. This raises the possibility that changes in Trk structure might be transmitted to neighbouring ion channels.

Transactivation through GPCRs

Although ligand-induced dimerization or oligomerization of receptors is a well-established mechanism for growth factor signalling, there is increasing evidence that biological responses can be mediated by two or more receptor systems. For some time, it has been appreciated that heterotrimeric G-protein-coupled receptors (GPCRs) produce similar responses (in terms of cell growth) to other growth factors that use receptor tyrosine kinases[84,85].

Activation of Trk neurotrophin receptors occurs after treatment with adenosine, a neuromodulator that acts through GPCRs. Trk receptor autophosphorylation is increased in hippocampal neurons and PC12 cells after treatment with adenosine. This transactivation requires adenosine A_{2A} receptors[86], and does not result from the production of neurotrophins. The increase in Trk activity was inhibited by protein kinase inhibitors, such as PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolol-[3,4-*d*]-pyrimidine, which is specific for Src family members) or K-252a. The pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide, can also transactivate Trk receptors in a manner similar to transactivation by adenosine[87]. PACAP occurs in two forms, one of 38 and one of 27 amino acids, and is a member of the vasoactive intestinal peptide/secretin/glucagon family. The two PACAP peptides also interact with GPCRs.

The effects of adenosine and PACAP are specific, as other GPCR ligands do not participate in crosstalk with Trk receptors. Bradykinin, carbachol, ATP, apomorphine, quinpirole and angiotensin II do not cause TrkA activation[86], even though receptors for these ligands are expressed on the same cells as those for adenosine and PACAP. By contrast, many of these ligands can stimulate epidermal growth factor (EGF) receptors and other mitogenic growth factor receptors. Conversely, adenosine and its agonists do not activate EGF receptors.

These GPCR transactivation events are unique in other ways. Both adenosine and PACAP require a long period of time (more than 1–2 hours) to activate Trk tyrosine kinase activity. Both ligands produce an activation of PI3K and Akt, which results in enhanced cell survival after withdrawal of NGF. These results provide an explanation for the neuroprotective actions of adenosine and PACAP, and point to a therapeutic use for small-molecule GPCR agonists in neurodegenerative disorders. For example, activation of Trk receptors by PACAP was also observed in primary cultures of basal forebrain cholinergic neurons, and administration of PACAP effectively rescued these neurons after fimbria–fornix lesion *in vivo*[88]. These results are significant, because NGF-responsive cholinergic neurons in the basal forebrain degenerate in Alzheimer's disease[89].

What is the physiological relevance to neurotrophin action of transactivation by GPCR signalling? Transactivation might explain why neuronal survival in the CNS is not adversely affected by the lack of neurotrophins — GPCR ligands might compensate by providing a survival function through a neurotrophin-receptor signalling pathway. Also, other essential activities, such

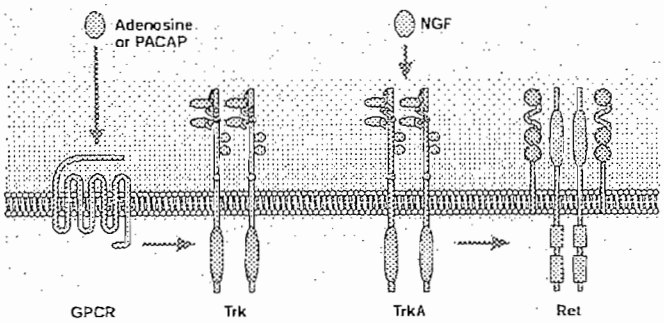


Figure 4 | **Transactivation of receptor tyrosine kinases.** Transactivation of Trk receptor by G-protein-coupled (GPC) ligands — such as adenosine and pituitary adenylate cyclase-activating polypeptide (PACAP) — results in neuroprotection[84,137]. In sympathetic neurons, binding of nerve growth factor (NGF) to TrkA results in the activation of Ret tyrosine kinase receptors[95]. GPCR, G-protein-coupled receptor.

as the regulation of ion channels, might be legitimate actions for transactivated receptor signalling. Indeed, dopamine–GPCR transactivation of platelet-derived growth factor receptors has an acute effect on NMDA ion channel activity in hippocampal neurons[90].

Importantly, mutations in components of the adenosine or PACAP signalling pathways give rise to behavioural problems in learning and memory[91,92] and heightened aggression[93], which are reminiscent of the effects of mutations in the BDNF and TrkB receptor genes[16,17,20,56,75]. These striking similarities imply that adenosine and PACAP signalling might work in parallel or converge with neurotrophin receptor action. These similarities also imply that Trk receptors act as convergence points for signals emanating from other receptor systems. In this manner, Trk receptors act to survey various inputs, in addition to those from neurotrophins.

Crosstalk between different transmembrane receptors might represent a more common signalling mechanism. Further to the influence that GPCRs exert upon Trk receptor activity, Trk receptors can activate other seemingly unrelated receptors. An unusual case is the Ret tyrosine kinase receptor (FIG. 4), which is a common signalling receptor for GDNF-related ligands that also include artemin, neurturin and persephin[94]. These ligands require specific GFRα subunits to confer ligand specificity. However, in postnatal sympathetic neurons, NGF produces an activation of Ret receptors over the course of 1–2 days, which does not require GDNF ligand binding[95]. Activation of Ret signalling provides additional survival advantages during postnatal periods when these sympathetic neurons become independent of NGF. Transactivation of Ret tyrosine kinases by binding of NGF to the TrkA receptor represents a new mechanism for transmitting survival signals within neurons.

Therefore, there are ways of activating Trk and Ret tyrosine kinase receptors other than direct ligand binding. Activation of the neurotrophin system through other receptor signalling systems is an alternative mechanism of communication in the nervous system, and examples of this crosstalk abound. For example, antidepressant agents that act through monoamine GPCRs can cause increased expression of both neurotrophins and neurotrophin receptors[96]. Notably, only the neurons that express the monoamine GPCRs have the capacity to enhance neurotrophin or Trk receptor levels. The results of studies with GPCR ligands raise the possibility of using small molecules to elicit neurotrophic effects in the treatment of neurodegenerative diseases[70]. This approach would allow selective targeting of neurons that express specific GPCRs and trophic factor receptors.

Regeneration

Proteins that modulate growth cone dynamics have an important role in axonal patterning during development, and in preventing regeneration of axons following injury. Considerable attention has been given to Nogo, myelin-associated glycoprotein (MAG) and semaphorin 3A — proteins that provide potent inhibitory signals for axonal growth. There is increasing evidence that places neurotrophin receptors in the realm of these inhibitory proteins.

Neurotrophins can modulate the response of growth cones to inhibitory axon-guidance molecules. For example, neurotrophins have been shown to affect the extent of the axonal response to MAG[97]. Moreover, semaphorin 3A induces the collapse of dorsal-root ganglion (DRG) and sympathetic growth cones[98], and neurotrophins can rapidly modulate the response of DRG growth cones to semaphorin 3A (REF. 99). The sensitivity of DRG growth cones to semaphorin 3A is influenced by BDNF and NGF in distinct ways — BDNF increases the sensitivity of DRG growth cones to semaphorin 3A, whereas NGF decreases it. These effects depend on Trk signalling, implying that TrkA and TrkB exert differential effects on semaphorin 3A signalling. Furthermore, the effects of NGF in opposing the inhibitory action of semaphorin 3A are highly dependent on ligand concentration and downstream signalling through the activities of protein kinase A and protein kinase G (REF. 100). These observations indicate the operation of a mechanism in which the receptors for neurotrophins and semaphorins are functionally linked.

Like the Trk receptors, p75 interacts with some unlikely partners. Nogo-A is an important inhibitory protein that is expressed in oligodendroycles. It binds to a glycosylphosphatidylinositol-linked receptor[101] that is recognized by a 66-amino-acid fragment of Nogo (Nogo-66). Unexpectedly, there are other ligands for this Nogo receptor, including oligodendrocyte myelin glycoprotein[102] and MAG[103,104]. The absence of a cytoplasmic domain in the structure of the Nogo receptor implies that other components are involved in signalling, and association of the Nogo receptor with the p75 receptor has proved to be a surprising and intriguing solution to this problem[105,106].

The identification of p75 as a co-receptor of the Nogo receptor was based on several key observations.

POLYMORPHISM
The simultaneous existence in the same population of two or more genotypes in frequencies that cannot be explained by recurrent mutations.

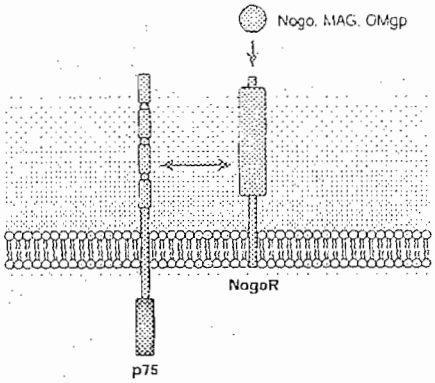


Figure 5 | **Neurotrophins and p75 undergo site-specific cleavages.** The Nogo and p75 receptors are found in a complex. Nogo[138], myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) are all ligands for the Nogo receptor (NogoR)[102,104]. Inhibition of neurite outgrowth by MAG is mediated by NogoR and the p75 receptor[105,106].

First, MAG serves as a ligand for the Nogo receptor. Second, the inhibitory effects of MAG were found to depend on the presence and action of the p75 receptor[107]. The Nogo receptor is closely associated with p75 through interactions between the extracellular domains of the two proteins (FIG. 5), and this association produces a repulsive effect on axonal growth. Together, these results indicate that myelin-dependent inhibition of axonal regeneration depends on the binding of MAG to a complex containing the Nogo and p75 receptors. Several different proteins bind to the cytoplasmic domain of p75 (FIG. 2); among them, RhoA is the most relevant to the inhibition of neurite outgrowth and growth cone collapse. Indeed, earlier work indicated that binding of p75 to RhoA influences axonal growth[108]. The inhibitory influence of MAG and Nogo might be explained by the recruitment of p75 into a complex that sends a repulsive signal in neurons. The participation of p75 in the prevention of regeneration following injury is plausible because the absence of p75 leads to sprouting and enhanced axonal growth and density[109].

Table 1 | **Induction of p75 receptor expression after injury**

Cell type	Injury
Motor neurons	Axotomy, regeneration[139–141]
Purkinje neurons	Traumatic injury[142]
Entorhinal neurons	Seizure[43]
Hippocampal neurons	Primary culture[143]
Striatal neurons	Ischaemia[144]
Cortical neurons	Zinc, ischaemia[145], Alzheimer's disease[146]
Schwann cells	Axotomy[147,148]
Oligodendrocytes	Spinal cord injury[42], multiple sclerosis lesions[40,149]

p75 expression is also induced in experimental allergic encephalomyelitis[150,151] and Alzheimer's disease[152].

The participation of p75 receptors in the axonal regeneration process provides further insight into the function of these receptors. There are many examples of elevated p75 expression in the adult brain and spinal cord after injury, inflammation and stress[6,110]. Interestingly, many cell types — including hippocampal and cortical neurons, oligodendrocytes and microglial cells — ordinarily show low levels of p75. However, after ischaemia, seizure, axotomy or other forms of stress, the expression of this receptor is considerably elevated in these types of cells (TABLE 1). Also, many cell types express p75 in culture, presumably owing to the change in environmental conditions. In fact, the magnitude of NF-κB signalling through p75 is highly dependent on whether cells have experienced stress, such as changes in serum, temperature or cell–cell contact[111].

Neurotrophins and disease

Few associations have been found between neurotrophin genes and neurological or psychiatric disorders, although a recent series of studies has linked a POLYMORPHISM in the pro-domain of BDNF with depression, bipolar disorders and schizophrenia. This polymorphism — which was identified from a single nucleotide polymorphism screen — is caused by a single amino-acid change, from valine (Val) to methionine (Met), at position 66 in the pro-domain of the BDNF protein[112–114]. In patients with bipolar disorder or depression, the *Val* allele seems to confer greater risk for the disease, whereas in patients with schizophrenia, the *Met* allele seems to be associated with impaired memory functions. The existence of mutations in BDNF — a highly conserved protein — implicates neurotrophins in the complex pathophysiology of psychiatric diseases[115], as well as neurodegenerative diseases such as Alzheimer's disease[116]. Cleavage of the p75 receptor has also been implicated in the pathogenesis of Alzheimer's disease (BOX 2).

An analysis of the Val→Met change in the pro-BDNF protein indicated that this alteration is responsible for abnormal sorting and secretion of BDNF[113]. The impact of this BDNF genotype was followed in human subjects who were examined for alterations in episodic memory. Individuals in which the pro-domain of BDNF has Met at position 66 performed relatively poorly in verbal episodic memory tests, and functional magnetic resonance imaging of hippocampal function showed an abnormal pattern of activation during cognitive tests. These effects of this polymorphism in BDNF indicate that neurotrophins can participate in hippocampal function and memory through a mechanism that relies on correct BDNF secretion. So, activity-dependent secretion of BDNF, and its subsequent effects on LTP and synaptic plasticity now have an important correlate in the human population.

An unexpected example of the involvement of neurotrophins in psychiatric disorders has come from the pathophysiology of depression, especially when depression is associated with stress. Several lines of evidence have implicated neurotrophins in depression. First, in animal models, restraint stress leads to decreased expression of BDNF in the hippocampus[117,118]. Second, the

LEARNED HELPLESSNESS
A commonly used model of depression in which animals are exposed to inescapable shock and subsequently tested for deficits in learning a shock-avoidance task. Learned helplessness is a rare example in which, rather than working from the psychiatric disorder to the model, the behavioural effect was originally discovered in experimental animals (dogs) and later invoked to explain depression.

administration of BDNF to the midbrain or hippocampus results in antidepressant effects in animal models of depression — forced swim and LEARNED HELPLESSNESS. This effect is comparable to chronic treatment with pharmacological antidepressants[119]. Third, BDNF has been shown to have trophic effects on 5-HT and noradrenergic neurons. Mutant mice with decreased levels of BDNF show a selective decrement in the function of 5-HT neurons and behavioural dysfunctions that are consistent with serotonergic abnormalities.

Many functions of the neurotrophic factors in the adult nervous system — other than their effects on neuronal survival — have now been elucidated. These functions include the maintenance of differentiated neuronal phenotypes, regulation of synaptic connections, activity-dependent synaptic plasticity, and neurotransmission. These additional functions show that neurotrophin receptors act as a point of convergence that might be involved in the integration of many environmental inputs. This can lead to alterations in neuronal circuitry and, ultimately, in behaviour. In particular, it has become clear that neurotrophins can produce long-term changes in the functionality of adult neurons through changes in transcription. As several psychotropic drugs affect neurotrophin signalling, this ability might help to explain the delay in therapeutic action of many psychiatric treatments.

Perspectives

To explain the complex behavioural effects that are related to the function of neurotrophins, an understanding of how local circuits and signal transduction pathways are integrated is required. Neurotrophins show both rapid and slow effects that are breathtaking in their scope and duration, but need to be further differentiated and defined. Cell-surface receptors are generally represented as isolated integral membrane proteins that span the lipid bilayer, with closely associated receptor components and with signal transduction proceeding in a linear stepwise fashion. This view of receptor function will undoubtedly be modified in the future. Neurotrophins provide an excellent example of how receptors can act not only in a linear manner, but can also influence the activity of other transmembrane molecules, either directly or through signalling intermediates. The description of these actions will require new methods of computational analysis, such as the effort to describe activity-dependent neurotrophic interactions by mathematical modelling[120].

Cell–cell communication represents the combined effects of many growth factors. Unlike studies that have been carried out *in vitro*, in which cell lines are treated with single factors, the growth and survival of cells *in vivo* are under the influence of the simultaneous actions of many polypeptide factors. Cooperativity between just two different transmembrane proteins implies that the possibilities for extracellular signalling are greatly expanded. In reality, the regulation of trophic activities is probably determined by the additive effects of many receptors and the duration of signalling events, as well as by protein cleavage events. The lack of a detectable effect on cell numbers in mice that are deficient in key growth factors also indicates that cell growth and survival are supported by multiple proteins. Interactions between neurotrophin receptors and ion channels and other cell-surface proteins provide a powerful mechanism for merging the actions of different ligand–receptor systems to achieve new cellular outcomes.

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HISTOLOGY OF THE PERIPHERAL NERVE AND CHANGES OCCURRING DURING NERVE REGENERATION

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Peripheral nerves are complex organs that can be found throughout the body reaching almost all tissues and organs to provide motor and/or sensory innervation. A parenchyma (the *noble* component made by the nerve fibers, i.e., axons and Schwann cells) and a stroma (the *scaffold* made of various connective elements) can be recognized.

Although morphological analysis is the most common approach for studying peripheral nerve regeneration, researchers are not always aware of several histological peculiarities of these organs. Therefore, the aim of this review is to describe, at a structural and ultrastructural level, the main features of the parenchyma and the stroma of the normal undamaged nerve as well as the most important morphological changes that occur after nerve damage and during posttraumatic nerve regeneration. The paper is aimed at providing the reader with the basic framework information on nerve morphology. This would enable the correct interpretation of morphological data obtained by many experimental studies on peripheral nerve repair and regeneration such as those outlined in

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several other papers included in this special issue of the *International Review of Neurobiology*.

I. Introduction

Peripheral nerves are organs that expand throughout the body, forming a complex arborization that very much resembles that found in blood vessels (Fig. 1), sharing with it developmental pathways (Zacchigna *et al.*, 2008). The peripheral nerves emerging from the central nervous system (CNS) are divided into two

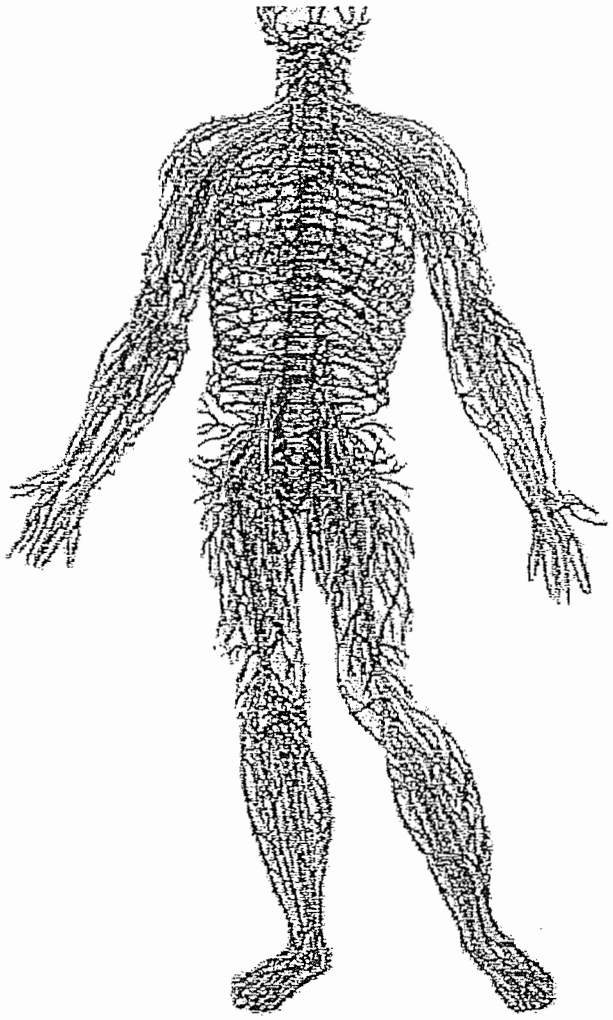

FIG. 1. Peripheral nerves expand throughout the body, forming a complex arborization that very much resembles that found in blood vessels. Taken from Vesalius (1514–1564).

categories: the cranial and the spinal nerves. Multiple branches originate from these main stems, and terminals reach all body districts. Although nerve trunks located in the various parts of the body differ with respect to the fiber-type composition (and thus functional significance) and the presence and number of fascicles (Sunderland, 1978; Sunderland and Bradley, 1949), the morphology of these nerve trunks is relatively similar in all districts (Lundborg, 2004) with the only exception being the first two cranial nerves, namely the olfactory and optic nerves.

Peripheral nerves are usually classified into three main categories, depending on fiber-type composition: (i) sensory, (ii) motor, and (iii) mixed nerves (Williams, 1999). With only few exceptions (VIII cranial nerve and the mesencephalic root of the V cranial nerve), sensory nerve fibers originate from pseudounipolar neurons located in the sensory ganglia. On the other hand, motor nerve fibers originate from somatic and autonomic motor neurons located in the CNS. While somatic motor fibers directly reach the target skeletal muscle fibers, autonomic motor fibers create synapses in an ortho- or parasympathetic ganglion where the second-order autonomic neuron is located and the axon of which eventually reaches the target visceral organs (Williams, 1999).

The aim of this paper is to describe and illustrate the main structural and ultrastructural features of the peripheral nerve. In addition, different diseases can affect peripheral nerves which spread their branches and endings throughout the whole body. This makes these organs particularly vulnerable to traumatic damage and thus, the second part of this article, we will point out the most important changes that occur during posttraumatic nerve regeneration. The technical issues concerning morphological analysis of nerves will not be addressed in this review because they are described in detail in an accompanying methodology-oriented paper (Raimondo *et al.*, 2009, this issue).

II. Structure and Ultrastructure of the Peripheral Nerve

It is possible to speculate that a nerve morphologically recall the same organization of a parenchymatous organ since, like other parenchymatous organs, a parenchyma and a stroma can be distinguished in the peripheral nerve. The former is represented by nerve fibers made by axons and the surrounding Schwann cells, the *noble* component of the nerve, while the stroma is composed by several connective elements some of which (the perineurial cells) are peculiar of the nerve.

In the remainder of this chapter, we will discuss the nerve morphology in normal conditions, while in chapter III the changes occurring during regeneration will be addressed. All descriptions refer to the adult since the developmental aspects of nerves are addressed in a dedicated article (Kaplan *et al.*, 2009, this issue).

A. The "Parenchyma" of the Nerve

The smallest functional unit of a peripheral nerve is the nerve fiber. Several schemes of classification of peripheral nerve fibers have been used, based on various parameters such as conduction velocity, function, fiber diameter, and other attributes. Anatomically, the strategy adopted from Schwann cells to enclose axons allows us to distinguish two subgroups of fibers: myelinated and unmyelinated nerve fibers (Fig. 2A–D). All larger mammalian axons are myelinated; myelin is responsible for the glistening whiteness of peripheral nerves and central white matter. Axons smaller than 1 μm in diameter are usually unmyelinated.

1. *Myelinated Nerve Fibers*

Myelinated nerve fibers consist of a single axon that is enveloped individually by a single Schwann cell. The membrane of this Schwann cell wraps around the nerve fiber to form a multilaminated myelin sheath.

Within the peripheral nervous system (PNS), myelin is produced by the Schwann cells. The myelin sheath can be thought of as a flat glial process that spirally wraps around the axon (Fig. 2A). The intracellular and extracellular spaces of the glial process are lost as the external and internal faces of the membrane become tightly apposed. In electron microscopy, the compacted external surfaces of myelin are seen as minor dense lines that alternate with the compacted inner cytoplasmic surfaces corresponding to the major dense lines (Fig. 2E and F). The inner and outer zones of occlusion of the spiral process are continuous with the minor dense line and are called the inner and outer mesaxons (Fig. 2G and H, arrows). The major dense line is continuous with the cytoplasmic face of the membrane at all regions where compaction is lost and appears to be quite stable. In contrast, the minor dense line appears to be labile (Blaurock *et al.*, 1986; Napolitano and Scallen, 1969; Williams and Hall, 1971).

In myelinated fibers, the territory of the Schwann cell defines an internode, the interval between internodes being the Ranvier's node (Williams, 1999). The internodal length varies directly with the diameter of the fibers, from 150 to 1500 μm (Kashef, 1966). In the PNS, the myelin sheaths on both side of a node terminate in paranodal bulbs, which often show an asymmetry related to growth. The surface of the bulbs is fluted as they approach the nodes. The grooves in the external surface of the myelin sheath that are produced by fluting are filled by Schwann cell cytoplasm, which is rich in mitochondria (Berthold, 1968; Landon and Williams, 1963). Each myelinated segment is separated from the enclosed axon by a narrow periaxonal space (15–20 nm), which, although nominally part of the extracellular space, is functionally isolated from the extracellular space at the paranodes.

