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Review

Diversity of vertebrate nicotinic acetylcholine receptors

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) are pentameric neurotransmitter receptors. They are members of the Cys-loop family of ligand-gated ion channels which also include ionotropic receptors for 5-hydroxytryptamine (5-HT), γ-aminobutyric acid (GABA) and glycine. Nicotinic receptors are expressed in both the nervous system and at the neuromuscular junction and have been implicated in several neurological and neuromuscular disorders. In vertebrates, seventeen nAChR subunits have been identified (α1–α10, β1–β4, γ, δ and ε) which can co-assemble to generate a diverse family of nAChR subtypes. This review will focus on vertebrate nAChRs and will provide an overview of the extent of nAChR diversity based on studies of both native and recombinant nAChRs.

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1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are excitatory neurotransmitter receptors and members of the super-family of ligand-gated ion channels (Changeux and Edelstein, 2005). They are complex pentameric transmembrane proteins assembled from a diverse collection of subunits. In this review, which will examine the extent of nAChR diversity, we will focus on vertebrate nAChRs, with particular emphasis on studies conducted with mammalian receptors. Although, nAChRs are expressed in both vertebrate and invertebrate species a discussion of invertebrate nAChRs is beyond the scope of this review. The diversity of nAChRs in invertebrate species has, however, been discussed in detail elsewhere (Jones et al., 2007; Millar and Denholm, 2007).

2. Nicotinic receptor structure

Recent structural studies have provided an insight into the three dimensional structure of nAChRs. In particular, a high resolution structure (4 Å) of the nAChR from the marine ray *Torpedo* (Unwin, 2005) has revealed important information about nAChR structure and has been invaluable in the interpretation of functional and pharmacological data (Fig. 1). In addition, higher resolution structural information has become available from studies of proteins which show close sequence similarity to nAChRs. These include a cytoplasmic acetylcholine binding protein (AChBP) from the pond snail *Lymnaea* (Brejc et al., 2001; Smit et al., 2001) and prokaryotic ligand-gated ion channels (Bocquet et al., 2007; Hilf and Dutzler, 2008). An atomic resolution structure of the AChBP from *Lymnaea* has been obtained by X-ray crystallography (Brejc et al., 2001) and has been used extensively as a model for the nAChR extracellular domain. In addition, the structure of a pentameric transmembrane ligand-gated ion channel from the bacterium *Erwinia chrysanthemi* has been determined by X-ray crystallography (Hilf and Dutzler, 2008) and has provided further insights into the structure of the super-family of ligand-gated ion channels. However, until recently, no atomic resolution structural information has been available for any nAChR. This situation has recently been rectified by the determination of an atomic resolution (1.94 Å) structure of the extracellular domain of the nAChR α1 subunit (Fig. 1) by X-ray crystallography (Dellisanti et al., 2007). Such information will, undoubtedly, be of considerable benefit to our understanding of these complex neurotransmitter receptors.

3. Subunit nomenclature

The nomenclature of nAChR subunits has arisen in a somewhat arbitrary manner. Early biochemical studies of nAChRs purified from the electric organ of fish such as the marine ray *Torpedo* and the freshwater eel *Electrophorus* identified four protein subunits (for a detailed review, see Popot and Changeux, 1984). The four subunits were assigned the Greek letters α, β, γ and δ on the basis of their increasing apparent molecular weights when resolved on polyacrylamide gels. Of these four subunits, only the α subunit could be labelled by quaternary ammonium affinity-labelling reagents, which led to the conclusion that the α subunit was the principal agonist binding site (Weill et al., 1974). The subsequent molecular cloning of the *Torpedo* α subunit (Noda et al., 1982; Sumikawa et al., 1982) identified two adjacent cysteine residues (Cys192 and Cys193), which were believed to be important in agonist binding. The convention which has been adopted

