Long-distance retrograde neurotrophic factor signalling in neurons

Anthony W. Harrington and David D. Ginty

Abstract | The specialized architecture of neurons necessitates unique modes of intracellular communication to allow for cell survival, the ability to detect and respond to injury and aspects of neuronal development, such as axon and dendrite growth, plasticity, and synapse and circuit formation. Many of these neuronal processes rely on signal transduction pathways and transcriptional programmes that are activated by retrograde signals originating from target-derived cues that act on distal axons. Here, we review the many functions of long-range distal axon-to-cell body signalling and discuss mechanisms of retrograde target-derived growth factor signalling.

Neurotrophic factor hypothesis
Neurons are overproduced during development. The neurotrophic factor hypothesis states that neurons that successfully compete for limiting amounts of target-derived survival factor gain a competitive advantage over others and survive, whereas those that fail to compete die.

The Solomon H. Snyder Department of Neuroscience and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA.
Correspondence to D.D.G.
e-mail: dginty@jhmi.edu
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The long distance that is often spanned by projecting axons presents neurons with a unique set of challenges, chief among which is how to detect and respond to events occurring far from its cell body. To meet this considerable challenge, specialized mechanisms of retrograde intracellular communication have evolved through which signals emanating from the farthest tip of the axon can be transmitted to the neuron's cell body, where an appropriate response can be initiated.

The huge importance of retrograde axonal signalling is perhaps best illustrated by the neurotrophin family of growth factors. Nerve growth factor (NGF), brain-derived growth factor (BDNF), neurotrophin 3 (NT3) and NT4 (also known as NT5) were first described as cues that are essential for neural development[1]. These growth factors can bind to and activate specific receptors called tropomyosin receptor kinases (TRKs; also known as NTRKs) that are present on the distal axons of neurons. Remarkably, neurotrophin signals can be propagated retrogradely along the axon to the cell body and are now known to be required for proper neuronal survival, axon growth, gene expression, neuronal subtype specification and synapse and circuit formation.

Retrograde signalling is important not only for normal development of the nervous system but also for helping neurons respond to axonal insults and injury. Indeed, retrograde signals that are initiated following axotomy and nerve crush injuries are required for proper axon regeneration[2]. The identification of the specific signals that are transported from distant injury sites and the mechanism of such transport should provide critical insight into the development of novel therapies to promote neuronal regeneration and functional recovery. In addition, defects in retrograde signalling may underlie the pathophysiological basis of several neurodegenerative diseases[3].

In this Review, we examine the functions of retrograde axonal signalling, summarizing current knowledge of the developmental and disease aspects of this important and distinctly neuronal process. In addition, we discuss the mechanisms that are used by different types of retrograde signal and highlight unanswered questions and current challenges in this area of research.

Functions of retrograde neurotrophin signalling

The discovery of the target-dependent nature of survival of developing neurons was first demonstrated by Victor Hamburger and Rita Levi-Montalcini in experiments conducted on chick embryos[4]. In these early experiments, the authors showed the existence of naturally occurring cell death in motor and sensory neuron populations and that limb bud extirpation greatly enhanced the amount of neuronal death. This discovery has important implications. The observation that cell survival is target-dependent implies that target-derived factors signal retrogradely from the innervating axon to the cell body to promote neuronal survival and that these factors are probably present in limiting amounts. In fact, a central tenet in developmental neuroscience, namely the neurotrophic factor hypothesis, stems from this idea.

The neurotrophic factor hypothesis posits that developing neurons are overproduced. Those that successfully acquire adequate amounts of target-derived neurotrophic factor will survive, whereas those that fail to do so will die[5]. This hypothesis is supported by the

fact that NGF and other neurotrophins act as target-derived factors that are essential for the survival of select populations of developing neurons. The Hamburger and Levi-Montalcini findings also argue for the existence of systems matching, a process by which the end organ ensures that the amount of innervation it receives is appropriate for its size and demands. The systems matching idea provides a conceptual framework for understanding why neurons of the developing nervous system are overproduced and undergo a period of naturally occurring cell death[6,7].

Regulation of neuronal survival by target-derived neurotrophins. Examination of gene knockout animals has provided *in vivo* evidence indicating that NGF, BDNF and NT4, and NT3, and their receptors — TRKA, TRKB and TRKC, respectively — are required for neuronal survival. In each of the neurotrophin- and TRK-knockout mouse strains, select populations of sensory neurons of the dorsal root ganglia (DRG) are markedly reduced in number[8], resulting from increased levels of apoptosis. Also in these animals, other neuronal populations in the peripheral nervous system (PNS) — including trigeminal ganglia sensory neurons and postganglionic sympathetic neurons in *Ngf*-null animals, and nodose ganglia neurons in *Bdnf*-, *Ntf3*- and *Ntf4*-null mice — have reduced cell numbers owing to the lack of neurotrophins and neurotrophin signalling. Conversely, exogenous overexpression of neurotrophins in the periphery of transgenic mice results in increased survival of certain neuronal subtypes, including sympathetic and sensory neurons[9,10], suggesting that developmental apoptosis is at least in part caused by a competition for neurotrophins that act on distal axons in the periphery.

Several additional lines of evidence suggest that neurotrophins operate in a retrograde manner to control the survival of PNS neuronal populations. First, neurotrophins seem to be synthesized and secreted at a considerable distance away from neuronal cell bodies and thus are exposed to distal axonal endings. For example, *Ngf* mRNA is expressed in targets of the sympathetic nervous system[11], with levels positively correlating with the amount of sympathetic innervation[12]. Similarly, *Ntf3* is expressed in developing muscle, which is the peripheral target of innervating TRKC-positive proprioceptors[13]. Second, axotomy of either sympathetic or DRG sensory neurons causes cell death in these neuronal populations[14,15] and, consistent with a loss of retrograde survival signals that are initiated by peripherally acting growth factors, exogenous administration of neurotrophins can prevent this cell death. Third, radiolabelled neurotrophins that are injected into target fields are internalized and retrogradely transported to neuronal cell bodies *in vivo*[16–19]. Last, neurotrophins acting exclusively on axonal endings *in vitro* are both necessary and sufficient to promote neuronal survival[20–22]. Together, these studies suggest that for select populations of PNS neurons, neurotrophins are produced and secreted in limited quantities by target tissues and act on distal axons, where they initiate a retrograde signal that promotes neuronal survival.