Along the interparanodal myelin in normal myelinated fibers, we see oblique interruptions in which the membrane compaction is lost. These oblique

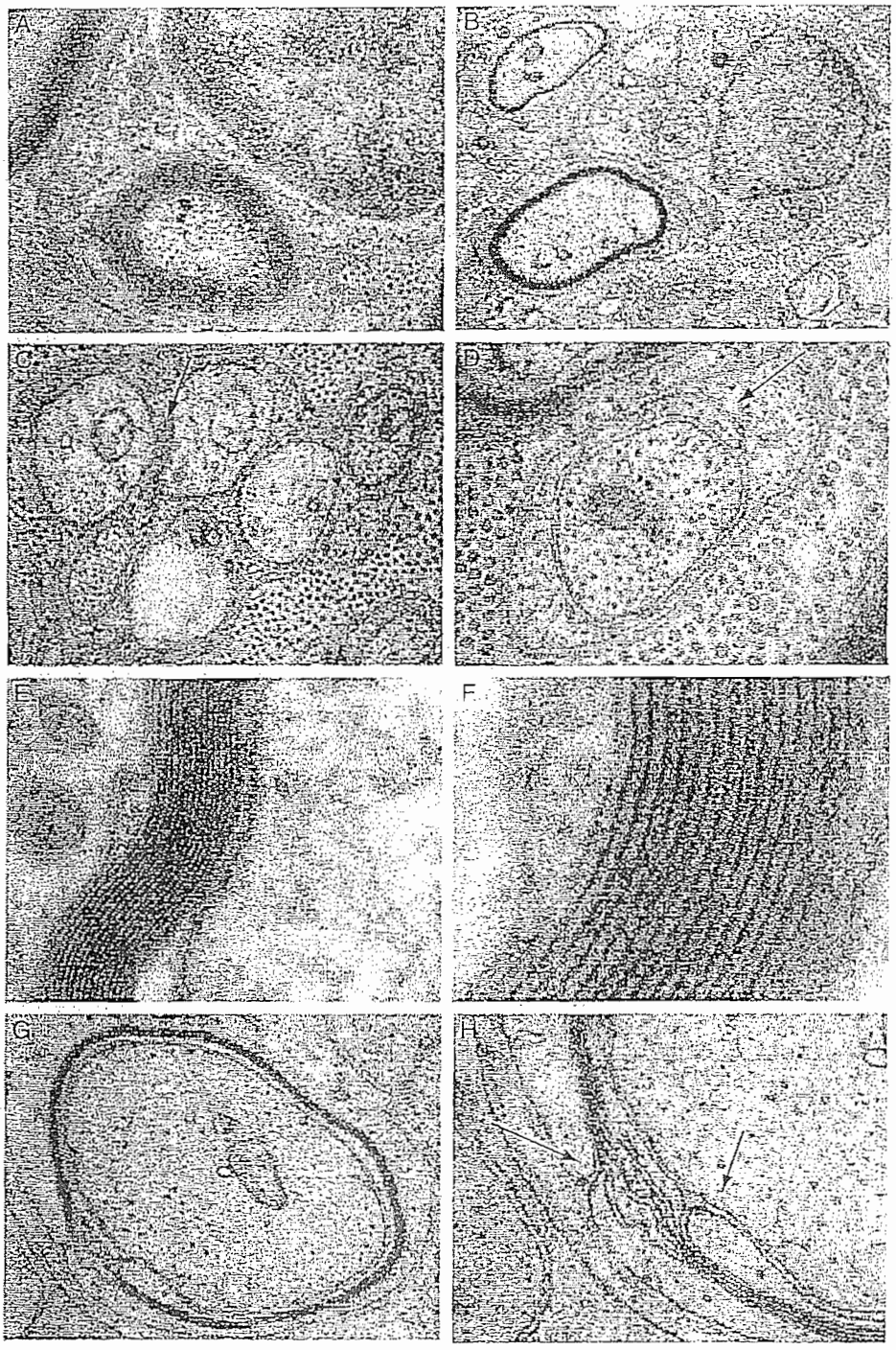

FIG. 2. Myelinated and unmyelinated fibers are shown in electron microscopy. The membrane of a Schwann cell wraps around a single axon forming a multilaminated myelin sheath (A). Unmyelinated fibers are shown in (C). The arrows point to tongues of Schwann cell cytoplasm that separate the axons from each other and ends forming mesoaxons (D, arrow). At higher magnification, the compacted minor dense lines alternated with the mayor dense lines forming the myelin are detectable (E, F). The inner and outer mesoaxons corresponding to the inner and outer zones of occlusion of the spiral process are shown (G, H, arrows).

interruptions are called Schmidt-Lanterman incisures (Williams, 1999). The dimensions and relationships of the myelinated segment features are altered to varying degrees in pathological conditions. Thus, following nerve crush or the induction of primary demyelination the paranodal myelin loses contact with the axon and the Schmidt-Lanterman incisures dilate as the adjacent minor dense line opens. This causes an irreversible collapse of the myelin periodicity (Hall and Gregson, 1971; Williams and Hall, 1971).

In general, myelination is seen only in axons above a certain diameter, about 1.5 μm in the PNS and 1 μm in the CNS (Matthews, 1968). Axonal diameter was thought to be critical in determining myelination; however, since there is considerable overlap between the size of the smallest myelinated and the largest unmyelinated axons, axonal caliber is unlikely to be the only factor.

2. *Unmyelinated Nerve Fibers*

Unmyelinated nerve fibers are composed of several nerve axons enveloped as a group by a single Schwann cell (Fig. 2B and C).

In cutaneous nerves and dorsal spinal roots, about 75% of mammalian axons are unmyelinated. They structure about 50% of the fibers of nerves projecting to muscles and 30% of the nerve bundles in ventral spinal roots. Autonomic postganglionic axons are almost exclusively unmyelinated. Unmyelinated axons are small (0.15–2.00 μm in diameter) and grouped within a sequential series of Schwann cells. In mature nerves, the mode of enclosure of each group of axons shows inter- and intraspecific variation. Axons are usually separated from each other by tongues of Schwann cell cytoplasm (Fig. 2C, arrow), but these axons are sometimes further isolated by separate processes of cytoplasm, that converge in the perinuclear region (Gamble and Eames, 1964). The line of invagination during development is marked by a mesoaxon (Fig. 2D, arrow), a double layer of Schwann cell plasma membrane. At the exterior of the Schwann cell, the layers separate and are continuous with the plasma membrane. Because of this arrangement, endoneurial tissue fluid reaches the periaxonal spaces between the mesoaxonal membranes. These intercellular spaces allow the movement of ions when action potentials are conducted along the enclosed axon. In the absence of a myelin sheath and nodes, salutatory conduction does not occur, and the interrupted passage of impulses is very slow, with velocity about 0.5–4.0 m/s.

A three-dimensional reconstruction from sections of somatic autonomic nerves revealed that the spatial relationships between axons and Schwann cells alter continuously within each cell (Aguayo *et al.*, 1973). The transfer of axons between Schwann cells usually occurs at the extremities of adjacent glial cells, where their cytoplasmic processes interdigitate (Gamble *et al.*, 1978).

B. The "Stroma" of the Nerve: Nerve Fibers

Unlike the CNS where connective tissue is mostly localized at the meningeal level all around the nervous tissue, in the PNS neurons, axons and glial cells are surrounded and supported by a reach connective scaffold as a support for an adequate resistance to stretch and compression forces applied during body movements.

Classically, nerve trunks, whether uni- or multifascicular, are surrounded by an epineurium; individual fasciculi are enclosed by a multilayered perineurium, which in turn surrounds the endoneurium or intrafascicular connective tissue (Fig. 3).

The epineurium is a supporting and protective connective tissue carrying the main supply channels of the intraneural vascular system: the vasa nervorum, which pass across the perineurium to communicate with the network of arterioles and venules within the endoneurium. Embriologically, the epineurium is derived from mesoderm. In human, the epineurium normally constitutes the 30–70% of the total cross-sectional area of the nerve bundle. As a general rule, the more fasciculi present in a peripheral nerve, the thicker the epineurium. The relative amount of epineurium varies among nerves, levels, and individuals (Sunderland, 1978; Sunderland and Bradley, 1949). Around the joints epineurium is often more abundant than elsewhere.

This connective tissue contains fibroblasts, collagen (types I and II), and variable amounts of fat, which seems to have a role in protecting the nerve this tissue surrounds.

The perineurium is a dense and mechanically strong sheath that surrounds each fascicle (Key and Retzius, 1876).

This sheath extends from the CNS–PNS transitional zone to the periphery, where it continues with the capsules of muscle spindles and the encapsulated sensory endings. At unencapsulated endings and neuromuscular junctions the perineurium ends open. This may be a critical point for the entry into the endoneurial space of substances that otherwise could not penetrate the perineurium along the course of the nerve. The perineurium consists of alternating layers of flattened polygonal cells and collagen: up to 15 layers are present around the fascicles of mammalian nerve trunks (Akert *et al.*, 1976; Thomas and Jones, 1967; Thomas and Olsson, 1984). Each cell layer is enclosed by a basal lamina. The cell layers are separated by spaces containing longitudinally oriented capillaries. Collagen fibrils and elastic fibers are located in the same spaces (Thomas and Jones, 1967). According to many studies, the epithelium-like flattened cells represent only an inner part of the true perineurium, whereas this cellular part of the perineurial sheath is encircled by an outer layer containing fibrous tissue gradually merging onto the connective tissue of the epineurium (Millesi and Terzis, 1984; Sunderland, 1978). This distinction is important from the surgical point of view, because it should be possible to place sutures in the perineurial membrane

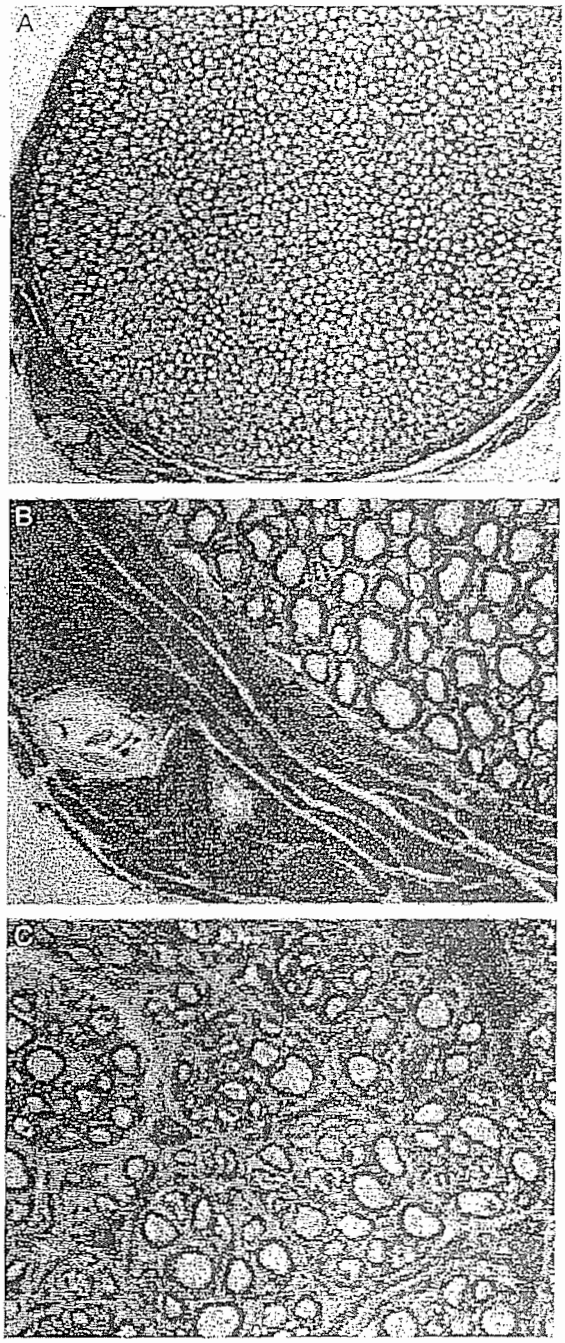

FIG. 3. Transverse sections of a rat peripheral nerve stained with toluidine blue. The nerve fasciculi, the epi-, peri-, and endoneurial connective tissue sheaths are shown at lower (A) and higher magnification (B, C). The epineurium supports and contains all the nerves carrying the main intraneural vascular system: the vasa nervorum (B). The perineurium and endoneurium are particularly evident in a distal stump of a regenerated nerve where compartmentation occurs (C).

without penetrating the layer. These cells characteristically contain numerous pinocytotic vesicles and often bundles of microfilaments. This finding, associated with the fact that perineurium cells are rich in phosphorylating enzymes, underlies the fact that perineurium functions as a metabolically active diffusion barrier. It is probable that the perineurium together with the blood–nerve barrier plays an essential role in maintaining the osmotic milieu and the fluid pressure within the endoneurium (Williams, 1999).

The mechanical strength of the perineurium is impressive. The intrafascicular pressure can be experimentally raised 300–750 mm Hg before rupture of the perineurial membrane occurs (Selander and Sjöstrand, 1978).

The endoneurium represents a loose, soft, connective tissue that embeds and protects the fascicles, cushioning them during the movements of an extremity, and protecting them against external trauma (Lundborg, 2004). The endoneurium is a loose collagenous matrix with large extracellular spaces. The matrix contains fibroblasts, macrophages, mast cells, extracellular matrix components (collagen fiber, mucopolysaccharide ground substance), and a capillary network (Thomas *et al.*, 1993). The fibrous and cellular components of the endoneurium are bathed in endoneurial fluid (Low, 1984). Endoneurial fluid pressure is slightly higher than that of the surrounding epineurium. It is believed that the resulting pressure gradients function to minimize endoneurial contamination by toxic substances external to the nerve bundle (Powell *et al.*, 1979).

Most of the cell population in the endoneurium consists of Schwann cells and endothelial cells, while fibroblasts make up only 4% of the total (Causey and Barton, 1959). In the endoneurium, the collagen fibrils are closely packed around each nerve fiber to form the supporting walls of the "endoneurial tubes."

III. Morphological Changes after Nerve Damage and Regeneration

Trauma to peripheral nerve trunks may result in various extents of nerve fiber injury. The axonal fate is a critical factor in determining the extent, time course, and recovery following nerve injury.

After a peripheral nerve sustains a traumatic injury, complex pathophysiologic changes, including morphologic and metabolic changes, occur at the injury site. These complex changes also occur in the nerve cell body, in the segments proximal and distal to the injury site, and in the distal endings of both muscle end-plates and sensory receptors. Changes in the nerve at the site of injury begin almost immediately. With crushing or transection of a nerve trunk, significant changes take place in normal morphology and tissue organization proximally and distally to the lesion. In the following sections, the main changes occurring in the segments proximal and distal to the injury site will be separately analyzed.

A. The Proximal Nerve Segment

Transection of an axon means amputation of a major part of the axoplasmic volume from the cell. It is therefore not surprising that such a traumatic event may lead not only to profound changes in cell body structure and function but also to cell death (Purves and Nja, 1978). These changes occur in both the dorsal root ganglia sensory neurons and in the motor neurons of the spinal cord anterior horn.

Changes can be seen in the nerve cell body as early as several hours after the injury. The series of morphologic changes that ensue in the cell body after injury are known as chromatolysis, and they entail cell body and nucleolar swelling, and nuclear eccentricity. All of these changes involve an alteration of the metabolic machinery from being primarily concerned with transmitting nerve impulses to fabricating structural components for reconstruction of the injured nerve (Ducker *et al.*, 1969; Lieberman, 1971). The neurons switch from a "signaling mode" to a "growing mode" (Fu and Gordon, 1997), and protein synthesis switches from neurotransmitter-related substances to those required for axonal reconstruction (Müller and Stoll, 1998; Terzis and Smith, 1990). Metabolic changes include altered synthesis of many neuropeptides (Hökfelt *et al.*, 1994) and changes in synthesis of cytoskeletal proteins (Fornaro *et al.*, 2008; Tetzlaff *et al.*, 1988) and growth-associated proteins (Schreyer and Skene, 1991; Tetzlaff *et al.*, 1991).

In the proximal segment, axons degenerate for some distance back from the site of injury, leaving the corresponding endoneurial tubes (the basal laminae of the Schwann cell) behind as empty cylinders. This retrograde degeneration may extend over one or several internodal segments, the length depending on the severity of the lesion (Cajal, 1928).

Within hours after injury, the axon in the proximal segment produces a great number of collateral and terminal sprouts that advance distally along the tube on the inside of the basal lamina (Fawcett and Keynes, 1990; Mira, 1984). The terminal sprouts arise from the tip of the remaining axon. Within hours of axotomy, small axoplasmic outgrowths have been observed from axoplasmic tips (Zelená *et al.*, 1968). This first wave of sprouts is followed by a second wave, appearing within the first 2 days (Cajal, 1928; Grafstein and McQuarrie, 1978; Mira, 1984). Early sprouts can apparently degenerate before the definitive sprouting phase occurs. The time required for the definite sprouts to appear has been called the "initial delay" (Sunderland, 1978). A recent study on rat regenerating sciatic nerve (Witzel and Brushart, 2003) showed that sprouts have great variability in their behavior. There were "direct" projections (i.e., single sprouts crossing the gap), often traveling laterally in the interstump gap before entering a distal Schwann cell tube. "Arborizing" projections, in contrast, sampled 5–10 distal tubes from among more than 100 within their 50- to 100-μm spread. A single axon traveling within distal Schwann cell tubes continued to sprout

collaterals, suggesting that the process of sprouting is a natural concomitant of regeneration. Schwann cell tubes in the distal segment were sometimes reinnervated by sprouts from several different parent axons.

Recent research shows that Schwann cells play an important role in nerve regeneration at the site of injury. Schwann cells elaborate processes that include physical conduits that guide axons to their targets. The rate of axon regeneration is limited by the extension of these Schwann cell processes rather than by axonal growth (Son and Thompson, 1995). The regenerating units will initially lack myelin even when the parent axon is a myelinated fiber. With time, these unmyelinated fibers will become myelinated (Flores *et al.*, 2000).

To reach the distal segment, the advancing sprouts have to pass a critical area between the proximal and distal stumps of the cut nerve: the interstump zone. The final success of the nerve regeneration is, to a great extent, dependent on what happens at this level and in what way local chemical and cellular reaction can influence the growth of sprouts toward their peripheral pathways.

1. *Perikaryal Phenotype Following Nerve Damage and Regeneration*

Axonal injury exposes the intracellular compartment to the extracellular environment, triggering ion fluxes and antidromic electrical activity that initiate pathways for neuronal death (Nadeau *et al.*, 2005; Navarro *et al.*, 2007; Zhang and Yannas, 2005). Moreover, damage to neurons or their axons induces phenotypic changes as indicated by alterations in mRNA transcription (Krekoski *et al.*, 1996; Salis *et al.*, 2007; Sebert and Shooter, 1993), protein synthesis (Ji *et al.*, 2007; Lundstrom *et al.*, 2005; Navarro *et al.*, 2007; Roglio *et al.*, 2008; Weragoda and Walters, 2007), and membrane receptor profiles (Karchewski *et al.*, 2004; Obata *et al.*, 2006; Oyelese *et al.*, 1995; Seniuk, 1992; Terenghi, 1999; Tonra *et al.*, 1998). Likewise, axonal transport (Hoffman and Luduena, 1996; Stone *et al.*, 2004), the secretion of neuropeptides and neurotrophic factors (Guseva and Chelyshev, 2006; Mulderry, 1994; Wang *et al.*, 2008; White and Mansfield, 1996) also are changed following injury to neurons or their axons. Finally, it is now apparent that an end result of injury is that a considerable proportion of all primary afferent neurons contributing to an injured nerve will die, with estimates ranging from 7% to 50%, depending upon the exact nature of the experimental model (Hiura, 2000; Hiura *et al.*, 1999; Navarro *et al.*, 2007). Hence, in addition to axonal regeneration, the potential for functional recovery after injury depends on restoration of neuronal numbers, and on development of appropriate neuronal phenotypes.

Previously reported studies reveal that peripheral nerve injuries induce a cascade of events progressing throughout the plastic changes to restoration of the damaged connections. In damaged neurons, axons begin to sprout after a delay of 3–42 days (Czaja *et al.*, 2008; Su and Cho, 2003). Nerve fibers grow by sprouting neurites that advance through the repair site only to be pruned down

when the endoneurial tubes of the distal stump are reached (Donnerer, 2003). Although neurite growth is facilitated by contact guidance from neurite outgrowth-promoting factors (Yoshii *et al.*, 2004), it also is dependent upon the neurons' inherent regenerative capacity. This is enhanced by adoption of the regenerative phenotype, partly in response to injury factors (Navarro *et al.*, 2007). As a result, axons preferentially reinnervate the distal stump over neighboring tissues, and display preferential reinnervation in the selection of endoneurial tubes (Brushart *et al.*, 1998; Kovacic *et al.*, 2007; Rajan *et al.*, 2003; Redett *et al.*, 2005). Moreover, several studies show that damage to the adult nervous system induces factors and mechanisms that control neuronal proliferation, migration, differentiation, and connectivity during development (Ghashghaei *et al.*, 2007; Navarro *et al.*, 2007; Taupin, 2006). The rate at which new neurons appear is not constant but can be increased or decreased in response to stress (Mirescu and Gould, 2006), activity (Bordey, 2006), drugs (Huang and Herbert, 2006; Perera *et al.*, 2007), or type of neuronal injury (Groves *et al.*, 2003; Kokaia and Lindvall, 2003; Zhang *et al.*, 2006).

B. The Distal Nerve Segment

After nerve transection, the distal segment undergoes a slow process of degeneration known as Wallerian degeneration (Fig. 4A). This process starts immediately after injury and involves myelin breakdown and proliferation of Schwann cells. Schwann cells and macrophages are recruited to the injury site, and over a period of 3–6 weeks they phagocytize all the myelin and cellular debris.

Within hours after transection, the axon membrane fuses and seals the ends. Disintegration of the axons starts within the first days. The first stages of this process are characterized by a granular disintegration of axoplasmic microtubules and neurofilaments due to proteolysis (Lubińska, 1982; Schlaepfer, 1977; Vial, 1958).

The loss of axon–Schwann cell contact is a signal that causes the Schwann cell proliferation. Schwann cells upregulate the synthesis of several types of neurotrophic factors as NGF (Heumann, 1987; Thoenen *et al.*, 1988). In addition to NGF, Schwann cells also produce and present the neurotrophins BDNF, NT-3, NT-4/5, and NT-6 to the outgrowing axons (Funakoshi *et al.*, 1993) and the glial growth factor neuregulin (Geuna *et al.*, 2007). Proliferating Schwann cells organize themselves into columns (named bands of Büngner) and the regenerating axons associate with them by growing distally between their basal membranes.

The advancement of regenerating axons in the distal segment is promoted by neurite outgrowth-promoting factors, such as laminin and fibronectin (Baron-Van Evercooren *et al.*, 1982; Hall, 1997; Liu, 1996). A number of cell adhesion molecules such as N-CAM, L1, the myelin-associated glycoprotein, and

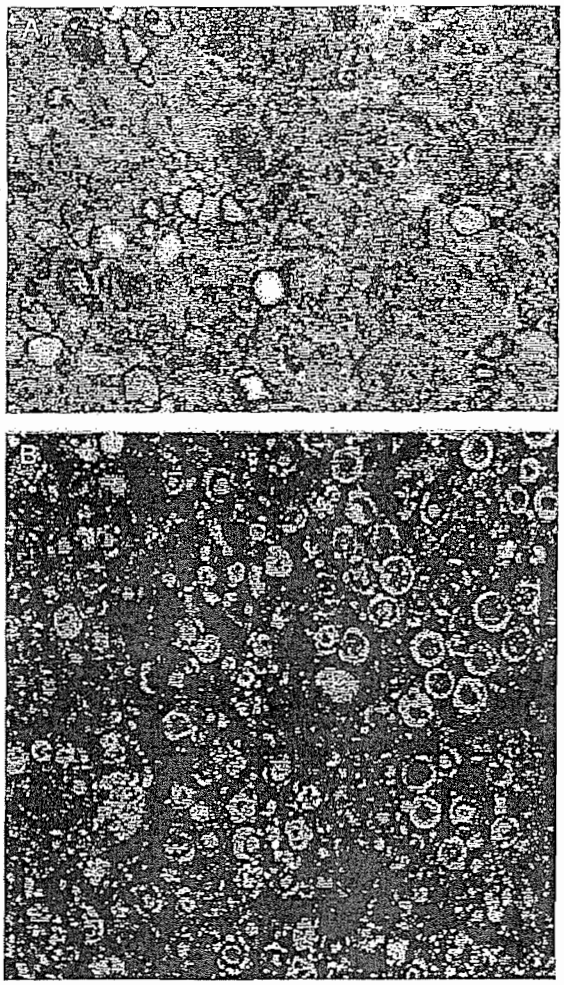

FIG. 4. After nerve transection, the distal segment undergoes a slow process of degeneration known as Wallerian degeneration (A). Signs of degeneration regarding axons and myelin disintegration are shown. After few days, few new regenerated fibers surrounded by new-formed thin myelin sheath are detectable (A, B). A double immunofluorescence shows different caliber of myelinated regenerated axons neurofilament-positive (green) surrounded by Schwann cells S100-immunopositive (red) (B).

tumor-associated glycoprotein (TAG)-1, also play an important role (Daniloff *et al.*, 1986; Walsh and Doherty, 1996). In the distal segment, axon sprouts (which do not take an extraneural course) either approach a Schwann cell column or may grow at random into the connective tissue of the nerve. The Schwann cell columns are invaded by axon sprouts arising from parent axons in the proximal segment (Fig. 4B).

Since an excess number of sprouts invade the distal Schwann cell columns (Aguayo *et al.*, 1973; Sanders and Young, 1946), the initial number of axons present in the distal nerve segment may considerably exceed the number in the

same nerve proximal to the lesion (Povlsen and Hildebrand, 1993). With time, some of the regenerated axons, which have reached appropriate distal targets, enlarge, mature, and regain a close-to-normal diameter (Sanders and Young, 1946) as result of a trophic supply from the target organs. Other branches that do not reach the target are pruned away and disappear (Griffin and Hoffman, 1993). After a few months of nerve regeneration, we will see a reorganization of the nerve trunk into a large number of miniature compartments, each surrounded by a new perineurium. Cajal (1928) described a process in which the distal stump of a divided nerve became separated into numerous nerve bundles, or "minifascicles," to replace the original large fascicle (Fig. 3C). This phenomenon is known as "compartmentation" (Morris *et al.*, 1972). Initially, it occurs also in the proximal stump of a cut nerve and in the gap between the two ends as the axons advance. The stimulus to compartmentation is probably a disturbance of the endoneurial environment resulting from damage to the perineurium. The formation of numerous miniature fascicles expresses the need for restitution of the normal endoneurium environment around the nerve fibers as quickly as possible by restoring the perineurial barrier (Lundborg, 2004).