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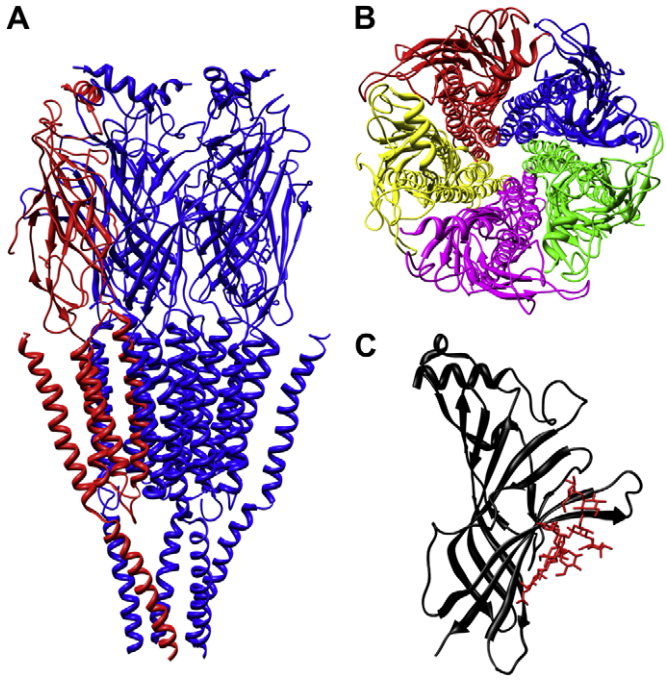

Fig. 1. Three dimensional structure of the *Torpedo* nAChR. The structure of the nAChR expressed in the electric organ of the marine ray *Torpedo* has been determined at 4 Å resolution (Unwin, 2005) and has been used extensively a model for other nAChRs. Representations of the *Torpedo* nAChR (as ribbon structures, representing only backbone amino acids) are shown viewed from the side (A) and from above (B). In the side view (A), one of the five subunits has been shaded a different colour (red) for clarity. In the top view (B) all five subunits have been shaded in different colours to indicate more clearly the boundaries between subunits. (C) The atomic resolution (1.94 Å) structure of the mouse nAChR α1 subunit, determined by X-ray diffraction (Dellisanti et al., 2007), is illustrated. Carbohydrates, linked to Asn141, are illustrated as a stick model in red.

subsequently for the nomenclature of nAChR subunits is that nAChRs which contain two cysteine residues at positions analogous to Cys192 and Cys193 in the *Torpedo* α subunit have been classified as α-type subunits. Non-α subunits expressed at the vertebrate neuromuscular junction have been assigned the names β1, γ, δ and ε, whereas non-α subunits expressed in the vertebrate nervous system have been assigned the names β2–β4. Nicotinic receptors expressed in either muscle cells and or within the nervous system are commonly referred to as 'muscle-type' and 'neuronal' nAChRs, although it has been suggested that such terms should not become a formal part of nAChR nomenclature or classification (Lukas et al., 1999). The term 'neuronal', for example, is used widely to describe the α2–α10 and β2–β4 subunits, despite evidence that some of these subunits are expressed in non-neuronal locations (Sharma and Vijayaraghavan, 2002).

On the basis of affinity-labelling experiments (Weill et al., 1974), it was assumed that α subunits are agonist binding subunits, whereas non-α subunits are 'structural' subunits. However, more recent studies indicate that nicotinic agonists bind at subunit interfaces (reviewed by Sine, 2002) and that both α and non-α subunits are able to contribute to the nicotinic agonist binding site (Luetje and Patrick, 1991). A consequence of this is that the terms 'agonist-binding' and 'structural' may be less appropriate descriptions of the roles performed by nAChR subunits. What may be more useful is a terminology which distinguishes between subunits which form what has been referred to extensively as the 'principal' and 'complementary' subunit interface of the agonist binding site (Bertrand and Changeux, 1995; Corringer et al., 1995, 2000; Arias, 1997).