In vitro culture systems have proven to be invaluable for determining the nature of the retrograde signals that are initiated by neurotrophins to promote survival. In compartmentalized culture platforms, distal axons and neuronal cell bodies can be exposed to distinct media conditions, resembling physiologically relevant conditions[20,23,24]. This approach also allows the direct testing of the specific pathways that are activated by neurotrophin signalling in the context of retrograde survival signalling. Much of this work has been conducted on TRKA-expressing sensory and sympathetic neurons, which depend on NGF for survival. In these neurons, treatment with NGF results in autophosphorylation of TRKA that is present on axons, which is followed by activation of downstream TRKA-mediated signalling pathways[1]. These pathways include the extracellular signal-regulated kinase 1(ERK1; also known as MAPK3) and ERK2 (also known as MAPK1), phosphatidylinositol 3-kinase (PI3K)–AKT and phospholipase Cγ (PLCγ) pathways.

Activation of TRKA and subsequent downstream pathways is required for the survival signal that travels from the axon to the cell body[25,26]. These signal transduction pathways probably promote survival by inhibiting cell death pathways partly through transcriptional upregulation of pro-survival genes[27,28] and by preventing translocation of the apoptosis regulator BCL-2-associated X protein (BAX) to the mitochondria, an event that is necessary for activation of the intrinsic cell death pathway[29]. Related to this, recent work provides evidence in support of the idea that TRKA and TRKC are dependence receptors, which promote cell death in their unbound states[30]. Thus, although some of the main players that are involved in neuronal survival have been identified, the precise mechanism by which these players collaborate to propagate the retrograde signal, inhibit apoptotic pathways and promote expression of pro-survival genes remains to be elucidated.

Recent genetic screens to identify NGF-regulated genes in sympathetic and sensory neurons have shed light on some of the molecules and mechanisms that are required for retrograde survival signalling[31,32]. In these studies, microarray analyses were conducted on mRNA from superior cervical ganglia (SCG) and DRG neurons from wild-type and *Ngf*-null mice that were kept alive by the concomitant deletion of *Bax*, which is required for apoptotic cell death of PNS neurons. These data sets are proving useful in identifying NGF-regulated genes that encode proteins that mediate the development and survival of TRKA-expressing sympathetic and sensory neurons. One interesting example of such a gene is *Trka* itself. In sympathetic neurons, NGF promotes transcriptional upregulation of *Trka*, thus enhancing NGF sensitivity and the duration of survival signals in neurons responding to NGF[31]. Interestingly, a high level of NGF–TRKA signalling in sympathetic neurons protects against apoptotic signals that are derived from neighbouring cells and, at the same time, promotes the expression of pro-death ligands that augment apoptotic signalling in neighbours. Thus, NGF promotes transcriptional upregulation of genes that sets in motion a

series of feedback loops enabling neurons that initially experience only slightly higher levels of NGF signalling to gain a large competitive advantage over neighbouring neurons for survival. This NGF-dependent transcriptional process triggers rapid and robust elimination of approximately half of the sympathetic neurons during the period of naturally occurring cell death[31]

Regulation of axon and dendritic growth. The actions of target-derived growth factors are clearly not limited to the promotion of cell survival. Studies conducted over the past two decades have revealed roles for neurotrophic factors in various aspects of neuronal development outside survival, including axonal growth and retraction[20,33], synapse and circuit formation[34,35], and the expression of certain peptides and neurotransmitters that are necessary for the proper functioning of mature neurons[36,37] (FIG. 1).

Neurotrophins that are secreted from intermediate and final targets can act directly on extending axons to promote growth. In compartmentalized chambers, NGF that is applied directly to distal axons can act locally to support their extension. By contrast, neurons grown in conditions in which only cell bodies are exposed to NGF fail to extend axons into a compartment that lacks NGF[20]. Although these experiments suggest that NGF acting solely on distal axons is sufficient to cause extension, a transcriptional response is required for axonal growth over long periods of time[38]. Several trophic factor-regulated transcription factors — including cyclic AMP responsive element-binding protein (CREB), serum response factor (SRF) and nuclear factor of activated T-cells (NFAT) — have been implicated in promoting *in vivo* axonal outgrowth[39–41]. *In vivo*, *Ntf3*- and *Ngf*-null mice display defects in sensory and sympathetic neuron final-target innervation[36,42–44]. The pro-apoptotic BCL-2 family member BCL-W is essential in DRG sensory neurons for innervation of the epidermis by small-fibre nociceptors *in vivo*[45]. BCL-W expression is increased in neurons by BDNF and NGF acting at distal axon terminals and is dependent on the transcription factor myocyte-specific enhancer factor 2D (MEF2D). MEF2D activity is stimulated by ERK5 (also known as MAPK7), a member of the ERK family that is implicated in the control of signal duration and retrograde signalling[28].

Dendrites typically develop before axonal target innervation. In sympathetic neurons, however, NGF signalling is required for dendritic arborization, with the size of a neuron's peripheral target field correlating with the degree of dendrite arborization[46]. Interruption of retrograde signalling by axotomy causes these dendrites to retract[47]. Target-derived cues mediate dendrite growth and morphology at least in part through activation of transcriptional programmes. In the case of motor neurons that innervate the cutaneous maximus and latissimus dori muscles, intermediate target-produced glial cell line-derived neurotrophic factor (GDNF) controls proper dendrite formation[48]. This control is achieved through a retrograde signal that leads to the expression of the transcription factor ETS translocation variant 4 (ETV4; also known as PEA3). In the absence of ETV4, these motor neurons display dramatically mislocalized dendrite arbors accompanied by misspecification of connections with presynaptic proprioceptors and severe impairment of movement coordination. The effect of the neurotrophic signal often depends on its site of action. In retinal ganglion cells (RGCs), the extent of dendritic arborization in response to BDNF depends on the source of neurotrophin, which can come from the retina or from the tectum. Local BDNF produced in the retina inhibits RGC dendrite arborization, whereas BDNF that is produced in the tectum and exposed exclusively to RGC axon terminals largely increases dendrite branching and length[49]. Thus, retrograde neurotrophic factors can control signalling and transcriptional events that promote both axonal and dendrite growth, patterning and connectivity.