Prolonged denervation of the distal segment results in a progressive increase in collagen content and extensive changes in the distribution of collagen types have been observed in the endoneurium and perineurium (Salonen *et al.*, 1985). Collagen production in the endoneurium may result from fibroblast activity but it may also be a result of Schwann cell activity (Barton, 1962; Thomas, 1964).

When assessing the rate of axonal outgrowth in experimental animals, several factors seem to play a role, such as the nature of the lesion, the species and the method of assessment. The quality of outgrowth obtained after transection and suture is always worse than that obtained after a crush injury. The regeneration rate in rat and rabbit nerves falls within the range of 2.0–3.5 mm/day after transection and repair and 3.0–4.4 mm/day after a crush lesion (Lundborg, 2004).

IV. Conclusions

Histological parameters are the far most used predictors of peripheral nerve damage and regeneration (Castro *et al.*, 2008; Vleggeert-Lankamp, 2007). Therefore, adequate knowledge on nerve histology is a prerequisite for peripheral nerve research. We have focused our attention on traumatic injury and regeneration of a "normal" nerve without addressing the neuropathological changes occurring as a consequence of various nerve diseases since this article is included in a special issue of the *International Review of Neurobiology* dedicated to peripheral nerve repair and regeneration and not to neuropathology of nerves.

This paper is aimed at providing the peripheral nerve researcher with the basic framework information on nerve morphology that can facilitate the correct

interpretation of the morphological data obtained in experimental studies. Yet, it may help researchers in selecting the best morphological technique for reaching their scientific goals. Finally, this work will hopefully lead the reader to appreciate how histology, carried out by both traditional and modern methods, can be a valuable tool for the scientific advancement in nerve repair and regeneration.

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ORIGINAL

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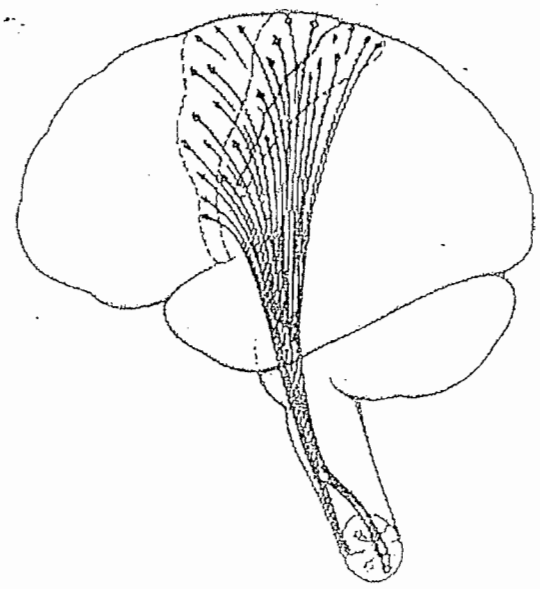
MYELIN BIOLOGY *and* DISORDERS 1

Robert A. Lazzarini



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CHAPTER

4

Functional Organization of the Nodes of Ranvier

Steven S. Scherer, Edgardo J. Arroyo, and Elior Peles

INTRODUCTION (FIGS. 4.1–4.4)

Myelinated axons are completely covered by myelin sheaths except at nodes of Ranvier, small gaps (less than 1 μm in length) that are directly exposed to the extracellular milieu. By increasing the resistance and especially by reducing the capacitance, myelin reduces current flow across the internodal axonal membrane (Blight, 1985; Funch and Faber, 1984), thereby facilitating saltatory conduction at nodes (Hille, 2001; also see Chapter 5 by Waxman and Bangalore). As shown in Figure 4.1, owing to their differential staining, Ramón y Cajal (1928) deduced that nodes, paranodes, and incisures contain different molecular components. He and his contemporaries recognized an acellular sheath ("the sheath of Schwann") that extended across PNS nodes, the reduction of the axonal caliber at nodes, as well as specializations around the node ("cementing disc of Ranvier") and the paranode ("spinous bracelets of Nageotte"). His observations presaged later ultrastructural studies, which, together with recent investigations of the organization of molecular constituents provide new insights on the molecular architecture of the node, are the subjects of this review.

MOLECULAR SPECIALIZATIONS OF THE PNS AND CNS NODAL AXOLEMMA (FIGS. 4.2–4.6)

By electron microscopy (EM), the nodal axolemma is more electron-dense and contains a higher density of intramembranous particles ($1200/\mu^2$)—the voltage-gated Na^+ channels (Na_v channels)—than the internodal axolemma (Rosenbluth, 1995). The flow of Na^+ through these channels is essential for the propagation of action potentials (Hille, 2001); also see Chapter 5 by Waxman and Bangalore). The $Na_v1.1$-$Na_v1.9$ α subunits belong to a gene family (designated *SCN1A-SCN9A* in mammals; Goldin *et al.*, 2000). The actual channel is composed of a single α subunit, a large, glycosylated, polytopic protein comprised of four homologous domains (Goldin *et al.*, 2000). Each kind of Na_v channel has distinct electrophysiological properties, including its sensitivity to tetrodotoxin (TTX). $Na_v1.6$, a TTX-sensitive channel, appears to be the main one expressed in mature nodes of the CNS and PNS (Arroyo *et al.*, 2002; Caldwell *et al.*, 2000; Tzoumaka *et al.*, 2000). $Na_v1.9$ is mainly associated with unmyelinated sensory axons, but has also been reported in some PNS nodes (Fjell *et al.*, 2000), and $Na_v1.2$ and $Na_v1.8$ are found at many CNS nodes (Arroyo *et al.*, 2002). In both the PNS and the CNS, $Na_v1.6$ replaces $Na_v1.2$ in some, but not all, nodes (Arroyo *et al.*, 2002; Boiko *et al.*, 2001; Kaplan *et al.*, 2001; Rasband and Trimmer, 2001).

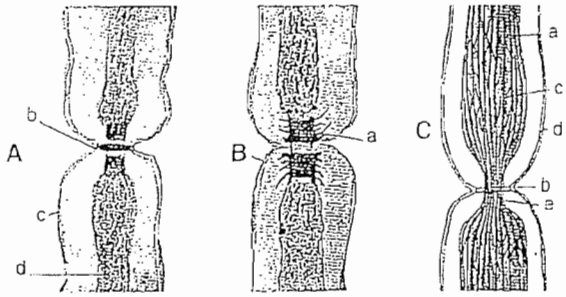

FIGURE 4.1
Ramón y Cajal's depiction of the nodal region. (A and B) "Node of nerve fibres. (A) Axonic impregnation often found in diluted and quick-acting silver solutions. (B) Impregnation with silver, after fixation in formol-pyridine-manganese. *a*, spinous bracelets of Nageotte; *b*, disc of Ranvier; *c*, Schwann's membrane; *d*, axon. (C) "Schematic drawing of the nerve fibre at the level of the node. *a*, fine oblique neurofibrils; *b*, cementing discs; *c*, longitudinal stout neurofibril; *d*, neurilemma; *e*, region of the axon which corresponds to the node." Figures and quotations are from (Ramón y Cajal, 1928), with permission of Oxford University Press.

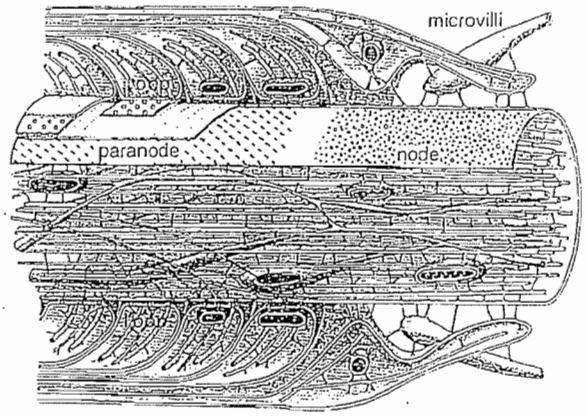

FIGURE 4.2
Schematic ultrastructure of the nodal and paranodal regions of a myelinated PNS axon. This drawing illustrates the transcellular bridges between Schwann cell microvilli and the nodal axolemma, which contains a high density of large particles (Na_v channels). The paranodal axolemma has rows of particles in register with the rows of particles in the paranodal loops; together these form the septate-like junctions. Modified from Ichimura and Ellisman, 1991, with permission of Kluwer Academic Press.

Mature channels are composed of one α and two β subunits (Fig. 4.5). Three genes (*SCN1B-SCN3B*) encode β subunits (β1-3), which are type I transmembrane domain proteins with a single extracellular immunoglobulin (Ig) domain (Catterall, 2000; Isom, 2002). The Ig domain of β2 is closely related to one of the Ig domains of contactin, a glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecule (CAM) of the Ig superfamily (Isom *et al.*, 1995). β1 and β3 are more closely related to each other than to β2. Both β1 and β2 have been localized to nodes (Chen *et al.*, 2002a; Ratcliffe *et al.*, 2001); whether β3 is localized to nodes remains to be determined, but its mRNA is widely expressed by CNS and PNS neurons (Qu *et al.*, 2001; Shah *et al.*, 2000). Because $Na_v1.6$ is the most common α subunit at nodes, it is their likely partner, but this remains to be shown directly; whether β1, β2, and β3 interact with $Na_v1.2$, $Na_v1.8$, or $Na_v1.9$ at nodes also remains to be determined. β2 is required for normal density of nodal Na_v channels (Chen *et al.*, 2002a).

A variety of functions have been proposed for β subunits. In transfected cells, β subunits increase the delivery to the cell membrane, and may alter their electrophysiological characteristics, of some but not all kinds of α subunits (Catterall, 2000; Meadows *et al.*, 2001). The Ig domain of β subunits enables them to act as CAMs and appears to mediate

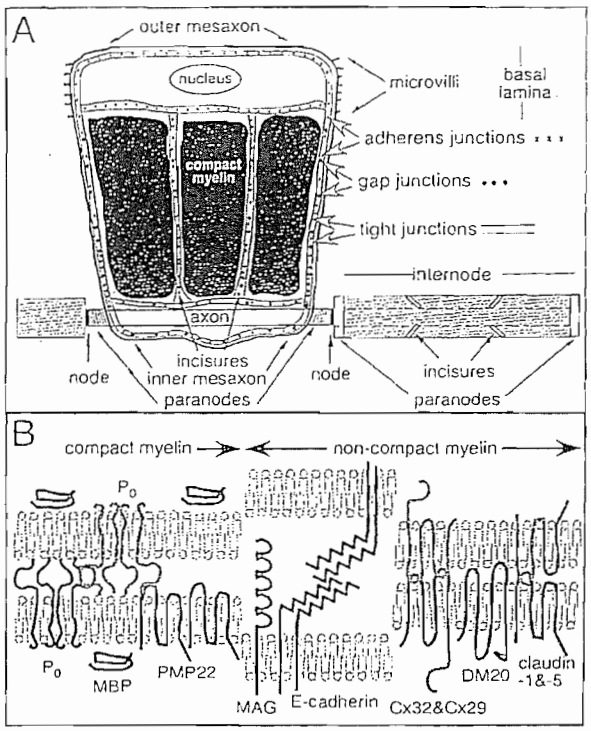

FIGURE 4.3
"Autotypic"/"reflexive" tight, gap, and adherens junctions in the PNS myelin sheath. (A) A myelinating Schwann cell has been "unrolled" to reveal its trapezoidal shape; the two lateral edges define the paranodes; the outside edge defines the outer mesaxon; the inside edge defines the inner mesaxon. The nodal microvilli are associated with the outermost aspect of the myelin sheath. Noncompact myelin is found in the paranodal region and in incisures. Tight junctions are depicted as two continuous lines; these form a circumferential belt and are also found in incisures. Gap junctions are depicted as ovals; these are found between the rows of tight junctions. Adherens junctions are depicted as "x"s. Modified from Kleopa and Scherer, 2002, with permission of W.B. Saunders. (B) Schematic representation of the proteins of compact and noncompact myelin. Compact myelin contains P_0, peripheral myelin protein 22 kDa (PMP22), and myelin basic protein (MBP); noncompact myelin contains E-cadherin, myelin-associated glycoprotein (MAG), DM20, Cx32, Cx29, claudin-1, and claudin-5. Modified from Arroyo and Scherer, 2000, with permission of Springer-Verlag.

complex interactions with various nodal components: β1 and β2 subunits can interact with the extracellular matrix molecules tenascin-C and tenascin-R (Srinivasan *et al.*, 1998; Xiao *et al.*, 1999); β1 and β3 (but not β2) can interact in *cis* with neurofascin and Nr-CAM (Ratcliffe *et al.*, 2001), both of which are found at nodes (see below); β1 (but not β2) can interact with contactin and phosphacan, a secreted isoform of receptor protein tyrosine phosphatase β (RPTPβ; Kazarinova-Noyes *et al.*, 2001; Ratcliffe *et al.*, 2000). Furthermore, β1 and β2 can also interact homophilically and their cytoplasmic domains can recruit ankyrin$_G$ (Malhotra *et al.*, 2000). In addition, the interaction of Na_v channels with ankryrin$_G$ at nodes could also be mediated by the α subunit (Bouzidi *et al.*, 2002). Although it is not clear which of these potential interactions play important roles in the formation of nodes, nodal Na_v channels are complexed with β subunits and CAMs that are also linked to the axonal cytoskeleton through ankyrin$_G$ (Bennett *et al.*, 1997).

Ankyrins are adaptor proteins that link many intrinsic membrane proteins to the spectrin cytoskeleton (Bennett and Baines, 2001). Two splice variants of ankyrin$_G$, 270 and 480 kDa, co-localize with Na_v channels at initial segments and nodes (Kordeli *et al.*, 1990; Kordeli *et al.*, 1995). These isoforms are distinguished by their membrane-binding domain composed of ANK repeats, a spectrin-binding domain, and a serine/threonine-rich domain (Zhang and Bennett, 1996). Ankyrin$_G$ 270/480 kDa interacts with the cytoplasmic domains of $Na_v1.2$ (Bouzidi *et al.*, 2002), NF186 and Nr-CAM, two Ig CAMs that are localized at the nodes (Davis and Bennett,

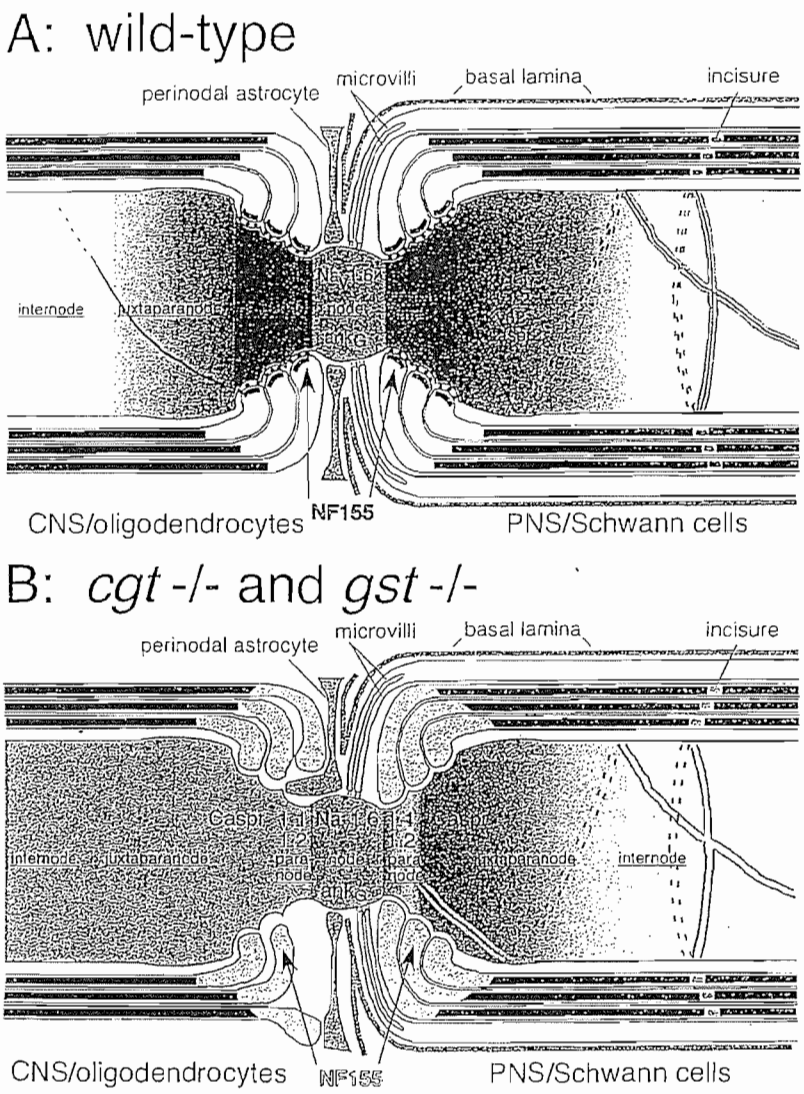

FIGURE 4.4
Schematic depiction of the node, paranode, juxtaparanode, and internode. Both panels depict the CNS and PNS myelin sheaths (meeting at a node) surrounding a single myelinated axon. The myelin sheaths have been bisected, revealing the surface of the axon. The relative intensity of the red and green indicates the relative abundance of the indicated proteins. (A) Wild-type pattern. This drawing illustrates that axonal membrane of nodes, paranodes, juxtaparanodes, and internodes are each characterized by their expression of a different set of molecules, in both the CNS and the PNS. Modified from Arroyo and Scherer, 2000, used with permission of Springer-Verlag. (B) Altered axonal organization in *cgt*- and *cst*-null mice. In each of these mutants, septate-like junctions are missing. Further, the components of septate-like junctions (NF-155, Caspr, and contactin) are mislocalized in a diffuse pattern, and the axonal proteins that are normally largely excluded from the paranodal region—Kv1.1, Kv1.2, Kvβ2, and Caspr2—are apposed to the nodal region. Similar anatomical changes (missing septate-like junctions, retraction of oligodendrocyte glial loops) have also been observed in *contactin*- and *Caspr*-null mice.

1994; Davis *et al.*, 1993, 1996; Srinivasan *et al.*, 1988). NF186 and Nr-CAM share an intracellular epitope (FIGQY) whose tyrosine must be dephosphorylated for them to bind to ankyrin (Davis *et al.*, 1993; Garver *et al.*, 1997; Zhang and Bennett, 1998). Bennett and colleagues (Bennett *et al.*, 1997; Davis *et al.*, 1996; Lambert *et al.*, 1997) have proposed that NF186 and Nr-CAM have heterophilic interactions in *trans* with other CAMs on the microvilli (Fig. 4.5), in accord with the ultrastructural data showing tethering of the microvilli to the nodal axolemma (Ichimura and Ellisman, 1991; Raine, 1982), as depicted in Figure 4.2.

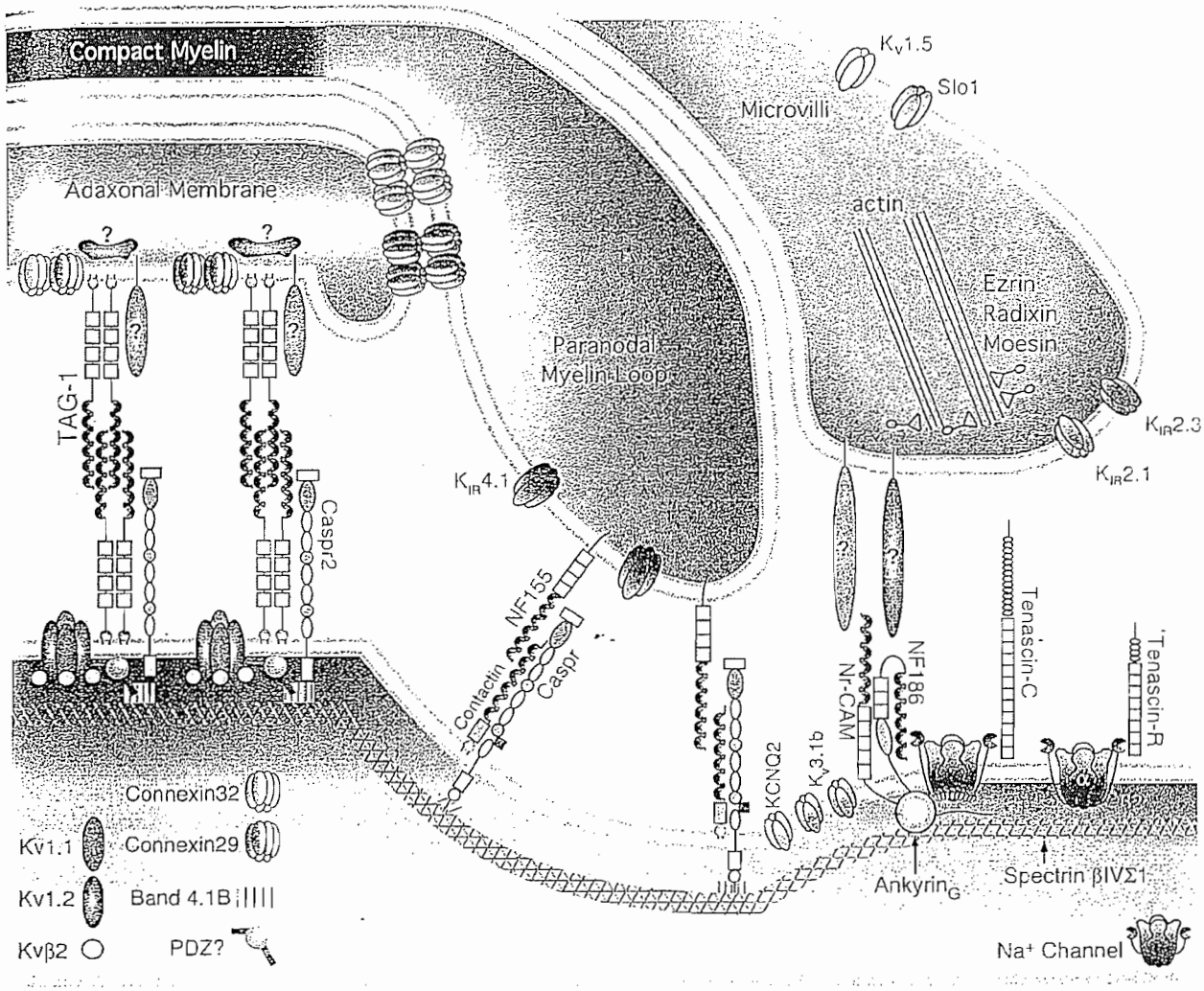

FIGURE 4.5

Possible *cis* and *trans* interactions in the PNS nodal region. This schematic drawing depicts the molecular organization of nodes, paranodes, and juxtaparanodes. At nodes, the ANK domains of ankyrin$_G$ are depicted encircling the cytoplasmic aspect of the Na_v channel α subunit (Michaely *et al.*, 2002); the Na_v channels β subunits as well as tenascin-R and tenascin-C are depicted as interacting with the globular domain of ankyrin$_G$, which in turn interacts with spectrin βIVΣ1. The extracellular domains of β2 subunits may interact with tenascin-R, tenascin-C, and NF186. Nr-CAM, NF-186, and β subunits may interact in *trans* with CAMs on the Schwann cell microvilli. At paranodes, Caspr and contactin multimers interact in *trans* with NF155. At juxtaparanodes, TAG-1 is depicted as dimers that interact homophilically in *trans* (Freigang *et al.*, 2000; Kunz *et al.*, 2002). On the Schwann cell side, TAG-1 may also participate in the formation of a complex that consists of Cx29 and other proteins. On the axon side, TAG-1 inteacts with Caspr2 (Poliak *et al.*, 2003), which also interacts with a multimeric PDZ domain protein that links Caspr2 and tetramers of Kv1.1/Kv1.2 channels (Poliak *et al.*, 1999). Protein 4.1B links the cytoplasmic tail of Caspr and Caspr2 to the spectrin cytoskeleton. Homotypic gap junctions comprised of Cx29 or Cx32 link the paranodal membranes of the myelin sheath; the paranodal loops contain Kir4.1, an inwardly rectifying K^+ channel, and the adaxonal juxtaparanodal membrane contains Cx29, putative hemichannels.