The system of nAChR subunit nomenclature, which is based upon the assignment of Greek letters, has led to some anomalies. As a consequence, the designation "α" and "β" subunit cannot necessarily be taken to imply functional equivalence of subunits. Whereas all heteromeric nAChRs contain at least one α-type subunit which can act as the principal subunit at the agonist binding site, not all α subunits can perform this role. The α5 and α10 subunits, for example, are only able to form functional nAChRs when co-assembled with another α subunit. In fact, the α5 subunit has greater sequence similarity to β3 than it does to other nAChR α or β subunits (Boulter et al., 1990). It has been suggested that the presence or absence of Tyr190 (rather than Cys192 and Cys193) might be a better basis for the classification of nAChR subunits (Abramson and Taylor, 1990), since this would place the α5 subunit in a group with other 'non-α' subunits. The problem of using methods such as these to classify nAChR subunits is illustrated by the fact that the recently identified α10 subunit would still be classified as an α-type subunit (using either of these two systems), despite its apparent inability to act as a principal subunit at the agonist binding site (Elgoyhen et al., 2001; Baker et al., 2004). Unfortunately, even greater anomalies concerning the use of Greek letters for nAChR subunit nomenclature have arisen for invertebrate species, as has been discussed elsewhere (Millar, 2003; Millar and Denholm, 2007).

Another long established convention is that nAChR subtypes are referred to by their subunit composition. For example, α4β2 refers to a nAChR subtype containing only α4 and β2 subunits (even though the precise subunit stoichiometry of subunits within the receptor may not be known). Also, by convention, when the precise subunit composition of a nAChR subtype is unknown, this uncertainty is indicated by an asterisk (Lukas et al., 1999; Luetje, 2004). For example, α4β2* indicates a nAChR which is known to contain α4 and β2 subunits but which may also contain additional subunit subtypes. In situations where both the subunit composition and also the subunit stoichiometry are known, the number of each subunit present in the assembled pentamer is indicated by subscript numbers (for example $(\alpha1)_2\beta1\gamma\delta$). Several authors have used subscript numbers for nAChR subunit nomenclature but this has been discouraged (Lukas et al., 1999), in part, because of the potential confusion that this can cause with the use of subscript numbers to designate subunit stoichiometry.

4. Subunit diversity

Seventeen nAChR subunits have been identified in vertebrate species (α1–α10, β1–β4, γ, δ and ε). All of these subunits, with the exception of α8 (which has been identified only in avian species), are found in humans and in other mammalian species. As will be discussed below, although some nAChR subunits such as α7 are able to generate functional homomeric receptors (containing five copies of a single subunit), most nAChR subunits form functional receptors only when co-assembled with other subunits to generate heteromeric receptors. Clearly, there are many possible combinations in which the sixteen mammalian nAChR subunits could potentially co-assemble. It appears, however, that assembly of nAChRs, like that of other oligomeric ion channels, is a tightly regulated and ordered process, which requires appropriate subunit–subunit interactions (reviewed by Green and Millar (1995) and Millar and Harkness (2008)). Although the potential exists for the formation of nAChRs with very many different subunit combinations, it appears that native nAChRs are assembled into functional pentamers of relatively restricted number of subunit combinations, whilst still generating considerable nAChR diversity (Table 2).

5. Subunit stoichiometry

As was mentioned above, some nAChR subunits (such as α7) are able to generate functional homomeric receptors (Couturier et al.,

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1990). In contrast, most nAChRs subtypes are heteromeric receptors, containing at least one type of α subunit and one type of non-α subunit. The subunit stoichiometry of heteromeric nAChRs has been the subject of considerable research. For some nAChRs, such as the muscle-type nAChR, subunit stoichiometry has been revealed directly by high resolution structural information (Unwin, 2005). Like the *Torpedo* electric organ nAChR, receptors expressed at the adult mammalian neuromuscular junction appear to have a fixed subunit stoichiometry of $(\alpha1)_2\beta1\delta\varepsilon$, in which two copies of the α1 subunit co-assemble with a single copies of the β, δ and ε subunits. In embryonic muscle, nAChRs contain a γ rather than an ε subunit, giving a subunit stoichiometry of $(\alpha1)_2\beta1\gamma\delta$. Muscle nAChRs have two non-equivalent agonist binding sites at the α–δ and α–γ/ε subunit interfaces (Neubig and Cohen, 1979; Sine, 1993), whereas the β1 subunit does not participate directly in the formation of an agonist binding site.