Lamina-specific axonal targeting and circuit formation. In addition to general axon and dendrite growth and patterning mediated by peripheral signals, increasing evidence suggests that proper lamina-specific axonal targeting of the central axonal projections of DRG sensory neurons is controlled by transcriptional activity that is initiated by retrograde signalling. For example, target-derived NGF activates TRKA that is expressed in small-diameter sensory neurons of the DRG and produces a retrograde signal that promotes maturation of both peptidergic and non-peptidergic nociceptors. Peripherally derived NGF controls the organization of a subset of peptidergic nociceptor axons through upregulation of the transcription factor homeobox protein HOXD1 (REF. 50). In the absence of HOXD1, mouse nociceptors display a central projection phenotype that is similar to that of non-mammalian nociceptors, suggesting that retrograde control of HOXD1 is required for mammalian nociceptor circuit formation. In another example, target-derived neurturin (NRTN) and one or more additional GDNF family ligands act on the rapidly adapting mechanoreceptors (RA-LTMRs) of the DRG, which express the receptor tyrosine kinase RET early during embryonic development. The signal initiated by RET activation is required for proper circuit formation, as in the absence of RET in this population of neurons, central collateral branches fail to develop and thus cannot form appropriate connections at the correct lamina in the spinal cord dorsal horn[51]. NRTN is essential for the peripheral innervation of at least one RA-LTMR target, the Pacinian corpuscle; however, NRTN is not required for the central projection innervation of RA-LTMRs. In a related example, peripheral NT3 promotes the expression of the transcription factor ETV1 (also known as ER81) in TRKC-positive proprioceptors[52]. In the absence of either NT3 or ETV1, central proprioceptor projections initiate normally but terminate prematurely, ending in the intermediate zone rather than ventral region of the spinal cord and hence failing to form proper connections with motor neurons in the ventral cord. Thus, central axonal targeting in various types of sensory neurons requires peripherally derived growth factors.

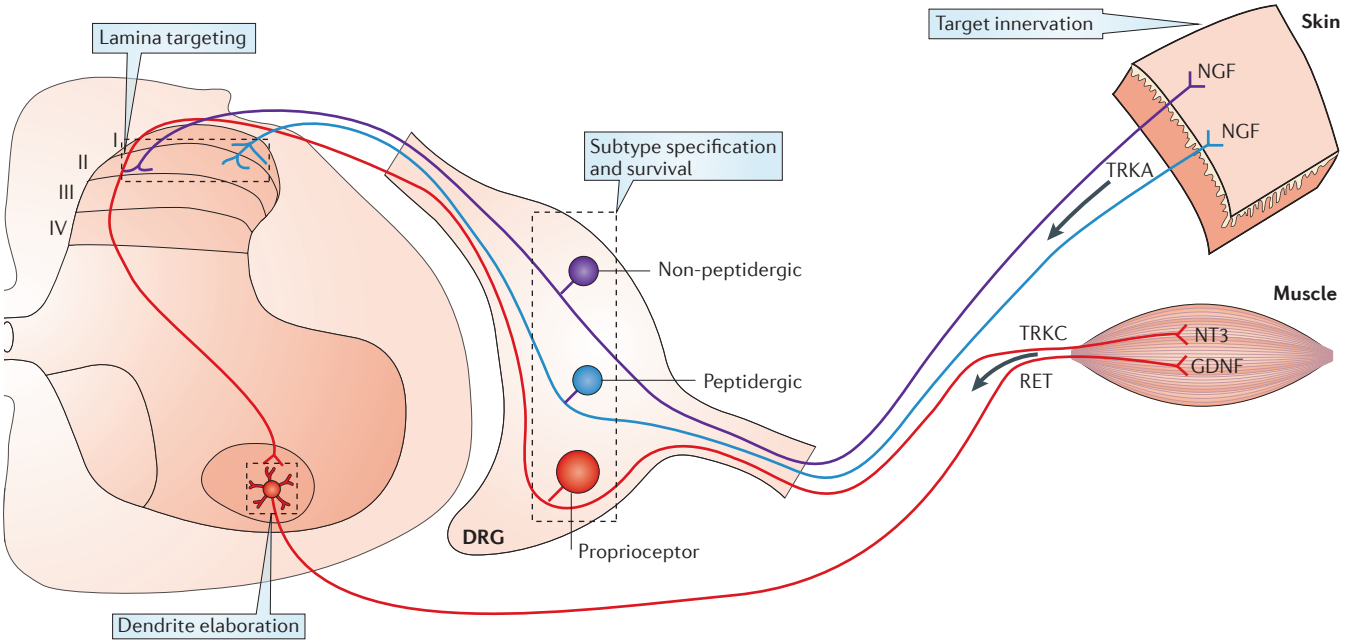

Figure 1 | **Retrograde signals controlling sensory neuron development.** Multiple neuronal developmental steps are controlled by peripherally derived cues that act on spinal motor neurons and dorsal root ganglion (DRG) neurons. Tropic factors, such as nerve growth factor (NGF), neurotrophin 3 (NT3), glial cell line-derived neurotrophic factor (GDNF) and target-derived neurturin (NRTN), that are produced in skin and muscle act on their receptors tropomyosin receptor kinase A (TRKA), TRKB, TRKC and RET, respectively. These receptors are expressed on peripheral axons of distinct subtypes of sensory neurons and motor neurons. Signalling endosomes, containing trophic factors and their receptors, are carriers of retrograde neurotrophic factor signals that support neuronal survival and circuit assembly. Transcriptional programmes activated by retrograde signals direct correct lamina targeting of the central axon branch, expression of genes required for subtype specification and proper dendrite elaboration — all of these events are required for circuit development and function.