In keeping with the idea that ankyrin$_G$ is an adaptor protein, inactivation of the ankyrin$_G$ gene in the cerebellum results in the failure of $Na_v1.6$, NF186, Nr-CAM, and spectrin βIVΣ1 to cluster in the initial segments of Purkinje cells (Jenkins and Bennett, 2001), which accordingly have a diminished ability to initiate axon potentials (Zhou *et al.*, 1998a). Although initial segments and nodes share many molecular characteristics, for unknown reasons, the nodal membranes of Purkinje cells are less affected, as they have clusters of $Na_v1.6$, neurofascin, Nr-CAM, and spectrin βIVΣ1 (Jenkins and Bennett, 2001). Ankyrin$_G$ also interacts with spectrin, likely the splice variant of spectrin IV (βIVΣ1) that is specifically localized to initial segments and nodes (Berghs *et al.*, 2000;

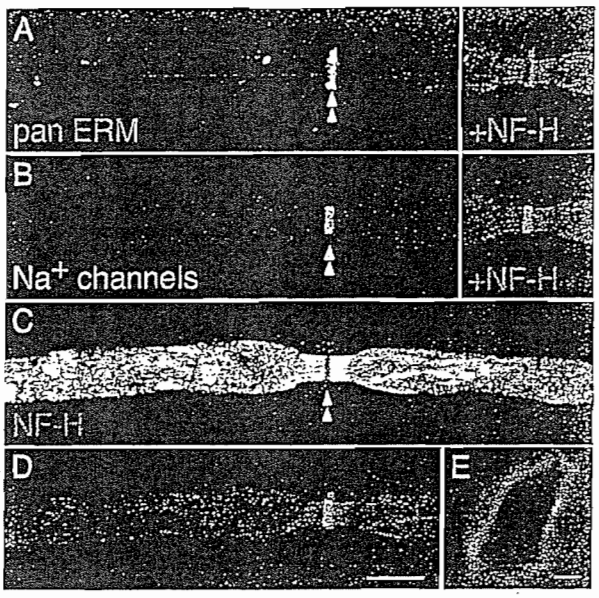

FIGURE 4.6
ERM proteins in Schwann cell microvilli. A–D: A confocal reconstruction of a teased myelinated fiber from a rat sciatic nerve, labeled with a pan-ERM antiserum (A), a mouse monoclonal antibody against Na_v channels (B), and a rat monoclonal antibody against a phosphorylated epitope of neurofilament heavy (NF-H, C); the merged image is shown in panel D. The insets in panels A and B show the superimposed NF-H staining. At the node (double arrowheads), note that the ERMs form a larger diameter disk than do Na_v channels. (E) A single 0.5 μm thick optical section of a node of Ranvier; taken from a section of an unfixed ventral root, double-labeled with a rabbit antiserum against ezrin (red) and a mouse monoclonal antibody against Na_v channels (green). Note that the ring of ERMs is larger than the ring of Na_v channels. Scale bar: A-D, 10 μm; E, 1 μm. From Scherer *et al.*, 2001, with permission of Wiley-Liss.

Koenig and Repasky, 1985; Trapp *et al.*, 1989b). Proof of a role for spectrin IV in this regard comes from the analysis of *quivering* mice, in which recessive mutations in the *spectrin 4* gene cause altered ion channel distributions in myelinated axons (Komada and Soriano, 2002; Parkinson *et al.*, 2001).

Other molecules have been localized to the nodal axolemma. An isoform of Na^+/K^+-ATPase was localized to CNS nodes in goldfish, and PNS nodes in mammals (Ariyasu *et al.*, 1985; Mata *et al.*, 1991; Schwartz *et al.*, 1981; Vorbrodt *et al.*, 1982; Wood *et al.*, 1977). The high concentrations of Na^+/K^+-ATPase is in keeping with the physiological function of the nodal membrane. Mammalian nodes have fast, intermediate, and slow K^+ conductances (Reid *et al.*, 1999; Safronov *et al.*, 1993); two of these K^+ α subunits, Kv3.1b and KCNQ2, have now been identified (Devaux *et al.*, 2003a, b), but their functional importance remains to be determined.

NODAL SPECIALIZATIONS IN THE PNS (FIGS. 4.2–4.6)

In the PNS, the extracellular matrix in the nodal region can be selectively stained with methylene blue and a variety of metal salts—the "nodal gap substance" (Hess and Young, 1952; Landon and Langley, 1971; Quick and Waxman, 1977). Hyaluronidase treatment abolishes staining by some kinds of metal salts (Landon and Langley, 1971), and hyaluronic acid and the hyaluronate-binding domain of versican are localized to the nodal gap (Abood and Abul-Haj, 1956; Apostolski *et al.*, 1994; Delpech *et al.*, 1982). The nodal gap is also stained by *Griffonia simplicifolia*-B4 isolectin and peanut agglutinin (PNA), lectins that recognize terminal α- and β-D-galactose, respectively (Apostolski *et al.*, 1994; Corbo *et al.*, 1993; Streit *et al.*, 1985). The actual molecule that *Griffonia simplicifolia*-B4 isolectin

recognizes in the nodal gap is not known. PNA binds to terminal galactosyl β1-3 N-acetylgalactosamine [Gal(β1-3)GalNAc], an epitope that is found on many glycoproteins including versican (Apostolski *et al.*, 1994; Delpech *et al.*, 1982). PNA also binds to glycolipids, including the gangliosides GM1, asialo GM1, and GD1b (Latov, 1990). GD1b and asialo GM1 have not been localized to nodes (Kusunoki *et al.*, 1993; Kusunoki *et al.*, 1997). Whether GM1 is present at nodes is not resolved, as cholera toxin (which binds to GM1) labels nodes (Corbo *et al.*, 1993; Ganser *et al.*, 1983; Goodyear *et al.*, 1999; Sheikh *et al.*, 1999; Thomas *et al.*, 1991), whereas antibodies against GM1 typically do not (Gong *et al.*, 2002; Molander *et al.*, 1997; Sheikh *et al.*, 1999).

The lateral borders of the Schwann cell cytoplasm have microvilli. By freeze-fracture EM, their membranes appear similar to the outer (abaxonal) membrane of myelinating Schwann cells (Blanchard *et al.*, 1985; Devor *et al.*, 1993; Ritchie *et al.*, 1990; Waxman and Black, 1987). Nevertheless, Kir2.1 and Kir2.3, two inwardly rectifying K^+ channels are enriched in the microvilli; these have been proposed to redistribute K^+ (Mi *et al.*, 1996). Two other kinds of K^+ channels, Kv1.5 and Slo1, are enriched in the abaxonal Schwann cell membrane, but not in the microvilli *per se* (Mi *et al.*, 1999; Mi *et al.*, 1996). The proximal portions of microvilli, even those from different Schwann cells, appear to be connected to one another by tight junctions (Berthold and Rydmark, 1983), and claudin-2 is specifically localized to this region (Poliak *et al.*, 2002). Like microvilli in other tissues, nodal microvilli contain F-actin (Trapp *et al.*, 1989b; Zimmermann, 1996), ezrin, radixin, and moesin (the defining members of the ERM family of proteins), as well as ezrin-binding protein (Hayashi *et al.*, 1999; Melendez-Vasquez *et al.*, 2001; Scherer *et al.*, 2001). ERM proteins bind to actin filaments mainly via their C-termini and can associate with a number of different integral membrane proteins via their N-termini. It remains to be determined what integral membrane proteins are associated with ERM proteins in Schwann cell microvilli and whether their phosphorylation regulates their function in this setting (Hayashi *et al.*, 1999). Both Nr-CAM and neurofascin can have homophilic and heterophilic interactions with a variety of CAMs (Volkmer *et al.*, 1996, 1998).

The nodal basal lamina and/or the nodal gap have also been reported to be enriched in several extracellular matrix proteins and CAMs, including tenascin-R, tenascin-C, NG2, N-CAM, and L1 (Daniloff *et al.*, 1986, 1989; Martin *et al.*, 1990, 2001; Mege *et al.*, 1992; Mirsky *et al.*, 1986; Rieger *et al.*, 1986). It is possible that some of these molecules may be associated with the Schwann cell microvilli rather than the extracellular matrix and/or the basal lamina.

NODAL SPECIALIZATIONS IN THE CNS (FIGS. 4.4 AND 4.7)

The axolemma of CNS nodes is thought to be similarly organized as those in the PNS. The main difference between CNS and PNS nodes is that processes from astrocytes (Black and Waxman, 1988; Butt *et al.*, 1994) and oligodendrocyte progenitor cells (Butt *et al.*, 1999) appose the nodal axolemma. These processes are not as well developed as the Schwann cell microvilli, especially for small axons (Bjartmar *et al.*, 1994; Raine, 1984), but they may be important for clustering Na_v channels, as clusters of Na_v channels are associated with astrocytic processes, even in demyelinating diseases (Arroyo *et al.*, 2002; Rosenbluth, 1985; Rosenbluth *et al.*, 1985). These astrocytic processes express the HNK1 epitope, as well as tenascin-R (Bartsch *et al.*, 1993; ffrench-Constant *et al.*, 1986), which can bind to Na_v channels (Srinivasan *et al.*, 1998), and alter their electrophysiological properties (Xiao *et al.*, 1999). These interactions may be the reason that myelinated optic axons in mice lacking tenascin-R have slowed conduction velocities (Weber *et al.*, 1999). The extracellular matrix of CNS nodes also contains versican, which is made by oligodendrocytes (Asher *et al.*, 2002; Delpech *et al.*, 1982), and Brail, a brain-specific link protein that binds to versican, which is made by neurons (Oohashi *et al.*, 2002).

CNS nodes also contain RPTPβ, which can interact with tenascin-R and both the α and the β subunits of Na_v channels (Milev *et al.*, 1998; Weber *et al.*, 1999; Xiao *et al.*, 1997).

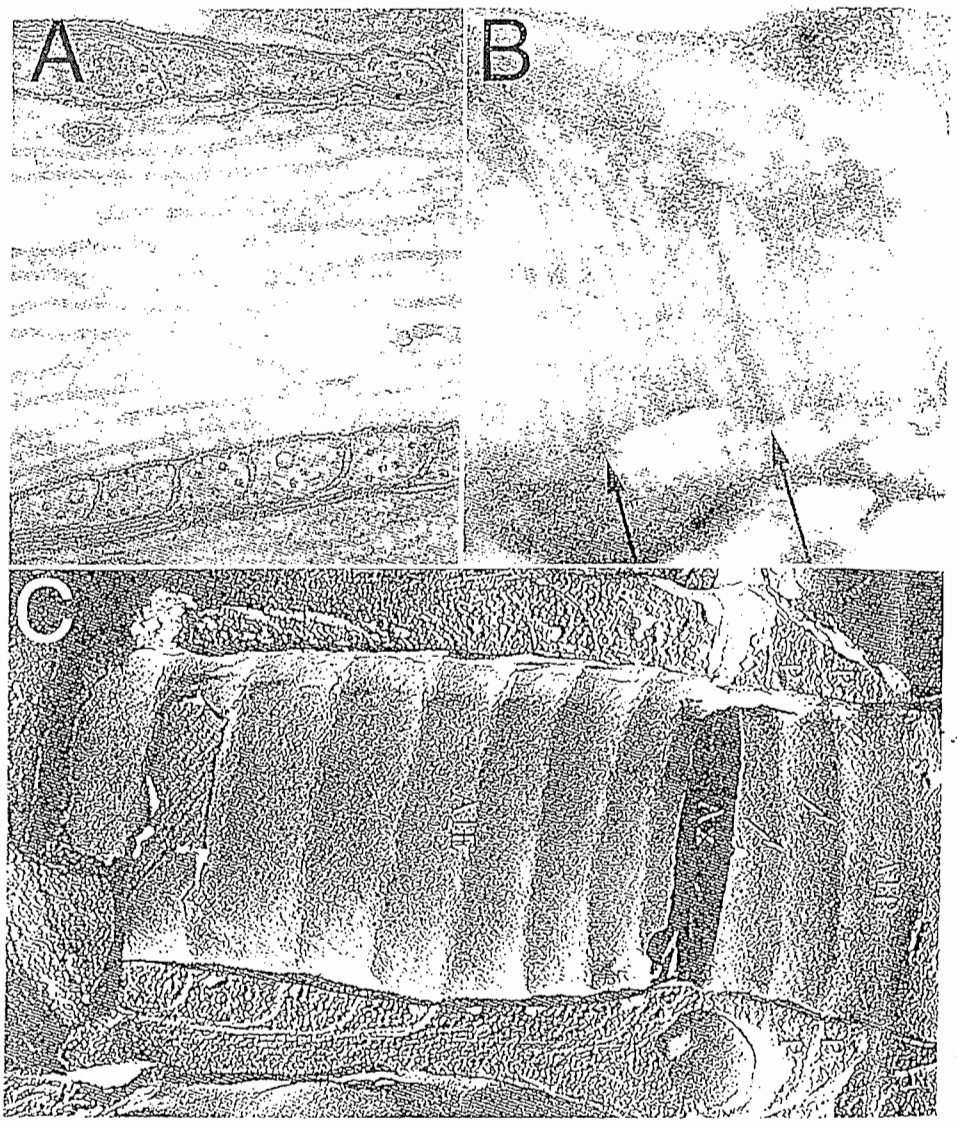

FIGURE 4.7
The ultrastructure of the paranode. (A) A transmission electron micrograph showing a CNS paranode in longitudinal section. The terminal loops of the myelin sheath (note the fluting of the axonal membrane in the upper aspect of the image) are connected to the axonal membrane by septate-like junctions. From Hirano and Dembitzer, 1982, with permission of Kluwer Academic Press. (B) A transmission electron micrograph showing lanthanum outlining the space between the septate-like junctions of CNS axons. The arrows indicate the locations of the spaces between adjacent glial loops; these spaces are larger than the spaces between adjacent rows of septate-like junctions. From Hirano and Dembitzer Hirano and Dembitzer, 1982, with permission of Kluwer Academic Press. (C) A longitudinal freeze-fracture EM through a CNS node and its paranodes. "In the middle of the field is the A face of the nodal axon (*NdA*), which is studded with randomly distributed membrane particles. On each side of this are the A faces of the paranodal axolemma (*AlA*). The one at the bottom shows the scalloping (*arrows*) produced by the pockets of paranodal cytoplasm that are visible at the sides (P). The fracture plane then passes across the axon (*Ax*) so that the B face (*AlB*) of the axolemma on the other side comes into view." From Peters *et al.*, 1991, with permission of W.B. Saunders.

The significance of these interactions are not clear, however, as the distribution of nodal Na_v channels, as well as conduction velocity of CNS myelinated axons, is normal in RPTPβ-deficient mice (Harroch *et al.*, 2000). Both tenascin-R and RPTPβ also interact with contactin (Peles *et al.*, 1995; Pesheva *et al.*, 1993), which, in contrast to the PNS, is found at CNS nodes (Rios *et al.*, 2000). Nodes contain a high MW form of contactin,

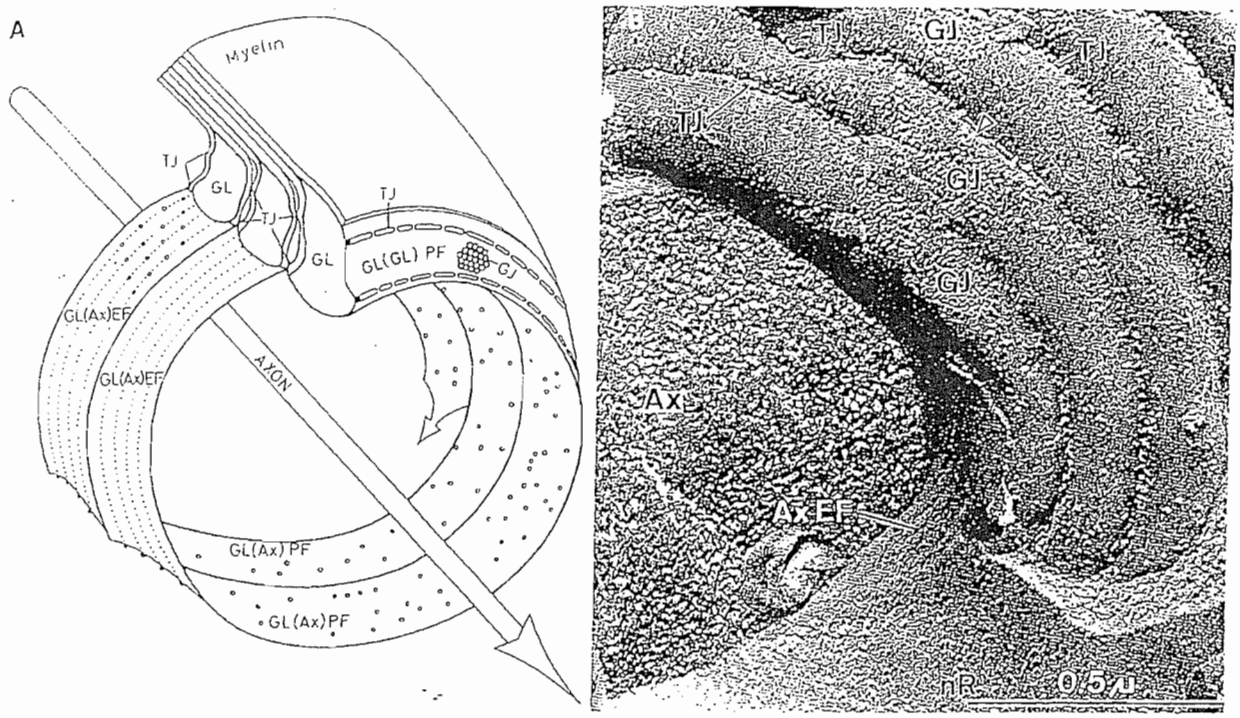

FIGURE 4.8
Paranodal gap junctions in the CNS myelin sheath. (A) "Junctions at the glial loops, diagram. The fracture plane inside the glial membrane has been illustrated as a plane which may be viewed from either side to disclose the glial P face and E face. The interglial junctions consist of junctions between consecutive gyres in the helix of glial loops (GL). One or two lines of *tight junctions* (TJ) follow the margin on the interglial P face (GL-GL PF). These ridges may consist of tightly arranged rows of particles or a series of bars of varying length. Occasional *gap junctions* (GJ) appear between the lines of tight junctions." (B) "Glial-glial junctions in rat spinal cord. The fracture has passed obliquely through the axon (Ax) at the junction of the last turn of the glial loop seen as the axonal E face (Ax EF) and the node of Ranvier (n R). Several glial loops which have piled up at the margin of the node without contact with the axolemma show lines of tight junctions (TJ) and dense particle accumulations representing gap junctions." Figures and quotations are from Sandri *et al.*, 1982, with permission of Elsevier Press.

whereas paranodes contain a low MW form (Rios *et al.*, 2000). The finding that Caspr induces a high to low MW switch and reduced levels of nodal contactin (Gollan *et al.*, 2003) is in keeping with the higher levels of nodal contactin in *Caspr*-null mice (Bhat *et al.*, 2001). Contactin regulates the surface expression of Na_v channels (Kazarinova-Noyes *et al.*, 2001; Liu *et al.*, 2001), and mice lacking contactin have reduced nodal Na_v channel clusters (Kazarinova-Noyes and Shrager, 2002).

SPECIALIZATIONS AT PARANODES (FIGS. 4.2–4.9)

At paranodes, the lateral edge of the myelin sheath spirals around the axon, forming the axoglial junctions. There are also "reflexive"/"autotypic" junctions between the paranodal loops themselves, including tight junctions, gap junctions, and adherens junctions (Arroyo and Scherer, 2000; Scherer and Arroyo, 2002; Spiegel and Peles, 2002). In the PNS, these reflexive junctions are also localized to incisures as well as to the inner and outer mesaxons; whether CNS "incisures" have any of these specializations remains to be demonstrated.

Paranodal glial and axonal membranes have distinct molecular specializations (Girault and Peles, 2002). MAG is enriched between the glial loops in the PNS but not in the CNS (Trapp *et al.*, 1989, 1989b). Glial loops are enriched in inward rectifying K^+ channel Kir4.1 (Chen *et al.*, 2002b; Wilson and Chiu, 1990), and Kir4.1-null mice develop profound CNS demyelination (Neusch *et al.*, 2001). PNS paranodes are enriched in oligodendrocyte-myelin glycoprotein (Apostolski *et al.*, 1994), and the gangliosides GD1b and

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Acetylcholine and regulation of gene expression in nerve system development

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ABSTRACT

The role of neurotransmitter molecules as modulators of early developmental events has received consistent experimental support in a number of different systems. We have investigated the role of acetylcholine in neuron differentiation as modulator of neurospecific gene expression. This problem was approached using two experimental models: developing sensory neurons of chick dorsal root ganglia and mouse neuroblastoma stable lines. As far as dorsal root ganglia are concerned is well known that their neurotrasmission apparatus is made of neuropeptides and aminoacids; however we have shown that at a very early stage they express a functional cholinergic system (including biosynthetic and inactivating enzymes and muscarinic and nicotinic receptor system), which has been suggested to be responsible for the modulation of genes involved in neuron differentiation (e.g. neurofilament components) and possibly for neuron-glia crosstalk. On the other hand transfection of neurotransmitter inactive neuroblastoma cells with a choline acetyltransferase construct has been shown to activate the expression of neurospecific genes, either upregulating those expressed at a low level or activating silent ones. It has thus been proposed that in this system an autoregulatory loop is activated, which may be active in the embryos. The data obtained in the two systems point to a role for acetylcholine among epigenetic factors acting on gene expression regulation in early stage of development.

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INTRODUCTION

The morphogenesis of the nervous system is dependent on a complex genetic program, responsible for the emergence of the large number of cell types present in adulthood; through their interaction and co-operation the co-ordinated function of the nervous system as an integrated structure is made possible.

Due to this complexity neural development and differentiation occur through a number of successive steps. Neural differentiation begins with the formation of the neural tube; cell proliferation and migration then takes place, gradually leading to the emergence of cell lineages, which then originate glial cells or neurones, with their multiple specific phenotypes, in response to a diversity of specific signals. Following the identification of proneural and neurogenic genes in *Drosophila*, homologous basic helix-loop-helix genes have been identified in vertebrates, such as *Neurogenin, Notch, Delta and Mash*, whose expression results in neural determination and control of the precursor pool size [1, 2]. During neural development downstream genes are then needed for the choice between a glial or neuronal fate; Neuro D genes have been identified as neuronal differentiation factors, which in turn activate specific neuronal genes, e.g. N-CAM [3]. A specific differentiation pathway is then selected by glial or neuronal progenitors and requires the activation of other regulatory genes, acting on the final steps of differentiation.

As far as neurone differentiation is concerned, after the emergence of neuronal precursors from neural stem cells and their proliferation, differentiation eventually occurs through the activation of several genes involved in neurite elongation, production of

specific adhesion molecules, assembly of a mature neurotransmitter synthesis apparatus. Neuronal differentiation is also characterised by the acquisition of cell polarity, dependent on the localisation of specific subsets of newly synthesised proteins in different cell compartments. Other gene products, by and large still unidentified, participate in the formation of mature synaptic structures. Thus differentiation of a specific neuronal population requires the activation of a rather large set of genes; some are shared by different neuronal types (e.g. those intervening in fibre formation and possibly in the basic neurotransmitter releasing mechanism), others are type specific.

One of the major questions related to nervous system development, and more specifically to neurone differentiation, is the identification of signals directing neuronal populations to specific phenotypes (e.g. cholinergic, adrenergic or peptidergic neurones). A number of factors are known, which can direct neuronal or glial differentiation [4]. Among these, neurotrophines play a major role; however the role of cell adhesion [5] and neurotransmitters [6, 7] has also been demonstrated. While growth factors have long been known for their function in development, the role of cell adhesion and neurotransmitters as modulator of gene expression, relevant for the acquisition of specific phenotypes, has only recently been recognized. A large body of evidence, emerging from diverse experimental systems and approaches, indicate that neurotransmitter molecules are present in a wide variety of animal species throughout development, thus giving support to their role as signal molecules controlling various basic cellular processes. In this view, as development proceeds, neurotransmitters take up new functions, ending up in the nervous system as mediators of synaptic communication [8].

Evidence for the ability of various neurotransmitter molecules to influence various cellular events, taking place during neurone differentiation has been obtained using different experimental systems [6]. We have focused our interest on acetylcholine (ACh) and its possible role on the regulation of neurone specific gene expression, using two systems: a) neuroblastoma cell lines, as a model of cholinergic neuron differentiation and b) dorsal root ganglia (DRG) sensory neurons, which use a peptidergic or aminoacidergic neurotransmitter apparatus; however they also activate the expression of a cholinergic system early in development. The two systems will be reviewed in the following chapters. First recent data on the neurotrasmitter developmental role will be shortly described.

NEUROTRANSMITTERS AS "MORPHOGENS"

The onset of neurotransmitter synthesis is known to occur in an early phase of development, during the elongation of the nerve fiber [9] and data are available showing the action of neurotransmitters on cell proliferation and survival of neural cells, as well as on neuritogenesis and expression of other neurospecific properties.

Stimulation of proliferation has been reported on various cell populations by different transmitters. Neurotransmitter effects on neural precursor proliferation have been extensively reviewed by Cameron et al. [10] and the possible interaction with growth factors has also been considered. More recently the action of neurotransmitters on proliferation has been analysed both in adult and during development. Serotonin action as a positive regulator of adult granule cell proliferation has been demonstrated by grafting raphe explants in rats, after hyppocampus serotonin input had been abolished by 5,7-dihydroxytryptamine injection [11]. Proliferation of rat cortical neural precursors can be enhanced by activation of muscarinic receptors [12]. Systemic injection of NMDA (MK 801) and AMPA (NBQX) receptor antagonists in adult gerbils increased BrdU labelled cells in the dentate subgranular zone (SGZ) [13]. NPY and PACAP38 (pituitary adenylate cyclase-activating polypeptide), two amidated neuropeptides, promote neuronal proliferation in the olfactory epithelium [14]. Conversely in cortical epithelium GABA receptor activation depolarises progenitor cells and elevates their cytosolic Ca^{2+} level, resulting in a decreased proliferation; on the other hand GABA stimulates progenitor cell motility via Ca^{2+} signalling [15].