Whist the subunit stoichiometry of other heteromeric nAChRs is less clearly defined, there is evidence that at least some heteromeric neuronal nAChRs co-assemble with two α subunits and three non-α (β) subunits. For example, heterologous expression studies performed with reporter mutations suggest that α4β2 and α3β4 nAChRs both contain two α subunits co-assembled with three β subunits (Cooper et al., 1991; Boorman et al., 2000) a conclusion which is also supported by more direct biochemical studies (Anand et al., 1991). However, more recent data indicate that this is an oversimplification. There is now strong evidence to indicate that some neuronal nAChR can assemble into nAChRs with alternate stoichiometries and that this can influence pharmacological and functional properties (Zwart and Vijverberg, 1998; Nelson et al., 2003). There is evidence, for example, that α4β2 nAChRs can assemble in two different stoichiometries $((\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2)$ which differ in properties such as agonist and antagonist sensitivity (Zwart and Vijverberg, 1998; Nelson et al., 2003; Moroni et al., 2006) and calcium permeability (Tapia et al., 2007). Recent studies are also providing an insight into the subunit stoichiometry of more complex neuronal heteromeric nAChRs. The use of reporter mutations has provided evidence that nAChRs containing the α3, β3 and β4 subunits assemble with a stoichiometry of $(\alpha3)_2\beta3(\beta4)_2$ (Boorman et al., 2000). Another potentially powerful approach to examining receptor stoichiometry has come from studies of artificially linked subunits (Zhou et al., 2003; Groot-Kormelink et al., 2004, 2006), although there are several problems which can arise with such approaches (Groot-Kormelink et al., 2004; Ericksen and Boileau, 2007).

6. Receptor diversity revealed by studies of recombinant nAChRs

The five muscle-type nAChRs (α1, β1, γ, δ and ε) co-assemble efficiently in heterologous expression systems into two distinct receptor subtypes ($(\alpha1)_2\beta1\gamma\delta$ and $(\alpha1)_2\beta1\delta\varepsilon$), an observation which, as is discussed below, is in agreement with studies of native nAChRs expressed in embryonic and adult muscle, respectively. There have been reports of functional recombinant nAChRs with fewer than four different muscle-type nAChR subunits (see Table 1), but these appear to be formed less efficiently than the fully assembled receptors (Charnet et al., 1992; Liu and Brehm, 1993) and have not been detected as native nAChRs (Table 2).

The α7 and α8 subunits are the only two vertebrate nAChR subunits (and α7 is the only mammalian subunit) which appear to preferentially form homomeric, rather than heteromeric, receptors in heterologous expression systems (Couturier et al., 1990; Gerzanich et al., 1994; Gotti et al., 1994). This is consistent with evidence that these subunits form homomeric nAChRs in native systems (Keyser et al., 1993; Chen and Patrick, 1997; Drisdel and Green, 2000). Whilst the α7 and α8 subunits both readily form functional homomeric nAChRs when expressed in *Xenopus* oocytes, they do so very much less efficiently in many types of cultured cell lines (Cooper and Millar, 1997, 1998; Kassner and Berg, 1997; Rangwala et al., 1997). Recent studies have revealed that co-expression of either α7 or α8 with a nAChR-selective molecular chaperone RIC-3 can facilitate the formation of functional homomeric nAChRs in otherwise non-permissive cell types (Castillo et al., 2005; Lansdell et al., 2005, 2008; Williams et al., 2005). RIC-3 is an ER-resident protein, with most studies indicating that it does not remain associated with assembled nAChRs on the cell surface (reviewed by Millar, 2008). In addition to the evidence that α7 can form a homomeric nAChR, there is evidence from heterologous expression studies that it can co-assemble with β2 to form a functional heteromeric receptor (Khiroug et al., 2002). There have also been reports of the co-assembly of α7 with β3, but this appears to lead to the formation of a non-functional receptor (Palma et al., 1999; Broadbent et al., 2006).