Regulation of neuronal specification and maturation. Proper neuronal specification and acquisition of correct neurotransmitter phenotype is crucial for the functioning of the nervous system. In sympathetic neurons, which release either noradrenaline or acetylcholine, non-neurotrophin peripheral signals in the form of ciliary neurotrophic factor (CNTF) ligands convert a subset of immature noradrenaline-producing neurons into cholinergic sympathetic neurons[53]. Moreover, NGF itself has a crucial role in neuronal subtype specification in certain subsets of sensory neurons. As mentioned above, for nonpeptidergic nociceptors, NGF is essential for the acquisition of the nonpeptidergic phenotype through upregulation of *Ret* and other nonpeptidergic genes. The NGF-dependent expression of *Ret* is in turn required for expression of a large subset of nonpeptidergic-related genes[37]. As for non-peptidergic nociceptors, target-derived NGF is also required for the expression of calcitonin gene-related peptide (CGRP) and thus the acquisition of peptidergic nociceptor specification[42]. Interestingly, defects in non-peptidergic nociceptor development that are observed in *Ngf*-null mice are similar to those seen in mice lacking the transcription factor RUNX1. Moreover, in the absence of *Runx1*, the central projections of these sensory neurons are abnormally shifted from lamina II to lamina I. The identities of RUNX1 target genes in nociceptors that control this process and how NGF controls RUNX1-dependent gene expression remain unanswered questions and represent key future directions for the field. Thus, the effect of NGF retrograde signalling in TRKA-expressing sensory neurons is cell type-specific and is required to initiate transcriptional programmes that dictate proper neuronal specification.

Retrograde control of synapse formation. Retrograde signalling by neurotrophic factors is also responsible for the development of correct synapse formation and maintenance. In postganglionic sympathetic neurons, retrograde NGF–TRKA signalling is required for the development of proper presynaptic and postsynaptic specializations[33], and axotomy, disruption of retrograde transport and application of NGF-blocking antibodies to adults can induce synaptic loss[34,47]. Remarkably, TRKA-containing 'signalling endosomes' (discussed below) are transported from distal axons to the cell soma and then into dendrites; this is a novel form of endosomal transport that has not been previously documented[34]. The presence of TRKA signalling endosomes in dendrites is probably a prerequisite for postsynaptic density and synapse formation with preganglionic sympathetic neurons[34]. Thus, in addition to affecting neuronal survival, retrograde signalling may confer an additional level of control for the target tissue to regulate the degree of connectivity it receives.

Retrograde transport
The directed, coordinated movement of proteins or vesicles from distal axons towards the neuronal soma.

Signalling endosome
A term referring to endosomes containing active ligand–receptor complexes that associate with and activate components of downstream growth and survival signalling pathways as they traffic within axons and cell bodies.

In summary, the functions of target-derived cues in the development of the nervous system are varied and many. These cues initiate signals in axons that travel in a retrograde manner to the cell soma and, as exemplified by sympathetic neurons, even into dendrites, where they influence signalling and transcriptional programmes that underlie cell survival and maturation, as well as circuit and synapse assembly.

Mechanisms of retrograde signalling

Retrograde signalling of target-derived neurotrophins has been the most widely studied form of retrograde growth factor signalling (FIG. 2). The first clues about the mechanism of retrograde neurotrophin signalling came from studies demonstrating that neurotrophins are taken up by axon terminals and transported retrogradely to cell bodies[19]. For example, injected NGF is transported retrogradely along sympathetic axons that innervate the eye, and BDNF and NT3 are transported from distal axons to cell bodies in DRGs following sciatic nerve crush injury[54]. These findings, together with the observation that ligand-bound TRK receptors are internalized into vesicles that are associated with downstream signalling molecules and undergo retrograde transport along microtubules[27,55,56], provided some of the early evidence in support of a model in which the retrograde signal initiated by neurotrophins involves the formation, trafficking and retrograde transport of an endosomal-based signalling platform. This idea, namely the signalling endosome hypothesis[57–59], has gained considerable support in the past several years, and recent findings provide key insights into TRK endosome formation, trafficking, transport and signalling. Many of the insights have come from the NGF–TRKA paradigm involving cultured sympathetic and sensory neurons, and a major, current challenge is to confirm and extend these findings in *in vivo* model systems. Although other models of retrograde signalling exist[60], there is considerable experimental support for the signalling endosome model and, therefore, the mechanisms of signalling endosome formation, retrograde trafficking and signal transduction are the focus of our attention.

Internalization and the formation of intracellular neurotrophin signalling platforms. Like other receptor tyrosine kinases, TRKA undergoes internalization following ligand engagement[61], and this internalization in distal axons seems to be essential for retrograde survival signalling. Controversy exists regarding the precise