GABA promotes survival but not proliferation of parvalbumin positive striatal neurons, as shown by exposure to GABA antagonist bicuculline during the proliferative and postproliferative phase of striatum neurogenesis [16]. The role of neurotransmitter release on neuron survival has also been shown in mice by deletion of Munc 18-1, a neuron specific protein of the SEC 1 family involved in membrane trafficking, which leads to loss of neurotransmitter release from synaptic vesicles throughout development. The ensuing abolishment of synaptic activity during development allows normal assembly of brain structures, but impairs neuronal survival during successive developmental stages [17].

As far as neuritogenesis, neurotransmitters can act either as positive or negative moduiator of fiber formation. Neurones from the buccal ganglion of the snail *Helisoma* in culture respond to serotonin by withdrawing filopodia and ceasing elongation [18]; on the other hand cultured thalamic neurones from newborn rats response to serotonin was positive and concentration dependent. In fact when increasing concentrations (0-100 μM) of serotonin were added neurite numbers and length and branching points were significantly higher in the presence of 25 μM serotonin, while other concentrations did not cause significant differences [19]. Neurite extension by mouse retinal ganglion neurons in culture was inhibited by ACh [20] while growth cones of *Rana Pipiens* DRG [21] or *Xenopus* spinal [22] neurons in culture turn and grow up concentration gradients of the neurotransmitter. Glutamate reduces dendrite formation in embryonic mouse cortical neurons and the effect is mediated by NMDA receptors [23]. In developing motor neurones dendrite arborization is dependent on glutamate receptor activation [24]; furthermore it has been shown that dendrite architecture can be remodeled, when a glutamate receptor subunit highly expressed in developing neurons is reintroduced in mature motor neurones [25]. Xenopus retinal ganglion cells express GABA receptors on their axons and growth cones; baclofen, a GABA agonist, has been observed to stimulate neurite outgrowth in cultured cells, while a GABA antagonist in vivo reduces the length of optic projections [26].

Finally as far as regulation of neurospecific gene products, noradrenaline has been shown to act early in neurogenesis, promoting the expression of proteins, as N-CAM and N-tubulin, in noggin-expressing ectodermal cells [27]. A number of data have also been obtained at later developmental stages. In slice cultures of olfactory epithelium expressing D2 receptors, dopamine enhances the expression of β-galactosidase driven by the promoter of the olfactory marker protein (OMP), a marker of mature olfactory neurones, in a dose dependent manner directly acting on epithelial cells [28]. Retinal cultures of 3 day rats treated with 20 μM glutamate showed higher neuronal survival and differentiation, as judged by the higher number of MAP2 positive neurones [29]. Glutamate and GABA stimulate the expression of BDNF and NGF in primary cultures of hyppocampal neurones [30]; the stimulation is dependent on Ca^{2+} influx and is enhanced by activation of adnylate cyclase [31]. A similar effect is elicited by serotonin in a glioma cell line [32]. The ability of neurotransmitters to act on glial cells, stimulating growth factor expression is in favour of their role as overall regulator of the developmental program. In this respect it is pertinent to mention that via stimulation of their receptors, both neurotransmitters and growth factors activate second messenger systems, such as cAMP, PLC-β, Ca^{2+} and βγ subunits of heterotrimeric G proteins. Utilization of common signalling pathways may provide a mechanism for interaction between neurotransmitters and growth factors [33].

This large body of evidence suggests that a single neurotransmitter may exert different action on neural cells, once they have been committed to a specific differentiation pathway.

CHOLINE ACETYLTRANSFERASE-TRANSFECTED NEUROBLASTOMA CLONES

Mouse neuroblastoma N18TG2 cell line and its FB5 subclone, isolated to reduce the intrinsic heterogeneity of neuroblastoma lines, are defective for neurotransmitter production [34, 35] and have been used as a model to study the ACh morphogenetic action on neurite outgrowth, avoiding the influence arising from endogenous production of neurotransmitters [35, 36]. A construct containing the cDNA for choline acetyltransferase (ChAT), the biosynthetic enzyme for ACh, has been transfected into N18TG2 cells and its FB5 subclone. Clones expressing high ChAT activity have then isolated [34, 35] thus providing an experimental system where the role of a functional neurotransmission apparatus for the progression of differentiation can be directly analysed.

Isolated clones expresses different ChAT levels; in particular, as expected, ChAT activity was undetectable in the N18TG2 cells and in its FB5 subclone, while the enzyme activity levels in the transfected clones, appeared similar or higher to that observed in the hybrid neuroblastoma-glioma 108CC15 cholinergic cell line. Differences in enzyme levels observed in the transfected clones most likely can be ascribed to a variable number of integrated constructs. Neomycin resistant clones isolated after transfection with a construct containing an antisense ChAT cDNA or with the empty vector do not express ChAT activity, as N18TG2 and FB5 cells [35, 36].

Expression of the enzyme was also shown by immunocytochemical analysis, using an antibody raised against ChAT from rat brain. Figure 1 (panel A) shows, by indirect immunofluorescence, the presence

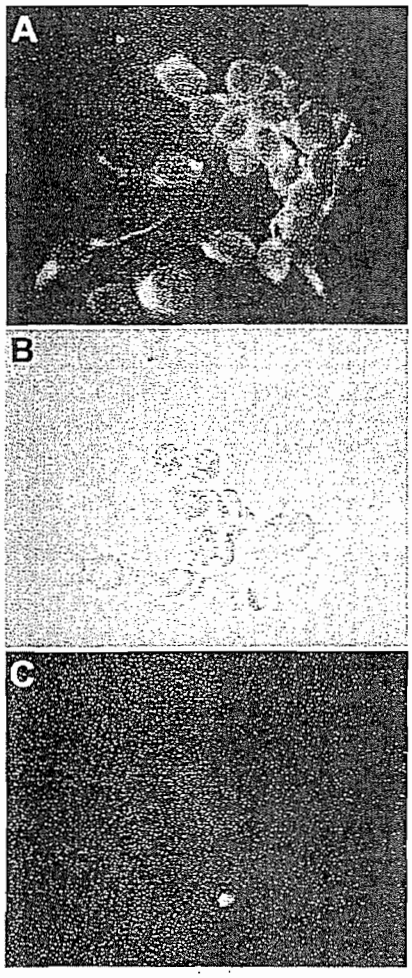

Figure 1. Immunostaining of (A) ChAT transfected clone 2/4 and (C) N18TG2 cells with an anti-ChAT monoclonal antibody. B: phase contrast micrograph of the same field showed in (C).

of the enzyme in the cytoplasm of the ChAT-positive 2/4 clone; similar results were observed in other ChAT-positive clones, while no immunoreactivity was detectable in N18TG2 cells (Figure 1, panel C) or in the other ChAT-negative cells [35].

In ChAT-positive clones acetylcholine was also detected and its level appeared to be related to the levels of enzyme activity, although a linear relation could not be observed [35]. Moreover the induction of ACh synthesis modified the expression of several neuronal markers; in particular increased levels of sinapsin I, high affinity choline uptake and voltage-gated Na^+ channels were observed while cellular and secreted acetylcholinesterase (AChE) levels were reduced [36].

N18TG2 cells, as many neuroblastoma clones, display little fiber outgrowth in basal culture condition; this can be significantly enhanced in response to various differentiating agents [37]. Thus, it appeared of interest to establish whether in ChAT transfected clones, outgrowth of processes would be modified and whether there was any relation between ChAT expression and morphological differentiation. Cell morphology was also analysed in the presence of retinoic acid or dibutyryl-cAMP, as differentiating agents. N18TG2 cells as well as transfected clones not expressing ChAT were characterised by an immature morphology, with cells bearing short processes. On the other hand, clones expressing ChAT activity showed a higher ability to respond to differentiating agents with the emission of a larger number of longer fibers [35]; in all cases the majority of the cells extended processes which appeared as a prominent feature of the culture, both for their length and branching. A morphometric analysis has shown that fiber extension per microscopic field was about 4-6 fold higher in transfected clones expressing ChAT activity as compared to those not expressing the enzyme (Figure 2). The ability to grow fibers was very high in ChAT-positive clones; lower values were observed for the hybrid 108CC15 clone, while ChAT-negative transfected clones showed values similar to those shown by N18TG2. As far as the presence of branching points on the elongating fibers, all the ChAT positive transfected clones showed considerably higher values as compared to N18TG2, 108CC15 cells and clones not expressing ChAT activity [35].

Muscarinic acetylcholine receptors (mAChR) are known to activate a number of cellular responses, being coupled to several G proteins [38, 39], which modulate fiber elongation and other neuronal properties. It is also known that promoters of neuronal proteins contain consensus sequences for various transcription factors among which the zinc-finger EGR-1 protein [40, 41] and that EGR family expression is increased by mAChR activation in different neuronal cells [42, 43, 44]. QNB binding studies on homogenate

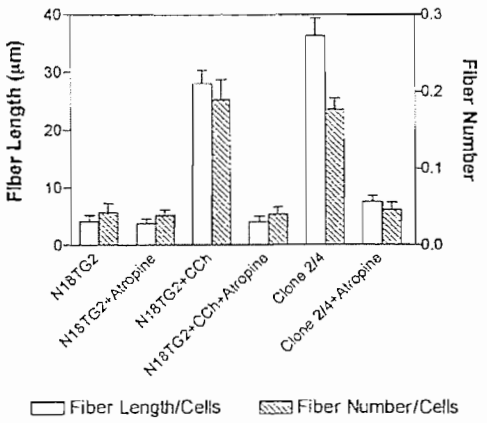

Figure 2. Quantification of fiber outgrowth, per microscopic field in N18TG2 and 2/4 ChAT transfected clone cultures maintained, for five days, in the presence of 10^{-3} M carbachol (CCh) and/or 10^{-6} M atropine. Fiber number and length were normalized to cell number present in each microscopic field. Micrographs of randomly selected areas of the culture dishes were taken from five independent 60 nim dishes (22 fields/dish); values are the mean ± SEM of about 110 observations for each culture condition. The observed differences resulted always statistically significant ($P<0.001$ Mann-Whitney test).

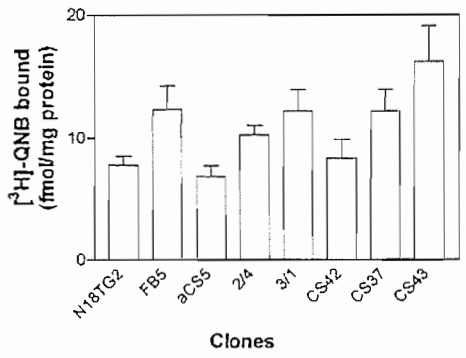

Figure 3. Muscarinic acetylcholine receptors in ChAT transfected clones. The presence of muscarinic receptors was evaluated using the non-selective muscarinic receptor ligand quinuclidinyl benzilate (QNB). [^{3}H]-QNB binding was evaluated in homogenates of non transfected N18TG2 and FB5 (a subclone of N18TG2) cells, in 2/4, 3/1, CS37, CS42, CS43 ChAT positive clones and in the aCS5 clone transfected with a construct containing the antisense ChAT gene. Values are the mean ± SEM of at least twelve observations.

of the neuroblastoma clones demonstrate the presence of muscarinic acetylcholine receptors in all examined clones (Figure 3) and no significant correlation between binding and ChAT activity was observed. Moreover although ChAT-positive clones showed different ability to accumulate ACh QNB binding appeared not related to the levels of ACh [36, 45].

The data summarized above are consistent with the hypothesis that the forced expression of ChAT in neurotransmitter inactive neuroblastoma cells brings about the activation of an autocrine loop via muscarinic receptors, which is responsible for the modulation of neuron specific trait expression, and prompted us to provide evidence for the existence of a direct modulation of fiber outgrowth and neuronal marker expression by muscarinic receptor activation, which may be related to EGR-1 levels. It thus appeared of interest to establish whether in N18TG2 and in ChAT-transfected clones, muscarinic agonist/antagonist could modulate neurite outgrowth. A morphometric analysis was undertaken and the results are shown in figure 2.

Figure 2 shows the results obtained when N18TG2 cells and 2/4 ChAT-transfected clone were cultured in the presence of 10^{-6} M atropine, a mAChR antagonist. Atropine treatment reduced the average fiber number and length normalized to cell number only in ChAT-positive clone ($P<0.001$ using the non parametric test of Mann-Whitney). Moreover the nonhydrolyzable cholinergic agonist carbachol (CCh) induced higher neurite outgrowth ($P<0.001$) in the parental non transfected N18TG2 neuroblastoma line with respect to untreated cultures; the effect of CCh was inhibited by the muscarinic receptor antagonist atropine, demonstrating that the effect of ACh on neurite outgrowth was mediated by muscarinic receptors.

Interestingly in ChAT-transfected clones the activation of synapsin I mRNA and protein synthesis, not detectable in the parental line, was also observed [35, 45]. Synapsin I belongs to a family of neuron-specific phosphoproteins and is highly concentrated at

presynaptic nerve terminals where it is associated to the cytoplasmic surface of synaptic vesicles playing a key role in neurotransmitter release [46]. However synapsin I is also believed to play a role in synapse formation; the injection of synapsin I in *Xenopus* blastomeres, accelerates synapse formation [47, 48] while its suppression in transgenic mice results in the inhibition of synaptogenesis [49]. Moreover cultured hyppocampal neurons, dissected from synapsin I-deficient mice, develop shorter and less branched axons than those observed in wild-type cells [49] suggesting that synapsin I may play a major role in axonal elongation and branching.

To evaluate the activity of transcription factors elicited by the endogenous ACh or by treatment with muscarinic agonist we transiently transfected N18TG2 and ChAT-positive clones with the the pSyCAT-10 plasmid containing the CAT reporter gene under the control of the human synapsin I promoter [50]. As shown in figure 4 the promoter activity was about 4

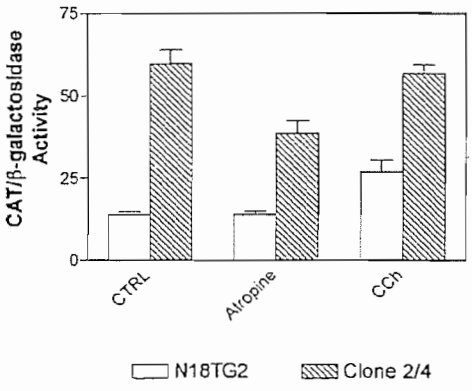

Figure 4. Muscarinic induction of synapsin I gene promoter activity in N18TG2 cells and 2/4 ChAT transfected clone. All transfections were normalized for variations in transfection efficiency, cotrasfecting the pSyCAT-10 plasmid, containing the CAT reporter gene under the control of the human synapsin I promoter, with a plasmid containing the β-galactosidase gene under the control of a constitutive promoter. Normalization was achieved by dividing CAT activity by β-galactosidase activity. Atropine was used at 10^{-6} M and carbachol (CCh) at 10^{-3} M final concentration. Values are the mean ± SEM of at least twelve observations.

fold higher in transfected clones with respect to the parental line and atropine treatment reduced its force [45]. Furthermore the nicotinic receptor antagonist mecamylamine resulted ineffective (data not shown), indicating a regulation of the synapsin I promoter mediated by muscarinic receptor activation. Moreover in parental N18TG2 cells, transiently transfected with synapsin I-CAT construct, the reporter gene expression was dose dependently induced by CCh and this effect was abolished by atropin, while mecamylamine resulted ineffective [45].

AChE has been considered as a modulator of neurite outgrowth, and various mechanisms for its action have been proposed [51]. Previous data showing changes of AChE cellular localization and secretion, related to developmental events [52, 53], would be in favor of such hypothesis. Furthermore it has been shown that the amount of AChE in DRG neuron cell bodies and neurites increases in parallel with neurite outgrowth [54] and the treatment with a monoclonal anti-AChE antibody produced a rapid detachment of DRG neurites, followed by regrowth upon removal of the antibody from the culture medium [55]. It has been proposed that AChE expression during development may modulate the extracellular levels of ACh establishing permissive pathways in which axons could elongate [56, 57]. Finally growth of different neuronal cells in the presence of soluble or coated AChE on culture dishes [58, 59, 60] and over-expression of AChE result in increased fiber outgrowth, suggesting that AChE may modulate cell adhesion [61, 62, 63, 64]. Starting from these considerations it appeared of interest to analyze the modulation of neuronal differentiation focusing on the role exerted by AChE in particular performing a comparative study of AChE secretion in the transfected clones.

AChE activity levels in ChAT transfected clones are shown in figure 5. AChE activity decreases about 2-4 fold in ChAT-positive clones with respect to FB5 cells and ChAT-negative aCS5 clone. Interestingly a roughly negative linear relation of AChE and ChAT levels seems to be present; CS43 clone which showed the highest level of ChAT activity, in fact, exhibits the lowest level of AChE activity [36]. Since the reduction of AChE secretion can be considered as part of cholinergic phenotype expression [52], AChE releasing ability of the different clones in culture was also evaluated. AChE secretion was significantly higher ($p<0.001$) in aCS5 transfected clone not expressing ChAT activity and in FB5 parental clone as compared with the transfected clones expressing ChAT activity,

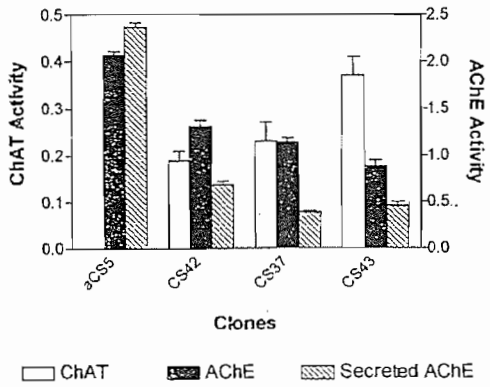

Figure 5. Expression of ChAT and AChE activity in transfected clones. FB5 non-transfected cells; aCS5 transfected clone with antisense ChAT gene construct; CS37, CS42 and CS43 transfected clones expressing ChAT activity. Cells in culture were routinely counted in a hemocytometer chamber before ChAT activity, AChE activity and protein content evaluation. ChAT activity was expressed as nmol ACh synthesized/mg prot/min and AChE activity as mU/10^6 cells (1 mU corresponding to 1 nmole of substrate hydrolyzed per min at 30°C, pH 8.0). The values are the mean ± SEM of at least eight independent experiments.

thus further suggesting that the forced production of ACh may induce a progression in the neuronal developmental program. Moreover sequential extraction of AChE activity revealed increased levels of AChE activity in membrane fraction from the transfected clones expressing ChAT activity with respect to FB5 and transfected ChAT negative aCS5 clone [36] supporting the hypothesis that, during development, a shift occurs in the destination of AChE from the secretory pathway to the pathway leading to AChE association with cell membranes [52].

It is known that murine AChE promoter contain consensus sequences for various transcription factors, among which the zinc-finger EGR-1 protein and it has been observed that this promoter shows a negative regulation in response to the EGR-1 factor [65]. It has also been observed that EGR-1 protein *in vitro* binds to two sites in the regulatory region of synapsin I gene and stimulates synapsin I gene transcription in transactivation assays [66]. Considering that *Egr* gene family expression is increased by muscarinic receptor activation in different neuronal cells [43, 44] it is possible to propose that in the ChAT positive clones an autocrine loop becomes active, leading to a a modulation of synapsin I and AChE expression, through the activation of *Egr* genes. In this respect it is relevant to mention that EGR-1 protein was detectable only in the ACh-producing clones [45].

Recently a gene encoding a zinc-finger protein functioning as a master regulator of the neuronal phenotype was identified. The protein: RE1 silencing transcription factor (REST/NRSF), being expressed in nonneuronal cells but not in neurons, negatively regulates the expression of neuronal type II voltage-gated Na^+ channels [67], synapsin I [68] and other neuron-specific proteins. Moreover REST mRNA is expressed in yet undifferentiated neural precursors, suggesting that it prevents the precocious expression of specific neuronal proteins during neurogenesis [69]. Therefore the observed increase in the level of voltage-gated Na^+ channels [36] and synapsin I [35, 45] in ChAT transfected clones may be also due to a decrease in the expression of REST consequent to the progression in the neuronal developmental program observed in ChAT transfected clones.

In conclusion stable transfection of neuroblastoma cells with ChAT gene construct is followed by the synthesis of ACh which brings about the activation of an autocrine loop via muscarinic receptors possibly inducing *egr*-1 expression, which is responsible for the modulation of neuron specific trait expression. At the same time the data described here raise a number of questions relevant for a better understanding of neuron differentiation; in fact it remains to be established whether the ACh modulation of synapsin I, voltage-gated sodium channels, choline transporters and AChE may be extended to other genes involved in neuron differentiation and whether other transcription factors are involved in the molecular machinery that control the neuronal response to ACh. In this respect, in fact, it is pertinent to recall that muscarinic receptor activation is also known to induce the transient expression of immediate early genes, as c-jun and c-fos [70]. Even more pertinent is the question whether this mechanism is actually occurring in embryogenesis. Although a direct demonstration is at the present lacking, it is interesting to mention that in chick DRG neurons, mAChRs are present at early developmental stages and are downregulated in the final phase of development [71] suggesting a functional role for ACh signalling in the regulation of sensory

neuron developmental program. On the basis of these considerations it is conceivable to propose that neurotransmitter modulation of gene expression, at least for the cholinergic system, may be operating in development and play a role for the acquisition of a specific neuronal phenotype.

SENSORY NEURONS

Dorsal root ganglia are structures of peripheral nervous system made of heterogeneous neuron populations differing for their morphological, biochemical and molecular properties; they transduce peripheral stimuli and convey through their central fibers the signals to the spinal cord [72, 73].

The number of neurons present in DRG is dependent on mitotic and apoptotic events. In chick embryo DRG, mitotic activity is arrested between E6 and E7; at later developmental stages (E8-E13), neuronal subpopulations undergo differentiation [73, 74]. DRG neurons utilise neuropeptides and aminoacids (e.g. Substance P, somatostatin, galanin, glutamate) as neurotransmitters. CGRP, aspartate and glutamate have been localised in *large latero-ventral* neurons while others neurotransmitters have been localised in *medium-dorsal* neurons [73, 75, 76]. Although a clear relation between morphological properties and functional role of the different neuronal types has not been established, it has been suggested that different neurotrasmitters may be involved in the transmission of different stimuli [77]. However the established coexistence of neuropeptides in a single neuron suggests that the transduction of peripheral stimuli may be dependent on a complex cooperation of more neurotransmitter molecules.

Sensory neurons during development respond to various neurotrophic factors. In particular nerve growth factor (NGF) acts as a survival and differentiation factor and promotes neurite outgrowth in *small dark* sensory neurons [78, 79]. Other neurotrophins such as BDNF, NT-3 and NT-4 act in the same manner on *large light* neuron subpopulations [80].

The presence of the ACh biosynthetic enzyme has been reported in DRG of different vertebrate species [81, 82]; however the possible function of ACh in sensory neurons until now is not clear. Over the last ten years we have accumulated significant evidence demonstrating the presence not only of ChAT but also of other cholinergic markers in DRG neurons [83]. The early expression of biosynthetic and degradative ACh enzymes (ChAT and AChE) suggested that ACh might be produced in sensory neurons in an early phase of gangliogenesis [84, 85]. Nevertheless the low amount of ACh present in DRG suggested that in these neurons it does not act as a neurotransmitter but rather as a modulator of sensory neuron functions [85].

DRG neurons are also cholinoceptive; in fact, they express both nicotinic and muscarinic receptors and are able to respond to ACh activating various signal transduction pathways [86-91]. The expression of the mAChR becomes evident at early developmental stages (E8-E12). At E12 a large number of neurons, differing in size and position in the ganglion, is positive for mAChR expression; however at later stages the number of positive neurons decreases and they are mainly restricted to the *medio-dorsal* region of the ganglion [87]. This distribution has suggested that during gangliogenesis mAChR could mediate ACh action on neuronal differentiation while, in the adult, they could be involved in the modulation of "*small size*" sensory neuron function. As regard to adult DRG it is known that in skin nerve preparations the ACh agonist carbachol causes stimulation of nociceptive C-fiber endings [92]; recently ACh has been shown to modulate the response to thermic and mechanical stimuli at sensory nerve endings [93] and cholinergic agonists have been shown to modulate CGRP release through activation of M_2 subtype receptor [94]. Furthermore both chick and rat DRG neurons express different mAChR subtypes (M1, M2, M3, M4 and probably M5), often co-expressed in the same neurons; this suggests that ACh stimulation on a single neurons may activate different second messenger pathways and modulate several sensory neuron functions [89].

Although sensory neurons are both cholinergic and cholinoceptive, there is yet no clear evidence of ACh storage and release in DRG. In fact, cholinergic vesicles so far have not been described either in the neuron soma or in sensory fibers, and ACh release has not been demonstrated. Recently the protein responsible for ACh accumulation in synaptic vesicles of cholinergic neurons has been identified. The vesicular ACh transporter (VAChT) coding sequence is located in the first intron of the ChAT gene, suggesting that the two proteins required for synthesis and accumulation of ACh are co-expressed and co-regulated in cholinergic neurons [95, 96, 97]. Since VAChT protein is localized in synaptic vesicles [98] it appears as a promising tool for identification of vesicles in cholinergic neurons and nerve terminals. A biochemical and molecular biology study together with immunolocalization at light and electron microscope

have allowed to demonstrate the presence and activity of VAChT protein in DRG providing evidence that sensory neurons are also able to accumulate ACh. It is thus likely that DRG neurons release this neurotransmitter with the same mechanism active in cholinergic neurons [99].