The nAChR α9 subunit, which is expressed predominantly in hair cells of the cochlear, can generate homomeric nAChRs when expressed in *Xenopus* oocytes (Elgoyhen et al., 1994), but it forms a functional nAChR very much more efficiently when it is co-expressed with the α10 subunit (Elgoyhen et al., 2001; Sgard et al., 2002). As is discussed below, this finding is consistent with evidence which indicates that native α9-containing nAChRs are heteromeric complexes with α10 (Elgoyhen et al., 2001; Katz et al., 2004; Vetter et al., 2007). Furthermore, there is evidence from

Table 1
Subunit combinations identified from studies with recombinant nAChRs

nAChR subtype		Selected references
Homomeric	α7	Couturier et al. (1990)
	α8	Gerzanich et al. (1994), Gotti et al. (1994)
	α9[a]	Elgoyhen et al. (1994)
Heteromeric (pair)	α1γ[a]	Liu and Brehm (1993)
	α1δ[a]	Liu and Brehm (1993)
	α2β2	Papke et al. (1989)
	α2β4	Duvoisin et al. (1989)
	α3β2	Papke et al. (1989)
	α3β4	Duvoisin et al. (1989)
	α4β2	Papke et al. (1989)
	α4β4	Duvoisin et al. (1989)
	α6β2	Fucile et al. (1998)
	α6β4	Gerzanich et al. (1997)
	α7β2	Khiroug et al. (2002)
	α9α10	Elgoyhen et al. (2001), Sgard et al. (2002)
Heteromeric (triplet)	α1β1γ[a]	Kullberg et al. (1990), Liu and Brehm (1993)
	α1β1δ[a]	Charnet et al. (1992), Liu and Brehm (1993)
	α1γδ[a]	Liu and Brehm (1993)
	α1δε[a]	Liu and Brehm (1993)
	α3α5β2	Wang et al. (1996), Gerzanich et al. (1998)
	α3α5β4	Wang et al. (1996), Fucile et al. (1997)
	α3α6β2	Kuryatov et al. (2000)
	α3α6β4	Fucile et al. (1998), Kuryatov et al. (2000)
	α3β3β4	Groot-Kormelink et al. (1998)
	α4α5β2	Ramirez-Latorre et al. (1996), Girod et al. (1999)
	α4β2β3	Kuryatov et al. (2008)
	α5α6β2	Kuryatov et al. (2000)
	α5α7β2	Crabtree et al. (1997), Girod et al. (1999)
	α5α7β4	Crabtree et al. (1997)
	α6β2β3	Tumkosit et al. (2006)
	α6β3β4	Kuryatov et al. (2000), Tumkosit et al. (2006)
Heteromeric (quadruplet)	α1β1γδ	Mishina et al. (1986)
	α1β1δε	Mishina et al. (1986)

[a] These subunit combinations generate functional recombinant nAChRs relatively inefficiently and appear not to be combinations expressed as native nAChRs (see Table 2). Due to constraints of space, only a limited number of references have been included in the table. See main text for additional references. The β3 subunit is able to co-assemble with several other nAChR subunits, including α7, but in most cases it has a strong dominant-negative effect (Palma et al., 1999; Broadbent et al., 2006). Such subunit combinations have not been included in the table.

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Table 2
Subunit combinations identified from studies with native vertebrate AChRs