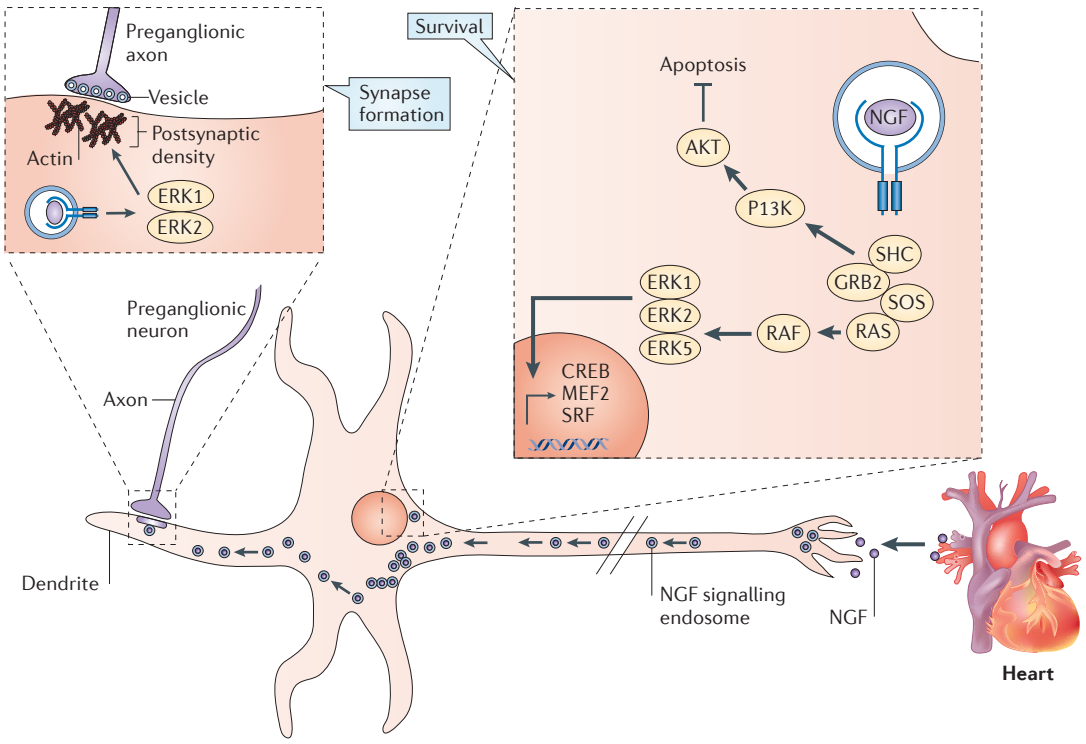

Figure 2 | **Retrograde NGF signalling controls sympathetic neuron survival and connectivity.** Sympathetic neurons of the superior cervical ganglion respond to target-derived nerve growth factor (NGF) through the formation of 'signalling endosomes' at their axon terminals. Once NGF is internalized, these endosomes are transported to the cell soma, where downstream signalling cascades, including phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinases (ERK1 and ERK2), can activate signalling events within the soma and transcriptional programmes that are necessary for survival and growth. The NGF-containing endosomes are also transported into dendrites, where signalling through ERK pathways (not shown) leads to formation of postsynaptic receptor complexes and synapses with preganglionic neurons. CREB, cyclic AMP responsive element-binding; GRB2, growth factor receptor-bound protein 2; MEF2, myocyte-specific enhancer factor 2; SOS, son of sevenless; SRF, serum response factor.

mechanism of TRK internalization. Receptor-mediated endocytosis mechanisms can be broadly classified as being either clathrin-dependent or clathrin-independent[62], and recent findings suggest that TRKA may be internalized by both mechanisms (FIG. 3).

A great deal of experimental evidence points to the existence of a clathrin-dependent mechanism for the endocytosis of TRKA. In PC12 cells, NGF causes a redistribution of the clathrin heavy chain to the plasma membrane and promotes the formation of protein complexes that contain TRKA, clathrin and adaptor protein 2 (AP2)[63]. Isolated clathrin-coated vesicles from NGF-treated cells were found to contain NGF, TRKA and downstream signalling components of TRKA signalling pathway[63].

One clathrin-independent mechanism underlying TRKA endocytosis is macropinocytosis, which involves the formation of plasma membrane protrusions that eventually fuse together and engulf large volumes of membrane and extracellular fluid. Pincher, an NGF-upregulated GTPase, is involved in this mechanism of TRKA internalization[64]. Interestingly, pincher-mediated endocytosis of TRKA and clathrin-independent internalization of epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR)[65] have some common features. For example, high concentrations of EGF cause the formation of circular dorsal ruffles (CDRs) into which EGFRs become concentrated and rapidly internalized within tubular structures. Likewise, immunoelectron microscopy analysis has shown that overexpressed TRKB localizes within the pincher-positive domains of CDRs[66]. The relative contributions of both clathrin-mediated and pincher-mediated endocytosis for the internalization of TRKA and initiation of the NGF retrograde signal in neurons *in vivo* are currently unknown.

The nature of the signalling events that underlie receptor-mediated endocytosis of TRKs is also poorly understood. As mentioned previously, three major effector pathways have been described for TRKA: the PI3K–AKT, ERK and PLCγ pathways. Of these, the PI3K and PLCγ pathways may have the most important roles in receptor internalization, as inhibitors of the RAS–ERK pathway cannot prevent TRKA endocytosis. PI3K can be activated either by RAS or through direct recruitment to TRKA via the adaptor protein GAB1 (GRB2-associated-binding protein 1)[67]. The activity of PI3K is necessary at an early stage of the endosome retrograde transport process, as blocking its activity in distal axons prevents the retrograde accumulation of NGF in cell bodies[26]. It is unclear how PI3K functions to support the retrograde transport of the TRKA endosome; however, PI3K itself participates in internalization and postendocytic sorting in other systems, and the phosophoinositide products of its enzymatic activity can bind and regulate several proteins implicated in endocytosis, including dynamin[68], RAS-related protein RAB5 (REF. 69), AP2 (REF. 70) and synaptotagmin[71]. Dynamin, a GTPase involved in pinching off the endocytic vesicle from the plasma membrane, is a required component for NGF–TRKA internalization and subsequent retrograde transport[21,72]. The pleckstrin homology (PH) domain of dynamin is necessary for its function in clathrin-mediated endocytosis. This activity probably requires the binding of phosphoinostides to the PH domain[73]. Phosphoinostides themselves participate in vesicle coat formation and vesicle targeting through recruitment of AP2. Thus, PI3K activity is probably required at multiple stages in TRK-mediated endocytosis.