The observations that DRG neurons are able to synthesize and store ACh and that the expression of mAChR is evident at early developmental stages, is in favour of the hypothesis that ACh is involved in the modulation of sensory neuron differentiation.

The possible morphogenetic role of the ACh was studied in cultures of DRG neurons from E12 and E18 chick embryos. In this experimental system we evaluated neurite outgrowth and neurofilament expression following cholinergic agonists and antagonists treatment. Evaluation of fiber outgrowth by morphometric analysis, revealed that DRG cultures obtained from E12 chick embryos treated with muscarine and charbacol grow longer fibers and higher number of fibers per neuron with respect to control cultures. The addition of the antagonists, atropine or mecamylamine, causes a decrease of fiber outgrowth, suggesting that the cholinergic agonist stimulation of fiber elongation was mediated by both muscarinic and nicotinic receptors [100]. The expression of neurofilament proteins was also evaluated. Western blot analysis showed that muscarine induces in E12 DRG neuron culture a significant increase of neurofilament expression and that this effect is inhibited by atropine addition (Figure 6) [100]. Moreover northern blot analysis has demonstrated a cholinergic modulation of neurofilament transcripts.

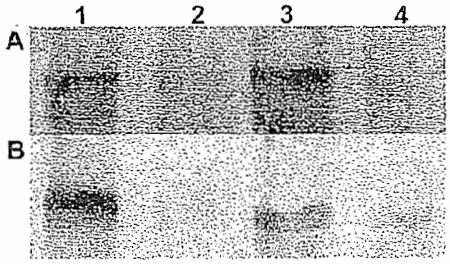

Figure 6. Immunoblotting analysis revealing neurofilament protein expression (panel A, 200 KDa subunit; panel B, 68 kDa subunit) in homogenates of E12 DRG neurons, plated on laminin substrate and maintained under different experimental conditions. Lane 1: neurons maintained in the presence of 50 ng/ml NGF; lane 2: neurons maintained in the absence of NGF; lane 3: neurons maintained for 72 hrs in the presence of 10^{-4} M muscarine; lane 4: neurons maintained for 72 hrs in the presence of 10^{-4} M muscarine plus 10^{-6} M atropine.

In conclusion the data above reported suggest that at early developmental stages, ACh activates an autocrine/paracrine loop, mediated by muscarinic and nicotinic receptors and contributes to sensory neuron differentiation and neurite outgrowth regulating neurospecific protein expression such as neurofilament.

CONCLUSIONS

The data described in this review demonstrate the role of ACh as modulator of neurospecific gene expression in two independent experimental systems. In DRG the early expression of cholinergic system components, e.g. neurotransmitter synthesis activation and muscarinic receptors, in the absence of a cholinergic neuronal circuit, has suggested a possible role for ACh as a cofactor of neuronal differentiation. The demonstration of the activation of neurofilament protein expression by cholinergic agonists in DRG cultures provides direct support to this hypothesis. On the other hand transfected neuroblastoma cells provided direct evidence that ACh is capable to modify the level of expression of several genes distinctive of the cholinergic phenotype. This system has also allowed to demonstrate that muscarinic receptor activation modifies the level of transcription factors, which act either as activator or silencing system in neurogenesis.

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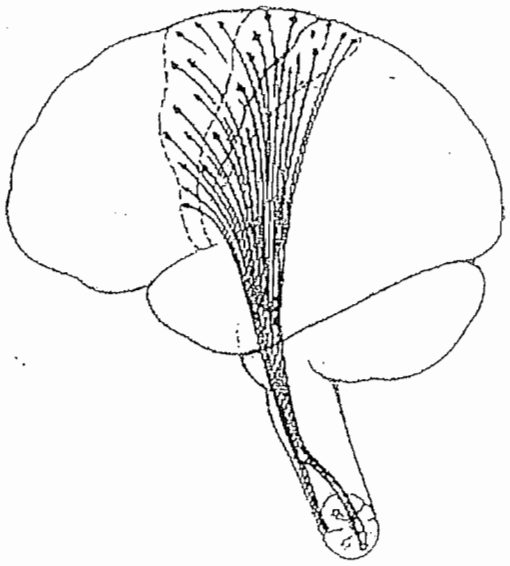
ORIGINALE

MYELIN BIOLOGY *and* DISORDERS 1

Robert A. Lazzarini



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CHAPTER

1

Structure of the Myelinated Axon

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INTRODUCTION

In his 1928 description in *Degeneration and Regeneration of the Nervous System* (Ramon y Cajal, 1928), Ramon y Cajal stated, "Myelin is an organ which is an adjunct of the axon, and as such, it is entirely foreign to Schwann cells, from which it is perfectly distinct in well fixed and stained preparations." This was one of the few misinterpretations in Ramon y Cajal's classic light microscopic observations. Ramon y Cajal's appreciation for the intricate relationships between the various components of the nervous system remains at the forefront of modern science. His major limitation in determining the source of myelin was merely technical. It was then, and is still today, impossible to resolve the cellular boundaries of the axon and myelin at the light microscopic level. It was not until the advent of the transmission electron microscope in the late 1950s that fundamental questions regarding the microstructure of the myelinated axons could be addressed. Initially, transmission electron microscopy (TEM) was a science unto itself. As the methods and electron microscopes improved, myelin ultrastructure was eloquently described. Myelin was unequivocally demonstrated to be a spiraled extension of the Schwann cell plasma membrane in the PNS (Geren, 1954) and of the oligodendrocyte plasma membrane in the CNS (Bunge *et al.*, 1962). Furthermore, oligodendrocytes were shown to form multiple myelin internodes, while Schwann cells formed but a single internode. Eventually, other concepts emerged. Myelin internodes were shown to consist of ultrastructurally distinct domains, and these domains were not identical in the PNS and CNS. These concepts are fundamental to current EM research that focuses on the molecular architecture of myelin internodes and on pathological changes that result from inherited and acquired diseases of myelin.

While TEM is no longer a science unto itself, it is currently the only road to many of the most momentous questions of modern molecular science. This chapter describes the molecular ultrastructure of myelin with a focus on normal function and pathological changes that result from myelin disease. For historical perspectives, the reader is directed to Peters *et al.*, 1991 and Rosenbluth, 1999. A discussion of the role of myelin in saltatory conduction is presented in Chapter 5.

MYELIN INTERNODE ORGANIZATION

To appreciate the ultrastructure of the myelin internode, one must understand its three-dimensional organization. In simple morphological terms, the myelin internode can be divided into two domains, compact myelin and noncompact myelin. Figure 1.1A is a diagram of a teased myelinated PNS axon observed and drawn by Ramon y Cajal (Ramon y Cajal, 1928). This myelin internode is 0.5 mm long and surrounds an axon

facilitates neuronal communication, but have little knowledge of how it modulates axonal integrity. Neurons communicate by depolarizing the "electrical potential" of their axons. This occurs by channel-mediated exchange of Na^+ in an energy dependent manner (see Chapter 5). Unmyelinated axons generate action potentials along their entire surface at a speed that is proportional to their radius. Rapid unmyelinated axonal communication would demand energy and space requirements that are not conducive to evolution of the large, complex mammalian brain. It was advantageous, therefore, to evolve a mechanism to propagate neuronal communication through thin axons in an energy efficient manner. Myelin-forming cells serve this function by producing a series of discontinuous insulation units called internodes along individual axons. Each myelin internode can be divided into two ultrastructurally and functionally distinct domains: compact myelin and the paranodal loops. Compact myelin inhibits ion exchange during nerve conduction by having a low capacitance and high resistance. The paranodes, which demarcate the longitudinal ends of each internode, facilitate ion exchange at the node of Ranvier, a small "bare patch" of axon that separates each successive internode. The nerve impulse jumps from node to node by saltatory conduction (Fig. 1.2B). Mammalian unmyelinated nerves have conduction velocities of less than 1 m/sec. Myelinated axons of the same diameter conduct 10 times faster and the largest fibers conduct up to 100 m/sec. For unmyelinated axons to achieve similar velocities to myelinated axons of the same size, they would need to be about 100 times thicker.

COMPACT MYELIN

Most of the myelin internode consists of compact myelin that appears by TEM as a lamellar structure of alternating dark and light lines that spiral around the axon (Fig. 1.3). Although compact myelin has a very simple ultrastructure, it is often difficult to comprehend. Compact myelin ultrastructure is best understood if it is considered a spiraled cellular process or sheet that contains two plasma membranes and no cytoplasm. Membranes visualized by conventional TEM have an internal translucent core and two electron dense lines or leaflets at the extracellular and intracellular surface (Alberts *et al.*, 1994). Figure 1.4A depicts a cross section of the theoretical myelin membrane sheet with cytoplasm (green). The lipid bilayer of the surface membrane is disproportionately increased in size: the red line represents the extracellular leaflet, while the dark green line represents the cytoplasmic leaflet. This process is spiraled in Figure 1.4B and spiraled and compacted in Figure 1.4C. In the compacted spiral, apposing extracellular leaflets (called intraperiod or intermediate lines) are separated by 2.0 nm in the CNS and 2.5 nm in the PNS (Fig. 1.4D). The cytoplasmic leaflets appear fused and form the major dense lines or intraperiod lines. The spiral membranes of compact myelin have a periodicity (distance from dense line to dense line) of approximately 13 to 14 nm when fixed with aldehydes and embedded in epoxy resins (Fernandez-Moran and Finean, 1957; Kirschner and Hollingshead, 1980) and is slightly greater in PNS myelin than in CNS myelin. *In situ*, however, these distances are slightly larger (17 to 18 nm) when measure by X-ray diffraction (Kirschner and Sidman, 1976).

Since the major function of compact myelin is insulation, it need not be biochemically complex or molecularly dynamic. Ultrastructure would predict membrane adhesion as the major function of compact myelin molecules. Furthermore, adhesion molecules cannot occupy large extracellular or cytoplasmic areas and it would be most efficient if they have a slow turnover rate. All of these predictions have been confirmed and are discussed in detail in other chapters of this book. A high lipid composition (~75%), imparts a high buoyancy to homogenized myelin membranes upon sucrose gradient subfractionation and separates it from other membrane fractions with the more common 1 to 1 lipid:protein ratios (Norton and Poduslo, 1973). Because of its abundance, invariable structure and unique biochemical composition, myelin was the first CNS membrane to be isolated and molecularly characterized. Abundant proteins include P_0 protein, the myelin basic proteins (MBP), P_2 protein and PMP-22 in PNS myelin (Greenfield *et al.*, 1973), and proteolipid protein (PLP) and MBP in CNS myelin (Lees and Brostoff, 1984). Immunocytochemistry

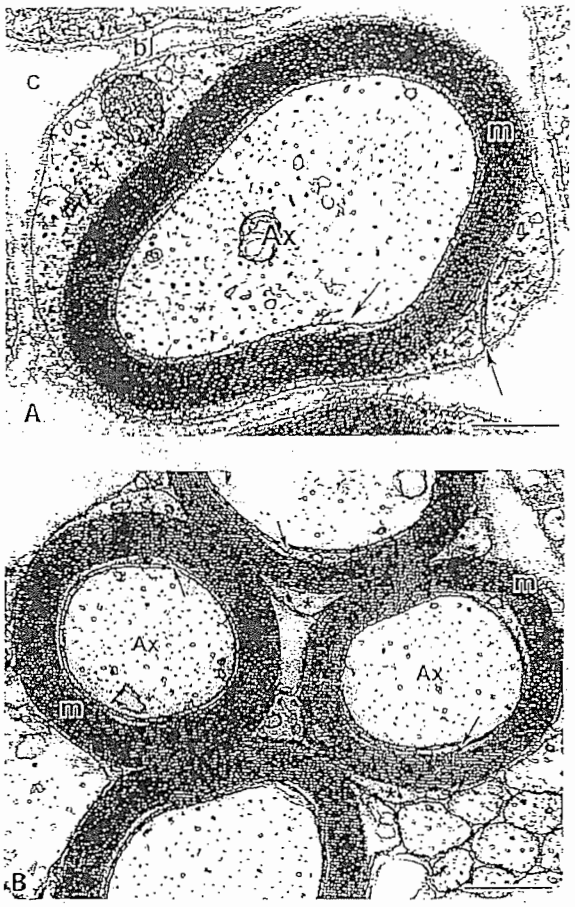

FIGURE 1.3
Ultrastructure of myelinated axons in the peripheral (A) and the central (B) nervous systems. In both PNS and CNS, axons (Ax) are surrounded by electron-dense compact myelin (m). The myelin spirals are bounded by the inner (periaxonal) and outer (abaxonal) mesaxons (arrows). In PNS myelinated fibers (A), organelle-rich cytoplasmic channels (*) occupy approximately 50% of the outer circumference, and are separated by nonchannel regions. Basal lamina (bl) surrounds the entire abaxonal surface, and extracellular collagen fibrils (c) separate adjacent fibers. Cytoplasmic channels of the CNS internode include inner and outer tongue processes (B, *). Unlike the PNS, no visible extracellular matrix separates myelinated fibers, and where compact sheaths lie adjacent to each other, a common intraperiod line forms. Panel B has been reproduced from Tabira *et al.*, 1978, with permission. Scale bars = 0.5 μm.

confirmed an enrichment of P_0, MBP, PMP-22, and P_2 protein in compact PNS myelin and PLP and MBP in compact CNS myelin (Hartman *et al.*, 1982; Snipes *et al.*, 1992; Sternberger *et al.*, 1978; Trapp *et al.*, 1979). Amino acid composition and biochemical properties identified P_0, PMP-22, and PLP as integral membrane proteins and MBP and P_2 protein as extrinsic membrane proteins. P_0, PMP-22, and PLP are positioned to maintain the periodicity of both the extracellular and cytoplasmic spacing of myelin membranes, while MBP and P_2 could influence cytoplasmic leaflet fusion.

A major focus of myelin research in the 1990s was the development of technology to eliminate these myelin proteins in mice. One unexpected finding of these studies is that significant amounts of multilammellar membranes form in the absence of P_0 (Giese *et al.*, 1992), PLP (Duncan *et al.*, 1988, 1989), MBP (Privat *et al.*, 1979), or MAG (Li *et al.*, 1994; Montag *et al.*, 1994), PLP/MBP (Stoffel *et al.*, 1997), or PLP/MBP/MAG (Uschkureit *et al.*, 2000). Spiral expansion of myelin membranes, therefore, does not depend on these molecules. Electron microscopy, however, has convincingly established that PLP and MBP are required for normal spacing of compact CNS myelin (Duncan *et al.*, 1988; Privat *et al.*,

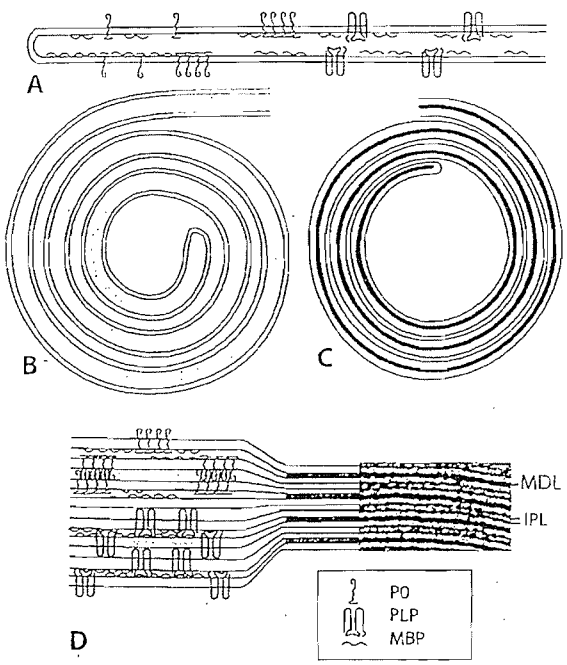

FIGURE 1.4
Schematics of compact myelin periodicity. Compact myelin can be viewed as a cell process (A). The process grows spirally (B) around an axon, and during myelin compaction (C), the cytoplasm (green) is excluded. Cytoplasmic membrane leaflets (dark green) fuse to form the major dense lines (D, MDL), while extracellular leaflets (red) of adjacent lamellae become closely apposed to form the intraperiod lines (D, IPL). The orientation of three proteins that play a dominant role in compact myelin integrity are depicted: For simplicity, both CNS and PNS proteins are shown here; see the text for their individual distributions.

1979), while P_0 is essential for normal spacing of PNS compact myelin (Giese *et al.*, 1992). When PLP and P_0 are removed, compact myelin membrane spacing is often variable. While the mistargeting of other proteins may play a role in this variability, it is most likely due to poor fixation. Interpreting compact myelin ultrastructure is not always straightforward. Larger fibers are prone to poor fixation even in the most experienced hands and can have split and disrupted lamellae. Mechanical damage during preparation can also produce artifacts. Great care must be taken in ascribing abnormal myelin ultrastructure to pathology. Fixation is a special challenge in PLP-null and P_0-null mutants since the majority of compact myelin proteins, which are the substrates for glutaraldehyde fixation, are missing. Individual chapters in this volume are dedicated to each myelin protein. Salient features of individual proteins as they relate to compact myelin structure are briefly summarized.

P_0 Protein

P_0 constitutes as much as 70% of the total proteins in PNS myelin. It is a type I transmembrane glycoprotein with a single extracellular immunoglobulin-like domain, one transmembrane domain, a cytoplasmic C-terminal, and an apparent molecular weight of 30 kD (Lemke and Axel, 1985). When transfected into nonadherent cells *in vitro*, the extracellular domain of P_0 protein mediated homophilic, but not heterophilic plasma membrane cell adhesion (D'Urso *et al.*, 1990; Filbin *et al.*, 1990). The molecular interactions responsible for P_0 homophilic adhesion have been delineated by 1.9Å resolution of P_0 extracellular domain crystals (Shapiro *et al.*, 1996). The extracellular domains of P_0 interact as cis-linked tetramers that bind through hydrogen bonds to P_0 tetramers in

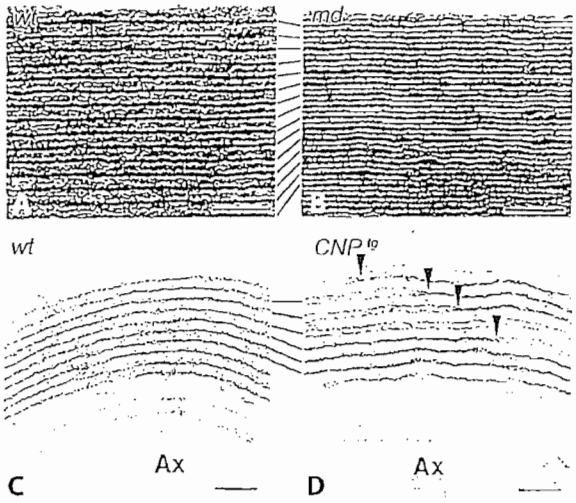

FIGURE 1.5
Alterations in the periodicity of compact myelin due to the absence of proteolipid protein (PLP) and the presence of 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (D). The periodicity of compact CNS myelin is compared in wild-type (A) and PLP deficient *md* (B) rats and in wild-type (C) and CNP overexpressing (D) mice. The absence of PLP results in the apparent "fusion" of the extracellular leaflets of compact myelin. Overexpression of CNP by oligodendrocytes results in mistargeting of CNP to compact myelin where it prevents fusion of cytoplasmic leaflets of compact myelin (D). Arrowheads in D mark transition from normal to abnormal major dense lines. Lines between panels A and B and panels C and D align major dense lines. Ax = axon. Reproduced from Duncan *et al.*, 1989 (A and B), and Yin *et al.*, 1997 (C and D), with permission. Scale bars = 50 nm.

opposite orientation on the opposing plasma membrane. Cis tetramerization occurs through polar interactions that may be stabilized by trans interactions. The intraperiod line of PNS myelin, therefore, is stabilized by P_0 homophilic interaction in the same and opposing compact myelin membranes. Spacing between the extracellular leaflets of compact PNS myelin is altered in P_0-null mice (Giese *et al.*, 1992), but the major dense line is ultrastructurally similar to wild-type mice.

Myelin Basic Protein

MBP is localized at the cytoplasmic surface of compact CNS and PNS myelin. It is a family of alternatively spliced, highly charged extrinsic membrane proteins (Greenfield *et al.*, 1973; Zeller *et al.*, 1984), which bind negatively charged lipids, especially phosphatidylserine residues. In MBP-deficient shiverer mice, the cytoplasmic leaflets of compact CNS myelin are not fused (Privat *et al.*, 1979). In contrast, the major dense line of PNS myelin is unaltered. MBP, therefore, maintains the major dense line of CNS but not PNS myelin. It has been hypothesized that the positively charged cytoplasmic domain of P_0 protein can maintain the major dense line of PNS myelin.

Proteolipid Protein

Proteolipid protein (PLP) is the most abundant CNS myelin protein. It is a hydrophilic protein with four membrane-spanning domains and a MW of 26 kD. In rodents with PLP mutations and little or no PLP in compact myelin (Duncan *et al.*, 1988, 1989), the extracellular leaflets of compact CNS myelin are fused (Figs. 1.5 A and 1.5B). In PLP-null mice, the extracellular leaflets of many CNS myelin lamellae were separated by distances greater than 2 nm (Griffiths *et al.*, 1998). These differences in MD rat and PLP-null mice may reflect differences in fixation or tissue processing. PLP lacks the homophilic

binding potential described for P_0 protein. It maintains the 2.0 nm spacing between extracellular leaflets of compact CNS myelin by electrostatic interactions with myelin lipids. As described later in this chapter, PLP may be essential for long-term survival of some CNS axons.

Peripheral Myelin Protein-22

Peripheral myelin protein-22 (PMP-22) is present in compact PNS myelin (Snipes *et al.*, 1992). It is a hydrophilic protein with four transmembrane domains and a MW of 22 kD. While absence has little effect on compact myelin ultrastructure, PMP-22-null mice initially exhibit a severe hypomyelination (Adlkofer *et al.*, 1995). PMP-22 may have additional roles in Schwann cells that are related to its function as a growth arrest protein (Bosse *et al.*, 1994; Zoidl *et al.*, 1995).

P_2 Protein

P_2 is an extrinsic membrane protein that is enriched in compact PNS myelin. P_2 has also been detected in CNS myelin of rabbits and humans (Trapp *et al.*, 1983), but not in mice or rats. In the CNS of those species that contain P_2 protein, it is abundant in spinal cord and brain stem myelin but not in cerebral cortex myelin. The reasons for the species differences and caudal to rostral gradient in CNS P_2 expression are unknown. P_2 protein can induce an autoimmune PNS demyelinating disease when injected subcutaneously with complete Freunds adjuvant (Kadlubowski *et al.*, 1980). P_2 protein has not been eliminated from the mouse genome and P_2 mutations in rodent or humans have not been identified. P_2 belongs to a family of proteins that function in fatty acid transport (Uyemura *et al.*, 1984). It may also participate in fusion of the major dense line of compact PNS myelin.

2′,3′-Cyclic Nucleotide 3′-Phosphodiesterase (CNP)

CNP is concentrated on the cytoplasmic side of noncompact myelin membranes (Braun *et al.*, 1988; Trapp *et al.*, 1988). As its name implies, CNP is capable of catalyzing the hydrolysis of 2′,3′ nucleotides into their corresponding 2′ nucleotides (Drummond *et al.*, 1962; Sprinkle *et al.*, 1978). A CNS substrate for this enzymatic activity has not been identified. When overexpressed in mice, CNP is mistargeted to compact CNS myelin membranes (Figs. 1.5C and 1.5D). These membranes are deficient in MBP and do not form major dense lines (Yin *et al.*, 1997). CNP, therefore, may help target MBP to wild-type compact myelin by inhibiting MBP binding to noncompact myelin.

Myelin Protein Dosage

Considering all the complex roles of compact myelin proteins, it is not surprising that their stoichiometry is as important as their presence. Alterations in myelin protein gene dosage often have chronic rather that acute manifestations. One allele of the human PMP-22 gene causes hereditary neuropathy with liability to pressure palsy (HNPP). Three copies of the human PMP-22 gene cause Charcot-Marie-Tooth disease type 1A. Duplication of the PLP gene (two alleles, PLP is on the X chromosome) causes the inherited CNS hypomyelination disorder Pelizaeus Merzbacher disease. Alterations in PMP-22 and PLP protein dosage are the major cause of inherited diseases of myelin.

NONCOMPACT MYELIN

When comparing the ultrastructure of PNS (Fig. 1.3A) and CNS (Fig. 1.3B) myelinated fibers, one is struck by the similar appearances of both the compact myelin and the axon.

While the periodicity of compact CNS and PNS myelin differs, they still remain difficult to distinguish without morphometric comparisons. CNS and PNS myelinated fibers can be easily distinguished by the presence of a basal lamina around PNS, but not CNS, myelinated fibers. They also differ by the distribution and ultrastructure of cytoplasmic channels at the outer margin of the internode. CNS fibers contain a single external or outer tongue process of oligodendrocyte cytoplasm, which occupies 5 to 20% of the outer circumference. This cytoplasmic channel longitudinally traverses the entire internode and connects oligodendrocyte perinuclear cytoplasm and paranodal cytoplasm. When viewed in cross section, the outer tongue process usually contains transversely sectioned microtubules, occasional mitochondria, smooth endoplasmic reticulum, and vesicles. The remainder of the outer membrane of CNS internodes appears identical to all other compact myelin lamellae: they form a major dense line and can form an intraperiod line with closely apposed neighboring internodes.