Subtype	Species	Localization	References
α1β1γδ	B	Muscle	Nelson et al. (1992)
α1β1δε	B	Muscle	Nelson et al. (1992)
α2α4β2*	C	Retina	Vailati et al. (2003)
	R	Retina	Moretti et al. (2004), Marritt et al. (2005)
	M	Cortex	Quik et al. (2005)
α2α5β2	C	Optic lobe	Balestra et al. (2000)
α2β2*	C	Retina	Vailati et al. (2003)
	R	Retina	Moretti et al. (2004), Marritt et al. (2005)
		Interpeduncular nucleus	Gotti et al. (2006b)
α2β4*	C	Retina	Vailati et al. (2003)
	R	Retina	Moretti et al. (2004), Marritt et al. (2005)
α3α4β2*	M	Cortex	Quik et al. (2005)
α3α4α6β2*	R	Optic nerve	Cox et al. (2008)
α3α5β2*	R	Superior colliculus	Gotti et al. (2005b)
	H	SH-SY5Y cells	Wang et al. (1996)
α3α5β2β4	C	Ciliary ganglia	Conroy and Berg (1998)
α3α5β4*	C	Ciliary ganglia	Vernallis et al. (1993)
	R	Superior cervical ganglia	Del Signore et al. (2002), Mao et al. (2006)
	R	Cerebellum	Turner and Kellar (2005)
α3α6β2*	C	Retina,	Vailati et al. (1999)
	R	Retina, optic nerve	Moretti et al. (2004), Cox et al. (2008)
α3β2*	R	Retina, optic nerve	Moretti et al. (2004), Cox et al. (2008)
	M	Striatum	Quik et al. (2005)
α3β2β3*	R	Superior colliculus	Gotti et al. (2005b)
α3β2β4*	R	Retina, cerebellum	Marritt et al. (2005), Turner and Kellar (2005)
	R	Superior cervical ganglia	Mao et al. (2006)
α3β3β4	R	Habenula, interpeduncular nucleus	Gotti et al. (2006b)
α3β4	R	Pineal gland, cerebellum	Hernandez et al. (2004), Turner and Kellar (2005)
α3β4°	R	Medial habenula	Quick et al. (1999)
α4α5β2	C	Brain	Conroy and Berg (1998)
	R	Striatum, SC, LGN	Zoli et al. (2002)
	R	Other brain areas	Brown et al. (2007)
α4α6β2β3	R	Retina, striatum	Zoli et al. (2002), Moretti et al. (2004)
	R	SC, LGN, optic nerve	Gotti et al. (2005b), Cox et al. (2008)
	M	Striatum	Quik et al. (2005)
α4β2	C	Brain	Whiting and Lindstrom (1986)
	R	Brain	Whiting et al. (1987)
	B	Brain	Whiting and Lindstrom (1988)
α4β2*°	R	Spinal cord	Marubio et al. (1999)
α4β4	C	Retina	Barabino et al. (2001)
α4β4*	R	Retina	Moretti et al. (2004)
α6β2*	R	Retina	Moretti et al. (2004)
α6β2°	R	Striatum	Salminen et al. (2007)
α6β2β3	R	Striatum,	Zoli et al. (2002)
		Retina, SC, LGN	Moretti et al. (2004), Gotti et al. (2005a)
	M	Striatum	Quik et al. (2005)
α6β2β3*	C	Retina	Vailati et al. (1999)
	H	Striatum	Gotti et al. (2006a)
α6β3β4*	C	Retina	Vailati et al. (1999)
α6β4*	C	Retina	Vailati et al. (1999)
α7	C	Retina, optic lobe	Anand et al. (1993), Gotti et al. (1997)
	R	Brain	Drisdel and Green (2000)
	H	IMR-32 cells	Gotti et al. (1995)
α7α8	C	Retina, optic lobe	Anand et al. (1993), Keyser et al. (1993)
α8	C	Retina	Anand et al. (1993), Gotti et al. (1997)
α9α10°	R	Cochlea	Elgoyhen et al. (2001), Katz et al. (2004)

In most cases direct evidence for the subunit combinations listed has been obtained by immunoprecipitation and/or immunopurification experiments. A small number of subunit combinations (indicated by a ° symbol) have been proposed based on evidence derived from alternative techniques. As discussed in the text, in situations where there is uncertainty as to whether additional subunits may be present in the assembled receptor, this is indicated by an asterisk (*). Multiple references are cited when data have been obtained from different species or from different brain regions. SC, superior colliculus; LGN, lateral geniculate nucleus; B, bovine; C, chick; H, human; M, monkey; R, rodent.

studies conducted in *Xenopus* oocytes that α9 and α10 co-assemble with the stoichiometry $(\alpha9)_2(\alpha10)_3$ (Plazas et al., 2005).