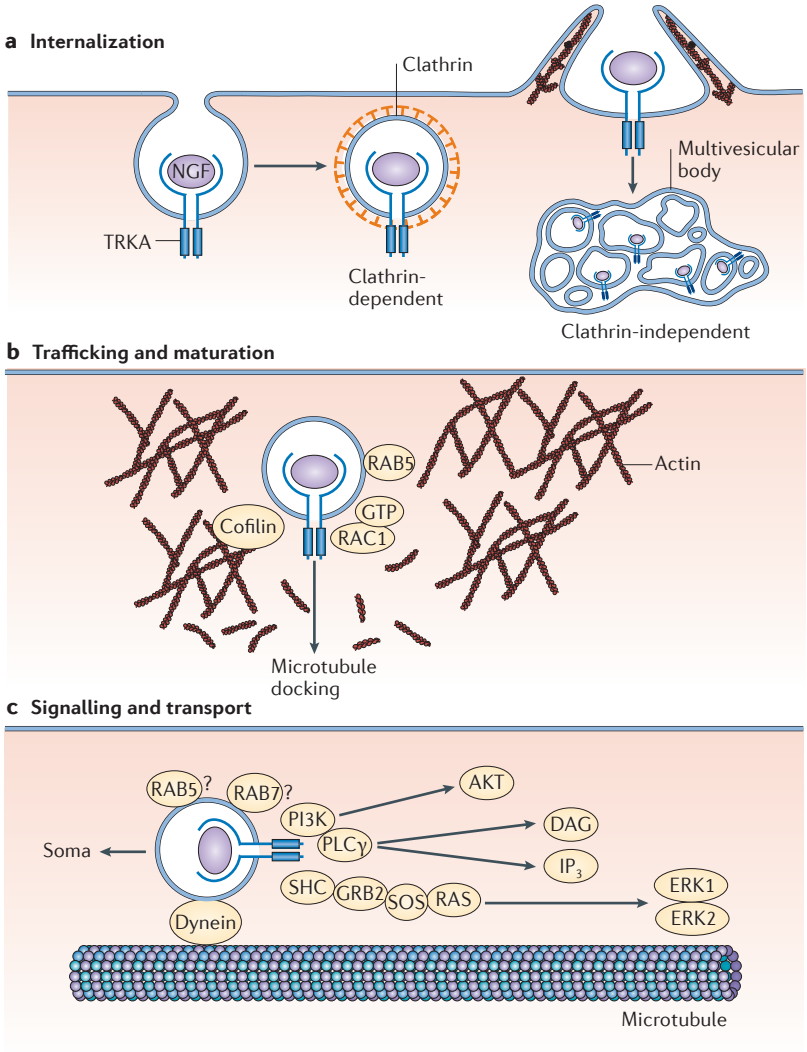

Figure 3 | **Mechanisms of neurotrophin internalization, signalling and retrograde transport. a** | It has been proposed that internalization of tropomyosin receptor kinase A (TRKA) following binding to nerve growth factor (NGF) occurs through clathrin-dependent and clathrin-independent mechanisms. Although clathrin-mediated endocytosis results in the formation of early endosomes, internalization via macropinocytosis leads to the generation of multivesicular bodies. **b** | Newly internalized TRKA endosomes must overcome an F-actin barrier before docking with microtubules for long distance transport. This is achieved by activation of RAS-related C3 botulinum toxin substrate 1 (RAC1), which leads to actin depolymerization through recruitment of cofilin to the signalling endosome. **c** | TRKA endosomes that are competent for transport are linked to the retrograde motor protein dynein and move towards the neuron's soma. Signalling from the phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK) and phospholipase Cγ (PLCγ) signalling pathways persists during endosome transport. The specific RAB composition of these endosomes remains controversial. DAG, diacylglycerol; GRB2, growth factor receptor-bound protein 2; IP_3, inositol 1,4,5-triphosphate; SOS, son of sevenless.

Phosphorylation of TRKA at Tyr785 following NGF binding leads to recruitment and activation of PLCγ[74]. PLCγ is a multifunctional enzyme, acting as both a lipase and as an interacting partner for additional receptor tyrosine effectors through its SRC homology domains[75]. The downstream effects of PLCγ can be categorized as those mediated by the second messengers of its lipase activity (that is, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3)) and those mediated by direct interactions. A recent study found that PLCγ activity is required for ligand-mediated TRKA internalization[38] possibly through the control of dynamin. Indeed, the release of Ca^{2+} from internal stores into the cytoplasm that is caused by PLCγ-mediated formation of IP_3 activates the phosphatase calcineurin, which in turn catalyses dephosphorylation of key dynamin residues that are required for receptor internalization[38]. In the context of EGFR signalling, the SH3 domain of PLCγ binds to and promotes nucleotide exchange on dynamin, increasing its membrane severing ability and activating effector molecules that are required for coated vesicle formation[76]. Thus, events that are essential for different steps of TRK internalization are probably controlled by the early effectors PI3K and PLCγ. Further studies into the mechanism of receptor-mediated internalization should be aimed at understanding how downstream signals recruit and activate the complement of proteins that are required for the formation of the endosome.

What type of vesicle is the NGF signalling endosome? Newly internalized vesicles undergo maturation from early endosomes to intermediate and late endosomes and are ultimately degraded following lysosomal fusion. Several members of the RAB family of small GTPases are enriched in endosomes at various stages of progression and have been implicated in controlling these maturation steps[77]. Evidence from studies in neuronal and non-neuronal cells indicates that RAB5 is a crucial early mediator of vesicle formation and the lateral homotypic fusion of early endosomes[78,79]. Another RAB protein, RAB7, seems to be associated with late endosomes and lysosomes and contributes to endosome maturation[80]. A principal mechanism for the maturation of endosomes, at least in non-neuronal cells, involves the loss of RAB5 on the vesicle membrane and recruitment of RAB7 (REF. 81).

Just as the type of internalization TRKs undergo is contentious, controversy surrounds the nature of the TRK endosome that is transported retrogradely along axons. Different investigators have argued that the active TRK signalling endosome is a multivesicular body (MVB) structure, a RAB7-positive late endosome, or a RAB5-positive early endosome. Two studies suggested that retrogradely transported NGF accumulates in MVBs[18,82]. Radioactive NGF that was exposed to distal axons of compartmentalized cultured sympathetic neurons was found to be transported to cell bodies and was detected almost exclusively in lysosomes and MVBs. Likewise, following injection of ^{125}I-NGF into the eye, NGF was detected localized to MVB structures in axons adjacent to sympathetic ganglia. Although these structures were infrequently seen, they were surprisingly large and, remarkably, caused the axonal diameter to expand and exceed its normal size by up to two and a half times[18]. Although in theory the MVB is an attractive structure owing to the protection it affords from lysosomal degradation, it is not clear whether this organelle would need to be broken down to permit signalling from individual endosomes to propagate within the cell body.