The external surface of PNS myelin fibers is surrounded by a basal lamina. In addition, the endoneurial or extracellular space of peripheral nerves is conspicuously abundant and contains collagen fibrils. Basal lamina and collagen are reliable criteria to distinguish PNS and CNS myelinated fibers. The Schwann cell basal lamina contains laminin-2 and collagens II and IV (Carey *et al.*, 1983; Cornbrooks *et al.*, 1983). The outer or abaxonal membrane of the PNS internode differs molecularly and structurally from the compact myelin. The outer surface of each PNS internode contains multiple cytoplasmic channels (Fig. 1.6A). A major channel that extends the entire length of the internode contains the outer mesaxon, which is comprised of the membranes that connect the abaxonal Schwann cell membrane and compact myelin. The cytoplasmic surface of the Schwann cell membrane that forms the channels contain CNP (Trapp *et al.*, 1988) and the microfilament components, filamentous actin (F-actin), spectrin, and ankyrin (Trapp *et al.*, 1989b). Schwann cell channel membranes are also rich in cavaeoli, which contain caveolin (Mikol *et al.*, 1999); cavaeoli are not a prominent feature of CNS internodes. The surface membrane of the nonchannel regions of the outer surface of PNS myelin internodes are separated from compact myelin by about 10 nm and is molecularly characterized by the presence of dystroglycan-related protein 2 and periaxin (Sherman *et al.*, 2001), but not by CNP, microfilaments or caveolin (Fig. 1.6B). Disruption of the molecular organization of the abaxonal membrane can cause dysmyelination. Periaxin-null mice have a peripheral neuropathy characterized by dysmyelination, demyelination, and remyelination (Gillespie *et al.*, 2000; Guilbot *et al.*, 2001). The abaxonal nonchannel regions of the PNS myelinated internodes were conspicuously absent in these mice. Normal Schwann cell myelination, therefore, requires the development of specialized abaxonal transport conduits.

Despite their differences, the inner surface of both PNS and CNS myelin internodes are separated from the axon by a 12 to 14 nm periaxonal space. The periaxonal membranes contain MAG, CNP, and microfilaments (Trapp *et al.*, 1988 1989b). MAG is also present in the inner mesaxon membrane, which connects compact myelin and the periaxonal membrane. The periaxonal membranes of all PNS fibers and developing CNS fibers are separated from the compact myelin by a gap of at least 10 nm. Portions of the periaxonal membrane of mature CNS fibers can form a major dense line with the compact myelin lamellae. A single cytoplasmic channel is present at the inner surface of both CNS and PNS internodes. These channels longitudinally traverse the entire internode and are distinguished from the remainder of the periaxonal region by increased cytoplasm, occasional organelles, and the presence of the inner mesaxon.

Compact and noncompact myelin membranes are contiguous with each other and with the plasma membrane of myelin-forming cells. However, they differ ultrastructurally and have distinct nonoverlapping protein compositions. Many noncompact myelin proteins serve specialized functions and are discussed later in this chapter. Others have more generalized functions and are enriched in all or some noncompact membranes. The distribution of proteins in the myelin internode was initially inferred from light microscopic immunocytochemistry. Figures 1.7A and 1.7B compare the light microscopic distribution of P_0 protein, the major structural protein of compact PNS myelin, with that of MAG. Based on these and other comparisons, Trapp and Quarles (Trapp and Quarles,

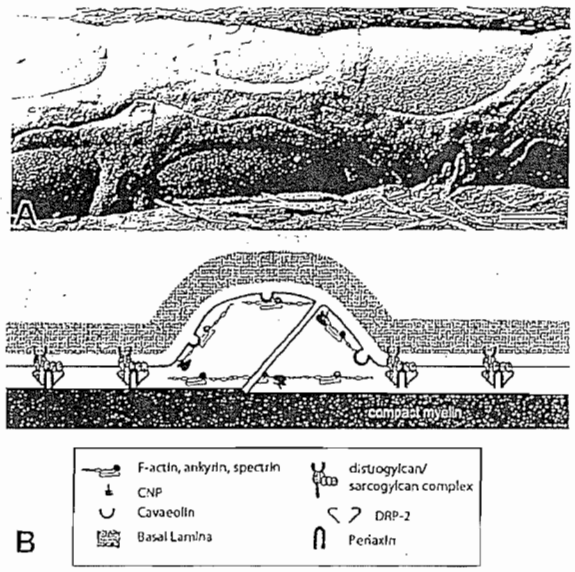

FIGURE 1.6
Cytoplasmic channels at the outer perimeter of PNS myelin internodes. In freeze fracture preparations (A), longitudinally and radially oriented cytoplasmic channels are raised above nonchannel regions, and the surface of the outer mesaxon (A, arrows) appears as a furrow in the large longitudinal channel. Numerous cavaeoli (A, arrowheads) pit the channel surface. Membranes in channel and nonchannel regions differ in molecular composition (B). Channel membranes are rich in actin-associated cytoskeletal components, cavaeolin, and CNP, while nonchannel membranes contain dystroglycan/sarcoglycan/periaxin-complexes that include dystrophin-related protein 2 (DRP-2). A basal lamina surrounds the entire surface of PNS internodes. Panel A has been reproduced from Mugnaini *et al.*, 1977, with permission. Scale bars = 1 μm.

1984) proposed that MAG was enriched in the periaxonal membrane, as well as in the membranes of Schmidt-Lanterman incisures, paranodal loops, and inner and outer mesaxons. In addition, they noted that all MAG-containing membranes apposed other membranes by 12 to 14 nm and did not form the major dense lines characteristic of compact myelin. MAG, therefore, is a molecular marker for the membranes that demarcate the cytoplasmic domains within the PNS internode. In CNS myelin, MAG is enriched in the periaxonal membrane and the CNS inner mesaxon, but not in paranodal membranes or outer mesaxons (Trapp *et al.*, 1989a). Because the size of the immuno-complexes is approximately 10 times larger than that of a single membrane (Trapp and Quarles, 1984), a comparison of P_0 and MAG distributions in 1 μm thick serial section is only suggestive of nonoverlapping distributions. However, electron microscopic immunocytochemistry, utilizing ultrathin cryosections and smaller gold-labeled immunoreagents (Trapp *et al.*, 1989a), clearly demonstrated the nonoverlapping distributions of P_0 and MAG inferred from light microscopic studies.

As the molecular structure of myelin is better characterized, the need for electron microscopic immunocytochemistry will increase. This is especially the case for molecules enriched in paranodal regions. Paranodal loops contain multiple subdomains that are difficult to resolve at the light microscopic level. Two general approaches, pre-embedding (staining before sectioning) and post-embedding (staining cut sections) are successfully used to localize antigens ultrastructurally. Each approach has its advantages and disadvantages. The trade-off is between tissue fixation and penetration of immunoreagents. Pre-embedding methods utilize tissue slices (30 to 100 μm thick) (Butt *et al.*, 1999) or teased nerve fibers fixed with low concentrations (0.1 to 0.5%) of glutaraldehyde. Glutaraldehyde is needed for optimal fixation and it irreversibly cross-links proteins. Too much glutaraldehyde fixation will inhibit penetration of immunoreagents into the tissue. Glutaraldehyde can alter the antigenicity of proteins, but this is usually the exception rather than the

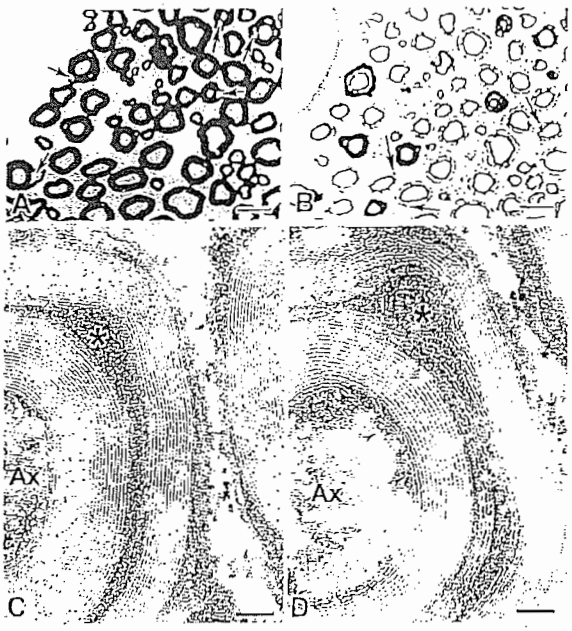

FIGURE 1.7
Light microscopic (A, B) and electron microscopic (C, D) distributions of P_0 (A, C) and MAG (B, D) in PNS myelin. By light microscopy, the distributions of P_0 and MAG appear different in the same fibers (arrows), but the section thickness and light microscopic resolution limit interpretation. Immunogold labeling of frozen sections indicates that P_0 is concentrated in compact membranes and excluded from incisural (*), periaxonal, and abaxonal membranes (C). MAG (D) is concentrated in incisures and periaxonal membrane (arrowheads) but not in compact myelin or at the cell surface. Reproduced from Trapp and Quarles, 1982 (A and B), and Griffin *et al.*, 1993 (C and D), with permission. Scale bars = 10 μm (A, B) and 0.2 μm (C, D).

rule. The use of post-embedding methods avoids the penetration limitation of stringent (2 to 5%) glutaraldehyde fixation. Immunostained ultrathin cryosections (Trapp *et al.*, 1989a) or etched embedded sections (Schérer *et al.*, 1995b) provide the best ultrastructure, and antigens exposed at the surface are accessible to immunoreagents. While post-embedding methods are technically more demanding than pre-embedding techniques, both approaches have determined the ultrastructural distribution of myelin proteins.

NODES OF RANVIER

CNS and PNS nodes serve identical functions, and are morphologically similar but not ultrastructurally identical (Fig. 1.8). From a functional and ultrastructural point of view, three regions can be distinguished: node, paranode, and juxtaparanode (Fig. 1.9A). Each region contributes to saltatory conduction and is maintained by complex molecular interactions that have been compared with those operating at the neural and immunological synapse (Pedraza *et al.*, 2001). We are just beginning to understand the complex molecular interactions at nodes of Ranvier; interactions in which many participants still remain to be identified.

Nodes

The node is a small stretch of bare axonal plasma membrane (axolemma) that separates myelin segments along individual nerve fibers. Easily identified in longitudinal sections, nodes separate the terminal paranodal loops of adjacent myelin internodes (Figs. 1.8A and

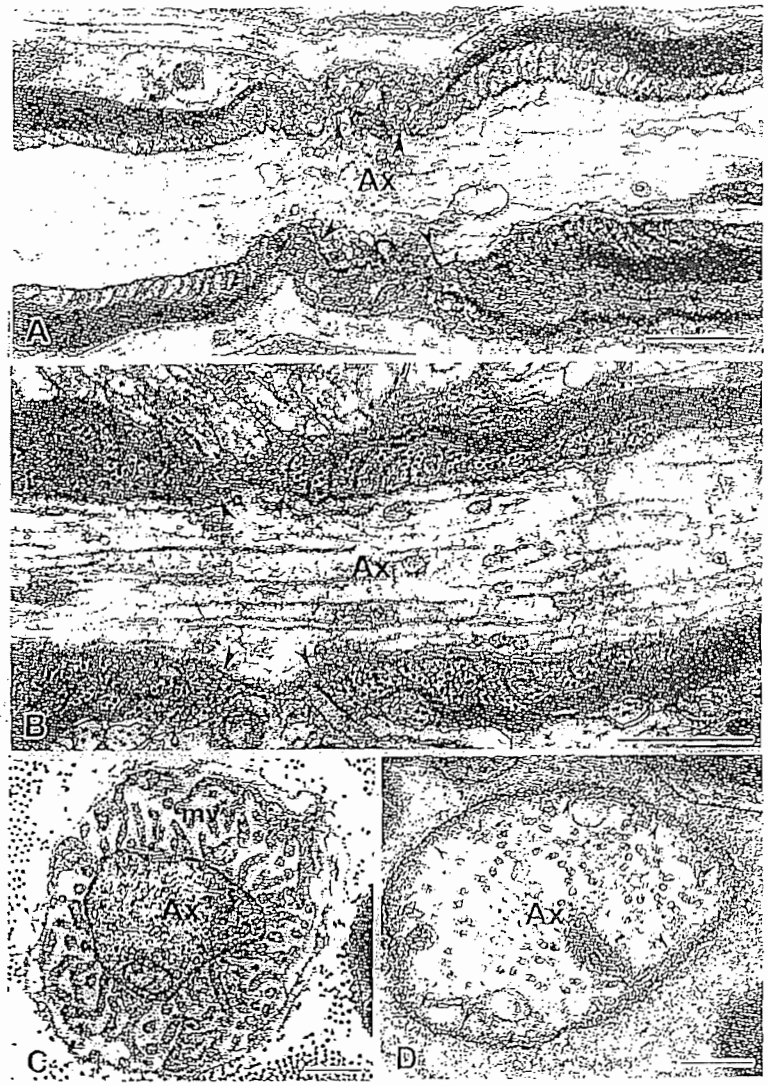

FIGURE 1.8
Nodes of Ranvier in longitudinal (A, B) and transverse (C, D) orientations from PNS (A, C) and CNS (B, D). The nodal axonal (Ax) membrane extends between the arrowheads (A, B), covering approximately 1 μm, and bulges outward in these relatively small myelinated fibers. To either side, the paranodal loops of the terminating internodes indent the axon and demarcate the paranodal region. In transverse section (C), the PNS nodal membrane is surrounded by numerous microvilli (mv), while CNS nodes (D) may be surrounded by glial processes or simply by amorphous extracellular matrix material. The nodal axolemma contains an electron dense undercoating (C, D small arrowheads). Panels A, B, and D have been reproduced from Peters *et al.*, 1991, and panel C from Boyle *et al.*, 2001, with permission. Scale bars = 0.5 μm (A, B) and 0.2 μm (C, D).

1.8B). Nodal length is related to the diameter of the axon and can vary from less than 1 μm in the small fibers of the optic nerve to more than 5 μm in the large fibers of the spinal cord (Fraher, 1973; McDonald and Ohlrich, 1971). Nodal axon diameter usually differs from paranodal and internodal, ranging from a conical bulge in small fibers of the CNS to constriction in large fibers of the PNS. When constricted, the density of microtubules, vesicles, and neurofilaments increases. Neurofilament spacing is reduced and reflects, in part, reduced neurofilament phosphorylation and reduced neurofilament sidearm extension (Mata *et al.*, 1992).

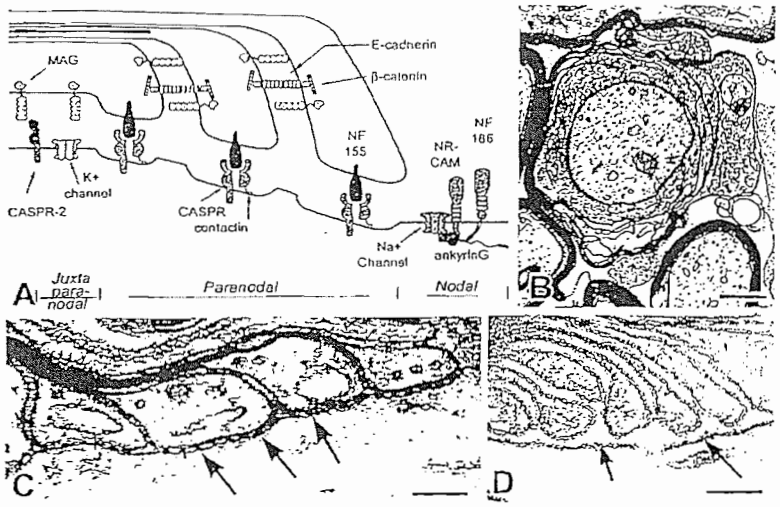

FIGURE 1.9

Molecular composition and structure of nodal regions in longitudinal (A, C, D) and transverse orientation (B). Three regions (nodal, paranodal, and juxtaparanodal) can be defined morphologically and each has a unique molecular composition (A). Nodal axolemma contains high densities of Na^+ channels and adhesion molecules such as Nr-CAM and neurofascin 186 (NF186), and an actin cytoskeleton enriched in ankyrin G. In the paranodal region, axoglial junctions involve complexes of axonal Caspr and contactin and glial neurofascin 155 (NF155). Adjacent paranodal loops are stabilized through E-cadherin/ß-catenin–based adherens junctions, and spacing is regulated by the presence of MAG. In transverse section (B), the paranodal loops appear as non-compacted membranes containing substantial cytoplasm. In longitudinal sections (C), the septate junctions (arrows) appear as punctate structures spanning the cleft between the axonal surface and paranodal loops. Contactin-null mice lack septate junctions (D, arrows). Juxtaparanodal axolemmae are enriched in K^+ channels and caspr2. Reproduced from Trapp and Kidd, 2000 (A), Bosio *et al.*, 1998 (C), and Boyle *et al.*, 2001 (D), with permission. Scale bars = 0.5 µm (B) and 0.1 µm (C, D).

The nodal axolemma is the functional ultimate of myelin formation as it contains voltage-gated Na^+ channels and thereby is directly responsible for saltatory conduction. The channels were initially visualized by freeze-fracture electron microscopy as 10 nm diameter axolemmal particles, at a density of 1000 to 1500/µm^2 (Rosenbluth, 1976). Although initial estimates suggested many more channels were present, the observed particle density matched the electrophysiological estimates of nodal Na^+ channel concentration (Chiu, 1980; Waxman and Ritchie, 1985).

Identification of nodes in cross section (Figs. 1.8C and 1.8D) is not as easy. One must rely on more subtle characteristics of the nodal axolemma and surrounding structures. One ultrastructural characteristic is a dense 20 nm thick coating beneath the axolemma. This coating is similar to that at the initial axon segment and correlates with the presence of high concentrations of Na^+ channels (Caldwell *et al.*, 2000; Ellisman and Levinson, 1982). It represents a specialized submembranous cytoskeleton that is connected to a multimolecular complex in the nodal axolemma (Fig. 1.9A). In addition to voltage-gated Na^+ channels, membrane proteins enriched in the nodal axolemma include an isoform of Na^+/K^+-ATPase, neurofascin-186 and Nr-CAM (Ariyasu and Ellisman, 1987; Davis *et al.*, 1996; Tait *et al.*, 2000). The submembranous cytoskeleton contains spectrin, f-actin, and two alternately spliced isoforms of the ankyrin$_G$ (270 and 480 kD) (Kordeli *et al.*, 1990, 1995). The dense undercoating is separated from the nodal axolemma by 10 nm; it helps to stabilize Na^+ channels at the node. Ankyrin$_G$ may also associate with Na^+ channels in the neuronal soma and assist in targeting Na^+ channels to the node (Kordeli *et al.*, 1995).

Although PNS and CNS nodes can appear similar by TEM, each is surrounded by distinctly different immediate environments. The nodal axolemma is surrounded by Schwann cell microvilli in the PNS and by glial cell processes in the CNS. The microvilli, which extend from the outer border of the adjacent myelin internodes, are

best visualized in cross sections, where they appear as thin processes (Fig. 1.8C). Nodal microvilli are enriched in actin filaments and members of the ERM family of actin associated proteins (Kordeli *et al.*, 1990; Melendez-Vasquez *et al.*, 2001; Scherer *et al.*, 2001). The Schwann cell basal lamina is continuous across the node but does not surround individual microvilli. The glial cell processes surrounding CNS nodes originate from astrocytes (Hildebrand and Waxman, 1984) and possibly oligodendrocyte progenitor cells (Butt *et al.*, 1999). These CNS glial processes do not, however, closely adhere to all regions of the nodal axolemma. Where absent, granular-appearing extracellular matrix, which may bind ions and participate in saltatory conduction, surrounds the CNS nodal axolemma. Little is known about the molecular characterization of the glial processes at CNS nodes.

Paranodal Regions

Paranodal loops are the lateral ends of the myelin internode. They consist of a longitudinally oriented spiral of Schwann cell cytoplasm that is tightly tethered to the axon and to each other by junctional complexes. Paranodal ultrastructure is best observed in longitudinal orientation where they appear on each side of the node as a series of Schwann cell cytoplasmic pockets (Figs. 1.8A and 1.8B). As compact myelin approaches the node, the major dense line "opens up" to accommodate cytoplasm. The extracellular leaflets of neighboring paranodal loops oppose each other by 12 to 14 nm. In this respect, they are similar to Schmidt-Lanterman incisures, and in transverse orientation (see Fig. 1.9B), they may be mistaken for incisures. They differ from incisures in that they usually contain more cytoplasm and organelles. In smaller fibers, paranodal loops are arranged in an orderly manner with the inner compact myelin lamellae terminating first and the outer lamellar terminating last, directly adjacent to the node. Paranodal loop alignment in larger diameter fibers follows a similar but less organized pattern, as some loops do not reach the axonal surface.

The paranodal membrane that apposes the axolemma is slightly curved, but separated from the axolemma by a constant distance of 2.5-3.0 nm (Peters *et al.*, 1991). The axolemma in paranodal regions, therefore, appears as a series of shallow troughs with each corresponding to a terminal loop. Between paranodal loops and axons are evenly spaced densities that appear to connect the two membranes (Fig. 1.9C). The densities are each about 15 nm long and 25 to 30 nm apart and because of their orientation were originally referred to as transverse bands. Based on a variety of experimental approaches, it is now clear that these densities represent septate-like junctions and are the major adhesive apparatus between myelin and axons. Paranodal membranes that oppose the axolemma contain large intramembranous particles that appose with a double row of smaller intramembranous particles on the axolemma (Wiley and Ellisman, 1980). Recent studies have begun to characterize the molecular composition of axoglial septate junction (Fig. 1.9A). The membrane proteins, contactin and Caspr, are enriched in paranodal axolemma in both the CNS and PNS (Einheber *et al.*, 1997; Menegoz *et al.*, 1997; Rios *et al.*, 2000). They function as heterodimers that may be assembled prior to insertion in the axolemma. Paranodal loops contain an isoform of neurofascin (NF155), which co-localizes with and possibly binds to contactin and Caspr (Charles *et al.*, 2002; Tait *et al.*, 2000). These molecules have homologues associated with septate junctions in invertebrates, and axoglial junctions are abnormal or missing in Caspr and contactin-null mice (Fig. 1.9D) (Bhat *et al.*, 2001; Boyle *et al.*, 2001). Clustering of Na^+ channels at the PNS node is not grossly altered in contactin-null mice. Shaker type Kv1.1 and Kv1.2 potassium channels, which are enriched in the juxtaparanodal axolemma and absent from paranodal axolemma in wild-type mice, are detected in paranodal axolemma in mice deficient in CGT (Dupree *et al.*, 1998), Caspr (Bhat *et al.*, 2001), or contactin (Boyle *et al.*, 2001). The paranodal axoglia junction, therefore, appears to play a role in spatially separating Na^+ and K^+ channel distribution at the node of Ranvier.

Juxtaparanodes

The juxtaparanodal region, is defined as the 10 to 15 nm of the myelin internode that is adjacent to the paranodal loops. Other than this spatial relationship to the paranodes,

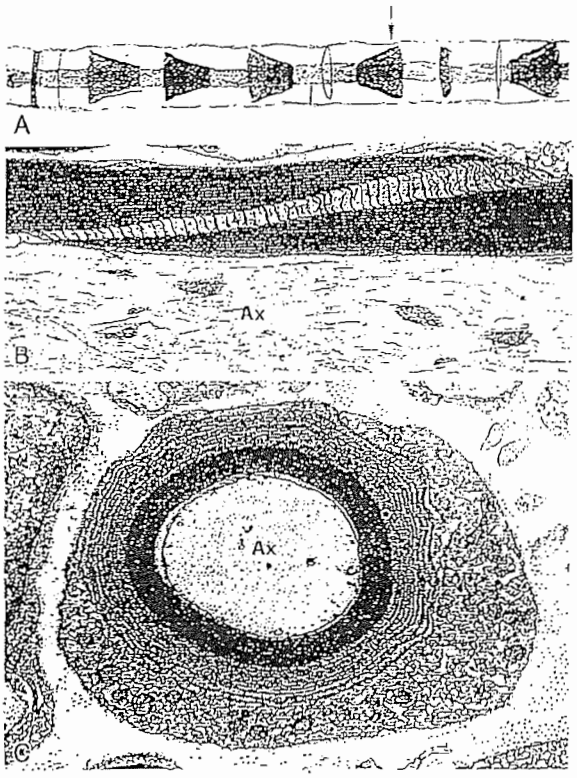

FIGURE 1.10
Ultrastructure of Schmidt-Lanterman incisures. Drawing by Ramon y Cajal (A) shows arrangement of "funnel-shaped" incisures in a teased PNS myelinated fiber. When incisures are sectioned longitudinally and examined by electron microscopy (B), the spiraled cytoplasmic channel transverses the compact myelin at an angle, but incisure membranes are in register with each other. When sectioned in transverse orientation (C), they appear as noncompacted membrane spirals (•) that are contiguous with compact myelin. Ax = axon. Arrow in panel A depicts plane of section for panel C. Reproduced from Ramon y Cajal, 1928 (A), Mugnaini *et al.*, 1977 (B), and Trapp and Quarles, 1982 (C), with permission. Scale bars = 0.5 μm (A) and 1 μm (B).