The neuronal α2–α6 and β2–β4 subunits fail to generate functional nAChRs when expressed individually in artificial expression systems such as the *Xenopus* oocyte (Boulter et al., 1987; Duvoisin et al., 1989; Luetje and Patrick, 1991), findings which are consistent with studies performed in cultured cell lines (Rogers et al., 1991; Whiting et al., 1991; Wong et al., 1995; Ragozzino et al., 1997). Heterologous expression studies have also demonstrated that, when these subunits are expressed alone, they are largely retained within the cell rather than being expressed on the cell surface (Cooper et al., 1999; Harkness and Millar, 2002). Despite their inability to form homomeric nAChRs, a subset of these subunits (notably α2–α4, β2 and β4) are able to form functional nAChRs when expressed as pair-wise combinations of one α and one β subunit (Duvoisin et al., 1989; Papke et al., 1989). Functional expression has been observed for pair-wise subunit combinations such as α2β4, α3β2, α3β4, α4β2 and α4β4 (Duvoisin et al., 1989; Papke et al., 1989). It appears that the α5 and β3 subunits (which, as discussed earlier, show greater sequence similarity to one another

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than to other nAChR subunits) are unable to generate functional nAChRs unless co-expressed as third subunits with another α and β subunit pair. For some time the α6 subunit appeared to be similar in this respect to α5 and β3 and, like the α5 and β3 subunits, had been described as being an 'orphan' subunit (Gerzanich et al., 1997). There have, however, been reports that the α6 subunit can generate functional recombinant nAChRs in a pair-wise combination either β2 or β4 (Gerzanich et al., 1997; Fucile et al., 1998), although the α6β2 subunit combination appears to be difficult to express in heterologous systems (Kuryatov et al., 2000).

Several heteromeric 'triplet' nAChR subunit combinations containing the α5, α6 or β3 subunits have been identified by heterologous expression studies, primarily as a result of differences in electrophysiological properties when compared with pair-wise subunit combinations (Table 1). Several triplet subunit combinations containing α5 have been distinguished from heteromeric 'pair' subunit combinations on the basis of altered electrophysiological properties. These include: α3α5β2 (Wang et al., 1996; Gerzanich et al., 1998), α3α5β4 (Wang et al., 1996; Fucile et al., 1997; Gerzanich et al., 1998) and α4α5β2 (Ramirez-Latorre et al., 1996). The β3 subunit has been shown to co-assemble with several nAChR subunit combinations, but in almost all cases (i.e. other than α3β3β4) it appears to have a dominant-negative effect which results in a lack of functional expression of the β3-containing assembled receptor complex (Palma et al., 1999; Broadbent et al., 2006). Co-assembly of β3 into functional α3β3β4 nAChRs has, however, been demonstrated by heterologous expression in oocytes using a reporter mutation approach (Groot-Kormelink et al., 1998). Heterologous expression studies have also demonstrated the preferential co-assembly of α6 into triplet subunit combinations. These include α6β3β4 (Kuryatov et al., 2000; Tumkosit et al., 2006) and also α3α6β4 and α3α6β2 (Fucile et al., 1998; Kuryatov et al., 2000).

7. Receptor diversity revealed by studies of native nAChRs

As was discussed above, nAChRs expressed at the vertebrate neuromuscular junction display close structural similarity (in terms of subunit composition) to the well-characterised nAChR from the electric organ of *Torpedo* (as reviewed by Lindstrom et al. (1991) and Kalamida et al. (2007)). In contrast to the considerable subunit diversity amongst native nAChR subtypes which are expressed within the vertebrate nervous system (see below and Table 2), nAChRs expressed at the neuromuscular junction are a largely homogeneous population, although some receptor heterogeneity exists due to developmental changes in gene transcription. The nAChR γ subunit is expressed preferentially in embryonic muscle (and also in denervated adult muscle), whereas the ε subunit is expressed preferentially in innervated adult muscle (Mishina et al., 1986; Witzemann et al., 1989). As a consequence of these transcriptional changes, nAChRs expressed in embryonic and in adult muscle have different subunit compositions, the stoichiometry of which is now well established ($(\alpha1)_2\beta1\gamma\delta$ and $(\alpha1)_2\beta1\delta\varepsilon$, in embryonic and adult muscle, respectively).