The organelle responsible for retrograde transport of neurotrophins and their receptors has also been suggested to be a late endosome. It has been shown that tetanus toxin is reterogradely transported in motor neurons in vesicles containing the low-affinity common neurotrophin receptor p75NTR (also known as TNFRSF16) and TRKB[83]. Analysis of tetanus toxin-containing endosomes in these neurons revealed that they are RAB7-positive but not RAB5-positive, which is consistent with them being late endosomes, and expression of dominant negative versions of RAB5 and RAB7 showed that only RAB7 is necessary for long distance axonal transport. It is possible that, to become transport-competent retrograde carriers, endosomes need to undergo a transition from expressing RAB5 to RAB7. A recent, related study found that the $P2X_3$ ion channel is retrogradely transported in DRG axons[84]. In this case, among $P2X_3$-containing endosomes, those positive for RAB7 showed higher movement velocity than those positive for RAB5. In addition, retrograde transport was inhibited in the presence of a dominant negative RAB7 mutant variant.

In further support of a model in which RAB7-containing endosomes are mediators of long distance transport, a recent proteomic analysis of the proteins associated with TRKA-containing endosomes detected both RAB7 and RAB7-interacting proteins[85], suggesting that RAB7 may indeed have a role in long distance transport and/or trafficking. The involvement of a RAB7-positive endosome in such transport alone, however, is not definitive evidence in support of the idea that signalling endosomes are late endosomes.

A good deal of evidence supports a model in which neurotrophins and their receptors are retrogradely transported from distal axons to cell bodies in early endosomes. Early studies conducted in PC12 cells showed that NGF treatment leads to an increase in TRKA in an endosomal fraction containing the early endosome markers RAB5 and EEA1 (REF. 86). Additionally, in DRG sensory neurons *in vivo*, immunostaining for NGF revealed distinctly labelled punctae in cell bodies that colocalized extensively with the early endosome marker RAB5 (REF. 87). Following the collection of proteins transported retrogradely from a dissected sciatic nerve section, protein analysis by western blot showed the presence of RAB5 and its effector EEA1, which correlated with the retrograde appearance of TRKA and NGF; however, RAB7 was not detected[87]. Electron microscopy analysis on this nerve segment identified vesicles that were consistent in size and appearance with early endosomes and that could be immunolabelled for both TRKA and RAB5. Collectively, these data suggest that NGF and TRKA are contained at least in a subset of early endosomes that are capable of retrograde transport in DRG sensory neurons.

How can the results of these studies, which have seemingly conflicting results, be reconciled? The findings summarized here that pertain to the identity of the transported neurotrophin endosome are not mutually exclusive. It is possible that retrograde transport of neurotrophins and their receptors proceed through the movement of early and late endosomes and MVBs within axons, and differences in experimental conditions account for the preferential use of one mechanism over the others. Consistent with this idea, one study examining the ultrastructural localization of activated TRKA in sciatic nerve segments observed the presence of this neurotrophin receptor on several, structurally distinct types of vesicles[88]. Endosomes ranging from 50 to 200 nm in diameter were shown to contain activated TRKA and consisted of coated and uncoated vesicles and in some cases multivesicular structures.

Another possible explanation for the differing results could be that distinct mechanisms are used in different neuronal types and for different receptor–ligand complexes. Retrograde transport of NGF and TRKA in sensory neurons may proceed differently to that in sympathetic neurons. Likewise, retrograde transport of p75NTR and TRKB in motor neurons may occur by a different means to that of TRKA in sympathetic and sensory neurons. Clearly, additional research is needed to gain a clear understanding of the nature of the retrograde signalling endosome for different receptor systems and different types of target-dependent neuronal populations. More thorough investigation of these transport vesicles will provide insight into the functions of neurotrophic factors as well as reveal new candidates for therapeutic intervention, as deficits in intracellular trafficking and transport are implicated in several neurodegenerative diseases.

Mechanisms of signalling endosome maturation and transport. The signalling pathways that are necessary for proper endosome trafficking and retrograde transport are an important topic of current investigation. The retrograde transport of TRKs requires their activation, as inhibitors of TRK autophosphorylation prevent the transport of such receptors to the cell body[21]. This activation is required for either TRK internalization or maturation of TRK endosomes into vesicles that are competent for long distance transport, as blocking TRK activity in a medial axon compartment has no effect on retrograde transport or neuronal survival. These findings suggest that downstream TRK effectors have essential roles in the initial trafficking and maturation processes but not retrograde movement of endosomes.

Recently, the importance of the actin cytoskeleton in retrograde TRKA-positive endosome transport was tested[85]. On the basis of experiments involving inhibitors of actin breakdown and assembly, it was determined that early TRK endosome trafficking requires disassembly of the actin cytoskeleton before long-distance, microtubule-based transport. From a proteomic analysis, two endosome-associated proteins — cofilin and RAS-related C3 botulinum toxin substrate 1 (RAC1) — were identified as being important in this vesicle trafficking step. Although the precise mechanism by which TRKA signalling activates RAC1 and cofilin remains to be determined, these studies provide evidence of how receptor activity in the endosome influences its own trafficking and subsequent retrograde transport.

The signalling events required for TRK endosome internalization, trafficking and transport are also dependent on the type of neurotrophin activating the receptor. TRKA shows affinity towards and can be activated by both NGF and NT3. NT3–TRKA signalling is thought to be important for axon outgrowth of sympathetic neurons early in development. Axon outgrowth caused by NT3 is probably a local growth-promoting effect and does not require a retrograde signal, as NT3 is incapable of promoting the retrograde transport of TRKA and retrograde survival signalling *in vitro*[43]. Owing to the reduced affinity of TRKA for NT3 at endosomal pH levels, endosomes that are formed following NT3 application *in vitro* lack the ability to recruit RAC1 and cofilin and thus are defective in the early trafficking that is necessary for retrograde transport[85].

Many questions pertaining to the neurotrophic signalling endosome remain, including how they are formed, transported and signal within axons, cell bodies and dendrites, and these issues could be addressed through a comprehensive analysis of TRK vesicle-associated proteins. One challenge in obtaining meaningful data from the proteomic analysis of endosomes comes from the difficulty in isolating pure populations of vesicles. If this challenge can be overcome, it will be fascinating to compare findings of prior early endosome-biased screens[85] against those of isolated TRKA-containing late endosomes and MVBs.