TEM characteristics of the juxtaparanode are indistinguishable from the remainder of the myelin internode. Freeze fracture electron microscopy, however, identified clusters of intramembranous particles in the juxtaparanodal axolemma (Rosenbluth, 1976). These particles correspond to the distribution of delayed rectifying K^+ channel. They have a similar size (10 nm) to that predicted for K^+ channels that function in myelinated fibers as mixed tetramers of the Kv1.1 and Kv1.2 subunits. Immunocytochemistry has localized Kv1.1, Kv1.2, and their associated subunit b_2 to juxtaparanodal regions (Rasband *et al.*, 1998). In addition, a homologue of Caspr, Caspr 2, is enriched in the juxtaparanodal axolemma (Poliak *et al.*, 1999). Potassium channels are thought to play a role in saltatory conduction by "dampening" the excitability of the node. This may be important in facilitating rapid, repetitive firing of the action potential.

SCHMIDT-LANTERMAN INCISURES

As part of the first reports on the morphology of PNS myelinated axons, Schmidt and Lanterman independently described a series of funnel-shaped clefts or incisures that interrupted the dense appearance of myelin (Figs. 1.1A and 1.10A). These clefts, called Schmidt-Lanterman incisures, traverse the thickness of the myelin internode at an average angle of 9 degrees. They are abundant between the perinuclear region and

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Therapeutic approaches enhancing peripheral nerve regeneration

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ABSTRACT

Peripheral nerve injury is a common occurrence and represents a major economic burden for society. The development of novel strategies to enhance peripheral nerve regeneration is, therefore, of great relevance. Conventional treatments include surgical repair of the damaged nerves for minor injuries, whereas autologous nerve grafts are required to recover longer interruptions. However, despite great surgical advances, functional recovery is often poor. Although it is well known that the peripheral nervous system has a greater regenerative capacity than the central nervous system and, considering the scientific advancements and knowledge in regenerative medicine, clinical applications appears still limited. This review provides an overview of the methodological approaches currently under study, aimed at enhancing peripheral nerve regeneration. In particular, tissue engineering, cell therapy and pharmacological approaches will be discussed.

Keywords: Nerve Fibers; Regeneration; Schwann Cells; Scaffolds; Cell Therapy

1. INTRODUCTION

Large part of peripheral neuropathies is due to traumatic events that include motor accidents, falls, sport-related injuries, among others. Transected nerves rapidly undergo Wallerian degeneration, therefore a delayed intervention may limit functional recovery. So far, the surgical treatment appears the first possible therapy; however, type of injury and correct nerve reapposition can dramatically influence the success of surgical interventions. Direct nerve repair (end-to-end) is preferred when the gap along a nerve is short and the two edges of the damaged nerve can be sutured with minimal tension [1]. However, when nerve gap is too long and does not allow an end-to-end repair methodology, the autologous nerve grafting may be necessary. On the other hand, autologous nerve graft is limited by the availability of donor nerves and donor site morbidity [2]. In even more severe cases, when the proximal stump of the injured nerve is unavailable, surgical alternatives may be used, such as end-to-side neurorrhaphy [3]. In any case, finding the right conditions to accelerate nerve regeneration allows to reach the best results, with complete recovery of sensory-motor functionality. An alternative approach for nerve repair is to develop synthetic nerve conduits to suture the gaps between the proximal and distal nerve stumps and promote nerve regeneration. A conduit for *in vivo* application should form a suitable environment for nerve regeneration by combining cellular stimulating factors and biodegradability. Moreover, it should be non-toxic and easily made-up in convenient sizes and shapes. In addition, its permeability, swelling and mechanical strength should be tunable, as it is known to play a relevant role for axon elongation *in vivo*. Finally, an ideal scaffold for nerve regeneration should combine mechanical properties to prompt Schwann cell (SC) adhesion and proliferation, as well as axon elongation.

2. STRUCTURAL COMPONENTS FOR NERVE REPAIR

Nerve conduits (NCs) represent a promising alternative

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to conventional treatments for peripheral nerve repair. Materials for NC production should be biodegradeable, possess adequate mechanical properties, and allow exchange of nutrients. NCs must fulfill several requirements, as they should 1) be biocompatible, 2) have sufficient mechanical stability during nerve regeneration, 3) be flexible, 4) be porous, to ensure supply of nutrients, and 5) degrade into non-toxic products after bridging the gap, to prevent long-term inflammation. Biodegradable NCs have been made out of various materials of either biological or synthetic origin. Natural-derived materials have good cell compatibility, but they often need extensive purification. Furthermore, the majority of them lacks adequate mechanical strength and water stability [4].

2.1. Natural Compounds

Natural polymers are advantageous materials for tissue engineering of nerves as they are biocompatible and favor migration of supporting cells avoiding toxic effects. However, their poor mechanical properties and the relatively fast biodegradation *in vivo* generally limits their applications as materials constituent of external tubular structures. Natural compounds preferentially used are: collagen, gelatin, silk fibroin, alginate, chitosan, agarose and hyaluronic acid [5].

We performed experiments using two types of experimental paradigms of sciatic nerve regeneration. In one modality, sciatic nerves were exposed at their exit from under the gluteus muscle (**Figure 1(A)**) and excised. In a first approach, we practiced the *end to end* regenerative condition, in which in the two stumps of the sharply cut nerve were sutured, perfectly aligned, with a nylon thread (9/0) (a procedure called neurorraphy) (**Figures 1(B)** and **(C)**). In same animals, the sutured site was surrounded by a sleeve made of a biological substrate, the ester of hyaluronic acid (HYAFF-11, or hyalophill; Fidia Farmaceutics, Abano Terme, PD, Italy) (**Figure 1(D)**), which was instead omitted in animals used as controls. In the second paradigm, we resected about 1 cm of the nerve (**Figures 1(G)** and **(H)**) and connected the two stumps with tube made of purified porcine collagen I and III (**Figures 1(I)** and **(L)**). Rats were killed three months after surgery and the part of the nerve distal to the injury site was cut into transverse semithin and ultrathin sections for light and electron microscopy investigation, respectively. At this time, nerves appeared reconstituted (**Figures 1(E)** and **(F)**), neurorraphy + hyalophill paradigm), as the two stumps were perfectly re-connected.

By morphological analysis on semithin sections, we observed a partial regeneration of myelinated and unmyelinated axons, in both neurorraphy without (**Figures 2(A)-(C)**) and with (**Figures 2(D)** and **(E)**) hyalophill application. However, a higher degree of axon myelination

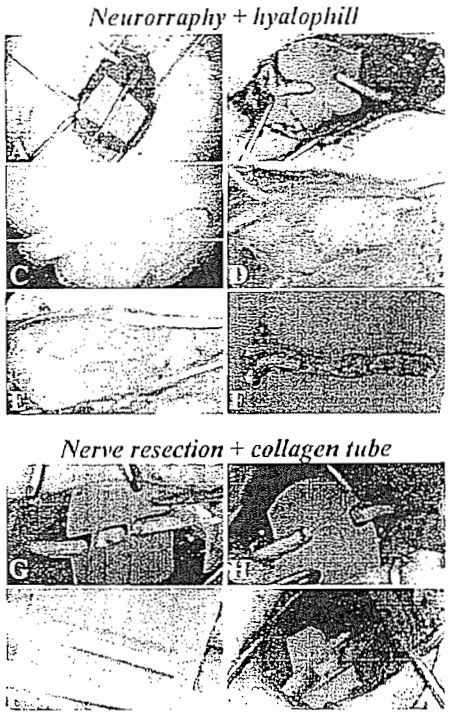

Figure 1. (A-F) End-to-end neurorraphy. Sciatic nerve, exposed at its exit from the gluteus muscle (A), is sharply cut in two stumps (B), later sutured in perfect alignment (C) and surrounded by a sleeve of hyalophill at the site of suture (D). Three months later, the nerve appears integer, surrounded by its perineurium. **(G-L) Nerve resection.** Nerves are resected, by cutting off about 1 cm (G, H), the two end stumps are inserted into a collagen tube (I, L), made of purified porcine collagen type I + III, and sutured.

and restoration of pre-surgery conditions was evident in those nerves treated with the biological compound. In both conditions, re-myelinated axons were more evident at the outer border of the nerves (**Figures 2(A)** and **(B)**, –hyalophill; **Figures 2(D)** and **(E)**, +hyalophill), respect to the center (**Figure 2(C)**, –hyalophill), possibly beneficing of factors released by the perineurium itself.

This characteristic was more evident in the hyalophill-treated specimens, as the amount of more centrally located unmyelinated axonal figures was predominant. Quantitative analysis, obtained by counting the number of myelinated axons over the total, showed a significant increase in axon regeneration and myelination in nerves treated with hyalophill, compared with the control (**Figure 2(F)**). Differently from these two conditions, sciatic nerves re-grown into the collagen tube appeared regenerated, with largely re-myelinated axons (**Figures 2(G)** and **(H)**). These observations were in accord with the fol-

Figure 2. Transverse semithin sections obtained from control (−hyalophill) and treated (+hyalophill) nerves after neurorraphy (A-E), and from resected nerves with successive application of a collagen conduit (G, H), three months after surgery. (A-E) Regenerated axons, myelinated and non-myelinated, are preferentially distributed in the outer areas of the nerve, closer to the perinerium, a condition more evident in control animals (A, B). Here, myelinated axons appear quite small in diameter (B), while the larger ones, not properly myelinated, are located more central in the nerve (C). In sciatic nerves treated with hyalophill, presence of myelinated axons is more evident (D, E), although figures of axon degeneration are easily recognizable (E). Count of myelinated axons over the number of total axons shows a significantly ($p < 0.01$ by Student's t-test) higher number in nerves treated with hyalophill respect to controls (F). (G, H) Three months after nerve resection and application of a collagen tube connecting the two nerve stumps, almost all axons appear regenerated in all nerve parts. Scale bars: A, D and G, 500 μm; B, C, E and H, 200 μm.

lowing ultrastructural analyses. Nerves sutured without addition of hyalophill showed several regenerated axons, mostly grouped inside one fascicle, usually surrounded by abundant collagen (**Figure 3(A)**), and intervalled by regenerated small unmyelinated fibers. Recovered myelin sheaths, however, appeared a bit loose at points, suggesting a non-perfect assembly, or an ongoing reorganization. Numerous axons were, instead, characterized by different degrees of suffering (**Figure 3(B)**), with some of them collapsed and encircled by disrupted or extremely disarranged myelin sheaths. These figures could represent different stages of abortive regeneration, as also suggested by numerous fascicles formed by completely degenerated axons embedded in remnants of myelin-based material (**Figure 3(C)**). Alternatively, they may be expression of an altered cross talk between neurons and SCs, which are not able to form a proper myelinating coat. In the experiments of neurorraphy with

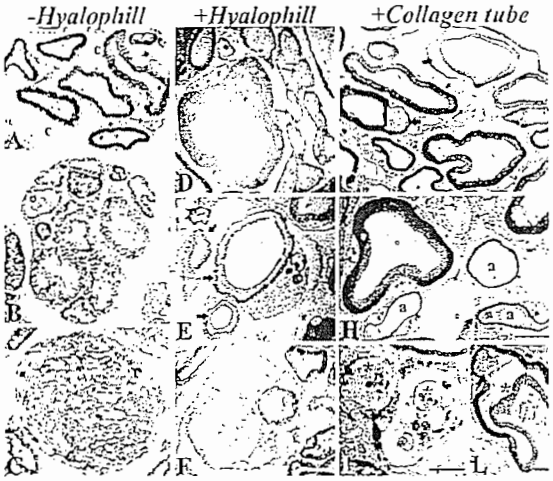

Figure 3. Electron micrographs of transversal sections of sciatic nerves subjected to neurorraphy, without (A-C) or with (D-F) hyalophill treatment, and of resected sciatic nerves with successive application of a porcine collagen (I + III) tube (G-L), three months after surgery. (A-F) Regenerated axons, with compact myelin sheath and healthy axoplasm, are present in both conditions (− and + hyalophill). However, in the −hyalophill specimens, myelin sheaths tend to be less compact than in the hyalophill-treaded nerves (A, −hyalophill; D, +hyalophill). Unmyelinated axons, intermixed with the myelinated ones, are also regenerated (arrows in A and D). In −hyalophill nerves, though, a high number of axons with different degrees of degenerations, often occurring in fascicles, are observed (B). Large figures of remnants of myelin sheaths are also present (C). In the hyalophill-treated nerves, numerous axons display an immature myelin coat in evident formation (block arrows in E), although a number of degenerating, or degenerated, axons are also present (F). (G-L) In resected nerves allowed to grow into a collagen tube, almost all axons, both myelinated and non-myelinated (arrows in G, H), are regenerated. Myelin sheaths are thick and compact, with only a few exceptions showing points of rearrangements (block arrows in G, L). A number of medium caliber axons (a in H and inset) have very thin myelin sheaths, in evident stage of formation. A few figures of phagocytosed axons into Schwann cells (asterisks in I) and of degenerating axons, often surrounded by a largely compact myelin sheath (asterisk in L) are observed. Differently long scale bars all correspond to 2 μm, with the following correspondence: A-C, G and L; D, H and inset to fig H; E, F; I.

addition of hyalophill, regenerated myelinated axons were characterized by a thick and compact myelin sheath (**Figure 3(D)**) and others were in the process to complete their myelination (**Figure 3(E)**). However, clusters of degenerating axons, usually intermixed with healthy ones, were also observed (**Figure 3(E)**). In the second experimental paradigm, which makes use of the collagen tube, myelinated axons were almost all perfectly regenerated, with thick and compact myelin sheaths. Some of them, usually the smaller diameter axon still displayed a thin rim of myelin, in the process of completing its formation (**Figure 3(H)** and inset). Phagocyte figures of axonal debris within SCs were occasionally observed (**Figure 3(I)**), as well as degenerating axons (**Figure 3(L)**). Differently from the other two treatments, however, degenerating axons were encircled by an apparently healthy myelin sheath, suggesting the intervention of connectivity problems only after axon regeneration had successfully occurred. All these results corroborate and reinforce what has been described in the literature, indicating a beneficial role of biological substrates applied to the site of damage and collagen scaffold on axonal regeneration. Moreover, our data strongly suggest that collagen conduits, although applied to severely damaged nerves (resection of a consistent portion of the nerve), are more efficient for axonal regeneration than external application of biological compounds.

In some cases, a combination between natural and synthetic polymers has been proposed, to join the biocompatibility of natural components with the mechanical performance of the synthetic material [6]. More natural polymers have been used as inner fillers for NCs, in the form of fibers, channels, porous sponges, or hydrogel matrices as delivery vehicles for cells, growth factors, or pharmacological drugs. Moreover, the inner layer can covalently bound RDG peptides (Arg-Gly-Asp), sequences typically present in extracellular matrix components (ECM) such as laminin and fibronectin. Adhesion of RDG peptides to natural or synthetic scaffolds may represent an alternative approach to the use of native ECM proteins able to promote cell adhesion and axon elongation [7].

2.2. Synthetic Polymers

Recent research has focused on the production of artificial nerve guides, which can degrade within the host with just a mild reaction. Biodegradable materials offer several advantages, such as the possibility of incorporating SCs, or bioactive molecules, through physicochemical modifications of the polymers, and deliver them during biodegradation. Another interesting property is that their flexibility, biocompatibility, mode of degradation, porosity and mechanical strength may be changed by modifying their chemical properties [5].

Scaffolds of polyglycolic acid (PGA), poly-DL-lactide-caprolactone (PLCL) and poly [(lactic acid)-co (glycolitic acid)-*alt*-(L-lysine)] (PLGL), are used as NCs, but present some limitations, such as induction of inflammation and local concentration of acid residues deriving from their biodegradability in the surrounding tissues [8]. In alternative, hydrogels can be considered an interesting option to rigid hydrophobic materials.

For example, poly(amidoamines) (PAAs), synthetic water-soluble polymers, are biocompatible and the products derived from their biodegradability are not toxic [9]. Several PAA hydrogels have been tested with success as substrates for culturing cells as SCs or dorsal root ganglia (DRG) neurons [10,11].

3. CELL AND TISSUE THERAPY

Although novel approaches have largely improved the knowledge on the type of nerve conduits more appropriate for nerve regeneration, their use is limited in the case of too long (>0.7 cm) nerve gaps. Under these circumstances, nerve auto-grafts, or the *muscle-in-vein* approach (described ahead) appear, so far, the best solutions for repairing this type of nerve injury [12]. Recently, great attention has been focused on the use of natural or synthetic conduits, enriched in different cell types, which may contribute to nerve regeneration.

3.1. Muscle-in-Vein

To improve nerve repair, the possibility of a conduit obtained combining a vein tract with fresh skeletal muscle fibers, has been largely experimented by several authors [13,14]. The rationale of the *muscle-in-vein* approach is that muscle prevents vein collapse, while the vein wall prevents axon dispersion and represent a natural tube, within which axon elongation can occur [13]. On the other hand, the use of fresh muscle fibers appear relevant, if considering that muscle basal lamina enhances the migratory properties of resident SCs. In fact, it has been demonstrated, by confocal microscopy, that SCs and regenerating axons successfully colonize the *muscle-in-vein* grafts already two weeks after surgery [15,16]. Moreover, in muscle fiber denervation, as well as in SCs deprived of their axons, over-expression of neuregulin (NRG)/erbB receptors has been observed [17]. Overexpression of this gliogenic factor and of its receptors appears relevant for supporting Schwann cell proliferation, indicating that the *muscle-in-vein* approach enhances nerve regeneration because it supports Schwann cell proliferation and migration.

3.2. Schwann Cells

It is well known that the peripheral nervous system (PNS) has a greater regenerative capacity than the central one

(CNS). It is thought that this different regenerative capacity is due to a greater permissive environment in the PNS respect to the CNS, which is likely to be provided by SCs. These have a repertoire of physiological roles, including protection and regulation of nutritive exchanges with axons, myelin production and digestion of axon and myelin debris under pathological conditions. SCs help peripheral nerve regeneration, considering their ability to proliferate, migrate and release growth factors during development or after nerve lesion. Nerve injury alters the SC-axon interaction, which results in SCs de-differentiation and activation of their growth-promoting phenotype. At the proximal stump of a cut nerve, SCs re-enter in proliferative state and produce several substances, such as laminin, integrins and growth factors [18,19]. Morover, SCs form the bands of Büngner, important structures for directing regenerating axons across the lesion site [20,21].

Tissue engineering techniques may offer a potential solution to clinical problems by favoring seeding of SCs in artificial nerve conduits [22]. In fact, several experimental evidences showed that SCs may be cultured and expanded on nerve conduits, such as the PLGL-RGD peptide conjugated or polyamidoamine hydrogels [8, 11,23]. However, cultured SCs have limited clinical applications. The requirement of nerve donor material evokes additional morbidity and the time required to culture and expand the cells would delay the treatment. Instead, the ideal transplantable cells should be easily accessible, proliferate rapidly in culture and successfully integrate into the host tissue with immunological tolerance [24].

3.3. Mesenchimal Stem Cells

Mesenchimal stem cells (MSCs) are an attractive cell source for nerve tissue regeneration, as they are able to self-renew with a high growth rate and possess multi potent differentiation properties. There are also some evidences indicating that the MSCs may be non-immunogenic, or at least hypo immunogenic [25]. MSC fibroblast-like cells can be isolated from the stromal cell population found in a number of tissues. In particular, bone marrow and adipose tissue have been identified as main sources of these cells [26,27]. Currently, adipose tissue appears the most promising source of MSCs, considering that it can be easily and non-invasively lipoaspirated from the patient. Recent studies have shown that, following differentiation with a cocktail of growth factors (bFGF, PDGF, GGF-2), MSCs express Schwann cell markers such as GFAP, low affinity neurotrophic factor receptor p75, and calcium binding protein S 100 beta [22]. It is of considerable clinical relevance to address the differentiation of human-derived MSC into SC-like cells. This alternative source of cells, which is relatively simple to isolate and expand in culture, may be easily obtained from the same patients affected by nerve injury and, once addressed to the SC phenotype, represents an excellent tool to support and guide nerve regeneration in combination with appropriate scaffolds.

3.4. Olfactory Ensheating Cells

Olfactory unsheathing cells (OECs) are glial cells associated to the olfactory system displaying SC or astrocyte-like cell properties [28]. They support axons originating from olfactory epithelium and projecting into the olfactory bulb. Considering the great difficulty of CNS to regenerate axons, it appears relevant that, after olfactory nerve transection, cell regeneration in the olfactory epithelium is facilitated by the close association with OECs [29,30]. OECs normally do not form myelin, but they can do so after transplantation into demyelinated lesions. OECs are also able to produce neurotrophic factors, such as nerve growth factor (NGF) and brain derived-neurotrophic (BDNF), and have great migratory ability [31]. For these and other reasons, transplantation of OECs has been considered as an alternative source of cell-supporting peripheral nerve repair. Several researchers have already collected OECs from the olfactory bulb, supporting the idea that these cells may provide a potential autologous source of cells to reduce the risk of immunological rejection in case of auto-transplantation [32]. Corroborating this hypothesis, is the demonstration that OEC engraftment into axotomized facial nerve enhances axonal sprouting [33,34].

4. ADDITIVE COMPOUNDS

Medical treatments have achieved limited success in restoring functions to severely injured nerves. The combination of synthetic conduits and chemical molecules able to promote neurite outgrowth (e.g. NGF) has been recently considered as an additional strategy to better enhance nerve regeneration [23].

4.1. Neurotrophic Factors

Neurotrophic factors are a family of growth factors playing important roles in the natural process of nerve growth and regeneration [35]. Principal factor of this family is the nerve growth factor (NGF), first isolated by Levi Montanlcini in 1950 [36]. NGF prevents, or reduces, neuronal degeneration in animal models of neurodegenerative diseases [37] and appears to be involved in several psychiatric disorders [38]. As suggested by *in vitro* and *in vivo* studies, NGF enhances peripheral nerve regeneration and promotes myelin repair [39]. Considering its multiple functions, it has been proposed its potential

use in peripheral nerve repair therapies. As above reported, the introduction of SCs into natural or synthetic scaffolds, in order to support and guide nerve regeneration, has been proposed based on the fact that SCs are main NGF producers [40]. However, studies orientated toward the identification of new and sophisticated NGF delivery system to mimic the natural process of secretion are emerging. First evidences indicate that a controlled release of NGF by microspheres, or by adenoviruses expressing this factor, increases the functional recovery of injured peripheral nerves, although the organic solvent used for the NGF-microspheres production might compromise NGF activity [41], NGF directly added within nerve conduits may be an alternative for local treatments. NGF immobilized on gelatin membranes, or PLGL scaffolds, promotes Schwann cell adhesion and survival *in vitro* and neurite outgrowth from pheochromocytoma (PC12) cells, indicating this approach potentially useful for the generation of nerve conduits for clinical nerve repair [23].

4.2. Glial Growth Factor

Glial Growth Factor (GGF) is factor produced by neurons, known to stimulate glial cells growth and differentiation and SC proliferation. Considering the role of SC in peripheral nerve development and regeneration, it appeared relevant to evaluate the ability of this factor in peripheral nerve regeneration [42]. GGF, introduced into conduits used to repair 2 - 4 cm peroneal nerve gaps in rabbit, caused an increase in SC number, accompanied by significant axonal regeneration [43].

4.3. Acetylcholine Mimetics

Acetylcholine (ACh), is the main neurotransmitter in the CNS and PNS. However, when released from growing axons, ACh is capable of regulating growth, differentiation and plasticity during nervous system development [44]. Moreover, it can act either both as stimulatory or inhibitory signal, as chemoattractant in spinal and sensory neurons [45,46] and a direct regulation of ACh on neurite elongation in chick sensory neurons has been reported [47,48]. ACh can also control SC development; in fact, when it binds to muscarinic receptors type-2 (M2), ACh can arrest SC proliferation and promote their differentiation [49,50]. Considering the dual role of ACh as modulating factor for neurite outgrowth and SC development, it may be relevant to investigate the potential effect of ACh in peripheral nerve regeneration. ACh is usually rapidly hydrolyzed by acetylcholinesterase and butirrylcholinesterase, largely produced in several tissues other than the nervous system. For this reason, addition of ACh to synthetic conduits may be not a successful strategy. In alternative, ACh mimetics are not hydrolyzed and for this reason they are more stable and may be used as additive compounds in combination with specific scaffolds. The potential application of ACh-like biomaterials in neural tissue engineering has been poorly investigated. More recently, it has been reported that ACh-like biomimetic polymers can promote neuritic sprouting and extension in explanted DRGs, and growth of hippocampal neurons [51,52]. Thus, ACh mimetics associated to appropriate scaffolds may be potential new biomaterials to further investigate nerve repair in PNS as well as CNS.

5. CONCLUSION

Improvement of nerve regeneration and the outcome of clinical microsurgery in the treatment of nerve injuries have a great relevance in term of social impact. Several aspects require specific attention in the clinical treatment of the patients, among which the timing of nerve reconstruction. In fact, delay of nerve injury treatment may cause neurobiological alterations in neurons and Schwann cells, impairing nerve functional recovery and affect neuron survival. Although conventional nerve reconstruction techniques used in clinical practice can largely contribute to treat nerve lesions, the outcome is generally insufficient. Tissue engineering and nanotechnology are suggesting new research therapeutic approaches, potentially orientated to accelerate nerve regeneration and recovery of nerve functionality. As discussed in this review, most significant advances in nerve repair and regeneration have been achieved. Therefore, each of the therapeutic approaches discussed appears limited. More recent studies report that combined treatments are more promising therapies. In fact, the use of an appropriate scaffold integrated with cells (*i.e.* autologous mesenchimal stem cells differentiated in SCs) and growth factors may represent the successful strategy to achieve better results in the clinical treatment of the peripheral nerve lesions.

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