Other models of retrograde growth factor signalling. Several lines of evidence support the idea that cytokines such as CNTF, leukaemia inhibitory factor (LIF) and interleukin-6 signal in a retrograde manner through a receptor tyrosine kinase JAK–signal transducer and activator of transcription (STAT) pathway[89,90]. These ligands are typically released by target tissues and glia following axonal injury, which must activate a retrograde signal to elicit a response in neuronal cell bodies. Application of CNTF or LIF to distal axons promotes an increase in activated STAT3 in the cell bodies of sympathetic neurons that are cultured in compartmentalized chambers. This STAT3 could be detected in neurites following distal axon application of LIF, and the retrograde appearance in the cell bodies was microtubule dependent[89]. Recent findings also suggest that the retrograde action of bone morphogenic proteins (BMPs) may proceed through similar mechanisms. Exposure of distal axons of the trigeminal ganglion to BMP4 activates a signal, resulting in the accumulation of SMAD proteins and BMP4 in the cell bodies[91]. In *Drosophila melanogaster*, this retrograde transport of BMP proteins has been shown to require the endocytosis of BMP receptors and the activity of dynein[92].

In addition to the signalling endosome model, other models have been proposed to explain the retrograde propagation of neurotrophin signals. These models include the lateral sequential activation of

plasma membrane localized receptors and the retrograde movement of downstream TRK effectors such as PI3K, ERKs and Ca^{2+}. In these models, retrograde transport of TRKA and NGF are not required for neuronal survival[22,60]. These findings are in contrast with evidence showing the necessity of TRK internalization and trafficking to the cell body for neuronal survival signalling[65,80,21,44,66]. Additionally, the signalling endosome model envisions the vesicle as a protective platform on which ligand and receptor can be continuously engaged, activating downstream signals as it is transported. This mechanism protects activated signalling molecules from being rapidly inactivated.

A recent report has observed the retrograde transport of a downstream transcription factor of TRKA, namely CREB[93]. Axonal translation of *Creb* mRNA following NGF stimulation, and subsequent retrograde transport of the activated protein back to the nucleus, may contribute to retrograde neuronal survival, although a recent report argues against the notion that *Creb* mRNA is present in axons[94]. Nonetheless, retrograde signalling initiated by neurotrophins acting on distal axons may require the active transport of more than one signalling entity. Ultimately, the relative contributions of the purported mediators of retrograde neurotrophin signalling will best be resolved by testing the requirement of the retrograde transport of NGF, TRKA and its downstream effectors *in vivo*. Evidence also exists suggesting that p75NTR is sorted into endosomes that undergo retrograde transport in the presence of NGF in motor neurons that do not express TRKA[95]. The physiological role of this process is not yet understood but it could provide an intriguing mechanism by which certain target-derived factors evoke cell death.

Retrograde neurotrophic factor signalling and implications in disease. Recent studies have investigated defects in the retrograde axonal transport machinery and their implications in several neurodegenerative diseases, including the importance of retrograde neurotrophin transport. In patients with Alzheimer's disease and in mouse models of this disease, levels of NGF are reduced in the basal forebrain, a site of major cholinergic neuron loss[96]. As there is no change in NGF levels in areas of the brain where it is synthesized, the decrease in NGF observed in the basal forebrain is probably caused by a decreased NGF retrograde transport[97]. Consistent with this assertion, the levels of TRKA in basal forebrain neurons are reduced in diseased human brains[98]. This finding is of great interest because cholinergic forebrain neurons are dependent on NGF–TRKA signalling; in the absence of NGF, these neurons exhibit somal hypotrophy, decreased fibre density and reductions in the levels of neurotransmitter-producing enzymes[99]. The causes of this decreased NGF transport in patients with Alzheimer's disease are not well understood at this point.

Mutations in amyotrophic lateral sclerosis 2 (*ALS2*), which encodes a guanine nucleotide exchange factor for RAB5 and RAC1, have been linked to rare forms of ALS[100]. The activation of both RAB5 and RAC1 are believed to be required for proper retrograde transport of endosomes[83,85]. In an attempt to create a mouse model for this form of ALS, knockout mice for *Als2* were generated. These mice have abnormal axonal endosome trafficking, which is consistent with a role of retrograde transport or signalling in the pathology of the disease[101].

Charcot–Marie–Tooth disease (CMT) is a sensory neuropathy disease that also may be caused by defects in axonal transport, as mutations in several proteins associated with movement along axons have been linked to the disease. Mutations in *RAB7* are associated with CMT type 2B[102]. Early *in vitro* studies suggest that these RAB7 mutants may disrupt axon growth and result in perturbed TRKA signalling[103,104], although whether defects in TRKA transport or signalling are directly involved in CMT type 2B is not known.

Conclusions

Retrograde signalling that is initiated in axons by target-derived cues or at an injury site is a principal means by which proper neuronal growth, survival, connectivity, differentiation and maintenance are mediated. Through mechanisms that are still being delineated, growth factors such as the neurotrophins bind to cognate receptors on innervating distal axons and initiate the transport of a signalling-competent complex to the cell body, where signal transduction events in the soma and even the dendrites can respond appropriately. The implication of the many neuronal processes controlled by retrograde signalling mechanisms is that target tissues have enormous control over the development, function, maintenance and regenerative capacity of neurons.

Even as new functions for retrograde signalling are being revealed, a great number of unresolved mechanistic issues remain. Although there may be overlap in the mechanisms used by different receptor systems, increasing evidence suggests that receptors of different families use unique mechanisms for retrograde signalling, and these mechanisms need to be understood. Likewise, it is possible that neuron type influences how retrograde signals are transmitted. Future work should focus on the roles and mechanisms of different endosomal types in transmitting retrograde growth-factor signalling; the nature of the signalling events controlling their formation, sorting and transport; non-signalling endosome mechanisms of retrograde signalling; and defects in these processes as they relate to disease.

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Competing interests statement
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