In contrast to the relatively homogeneous nature of nAChRs expressed at the neuromuscular junction, there is considerably greater diversity amongst nAChRs expressed throughout the central and peripheral nervous system (as summarised in Fig. 2 and Table 2). The fact that many neuronal cells express multiple nAChR subunits has hindered the identification and characterisation of native neuronal nAChRs. However, the increasing availability of knock-out or knock-in mice, subunit-specific antibodies and subtype-specific nicotinic ligands has made it possible to explore the diversity of subunit composition amongst neuronal nAChRs and examine the location of these subtypes within the nervous system. The picture which is emerging is one of considerable receptor diversity (see Fig. 2 and Table 2), but this information is starting to provide new insights into the role of nAChRs in normal and diseased brain (Paterson and Nordberg, 2000; Hogg et al., 2003; Gotti et al., 2006b). The important contribution which has been made by studies of knock-out and knock-in mice has been discussed in several recent reviews (Picciotto et al., 2001; Champtiaux and Changeux, 2002; Drago et al., 2003; Gotti and Clementi, 2004). These studies have, for example, helped to confirm earlier pioneering work which was preformed with nAChRs expressed in rat and chick brain (reviewed in Lindstrom (2000)) and which revealed that the α4 or β2 subunits contribute to the high affinity nicotine binding sites in the brain (and are distinct from α-bungarotoxin-binding nAChRs in the brain).

It is now well established that the most abundant nAChR subtypes in the nervous system are homomeric α7 receptors and heteromeric receptors containing only one type of α and one type of β subunit. Of these pair-wise heteromeric subtypes, α4β2 is the most abundant and widely distributed in mammalian brain, whereas the α3β4 is the predominant nAChR subtype in the autonomic ganglia, adrenal medulla, and in subsets of nerve cells in the medial habenula, nucleus interpeduncularis, dorsal medulla, pineal gland and retina. Interestingly, *in situ* hybridisation studies have revealed a more abundant expression of α2 mRNA in primate brain than in rodents (Han et al., 2000). This suggests that α2-containing nAChRs may be more abundant in primate than in rodent brain, a conclusion which is supported by radioligand binding studies (Han et al., 2003). In agreement with data obtained from studies with recombinant nAChRs (as was discussed earlier), there is now extensive evidence to indicate that native neuronal nAChRs can contain more than two types of subunit. For example, amongst α4β2* nAChRs (a subtype which is very widely distributed within the brain), approximately 20% of these receptors also contain the α5 subunit (Brown et al., 2007).

In some brain areas, minor nAChR subtypes such as α6β3* (Champtiaux et al., 2002; Cui et al., 2003) or α3* (Whiteaker et al., 2002) have been detected but appear to be restricted to localised areas of the brain (reviewed in Picciotto et al., 2001; Drago et al., 2003; Gotti et al., 2006b). Such receptor subtypes do, however, constitute a high proportion of nAChRs which are expressed within these localised brain areas (Zoli et al., 2002; Moretti et al., 2004; Gotti et al., 2005b; Marritt et al., 2005). Within rodent meso-striatal (midbrain and striatum) pathway and visual (retina, superior colliculus and lateral geniculate nucleus) pathway, the α6 and β3 subunits are present in two major subtypes (α4α6β2β3 and α6β2β3), with the former representing 40–60% of all α6* receptors. These two α6* subtypes have different affinities and sensitivities for the antagonist α-conotoxin MII (Zoli et al., 2002). Indeed, studies of wild-type and knock-out mice have shown that the α4α6β2β3 subtype has both α4β2 and α6β2 interfaces within the same receptor molecule, each with different affinities for α-conotoxin MII (Champtiaux et al., 2003; Salminen et al., 2007). The β3 subunit in this nAChR subtype is not directly involved in the formation of the ligand binding site and is thought to be located in a position equivalent to that of the β1 subunit in muscle nAChRs (Gotti et al., 2005a; Drenan et al., 2008).

Striatal α6* nAChRs, which have high affinity for α-conotoxin MII, are conserved across vertebrate species (being present in rodents, monkeys and humans), although with slight differences in subunit composition and subtypes ratios (Quik et al., 2005; Gotti et al., 2006a; Bordia et al., 2007). Retinal α6* receptors are almost exclusively associated with β2 subunit in mammalian subtypes (Keyser et al., 2000; Moretti et al., 2004; Marritt et al., 2005), whereas in adult chick retina α6* receptors are almost exclusively associated with the β4 subunit (Vailati et al., 1999).

Expression of α3-containing receptors within the brain is limited (Whiteaker et al., 2002), but α3 subunits are present as

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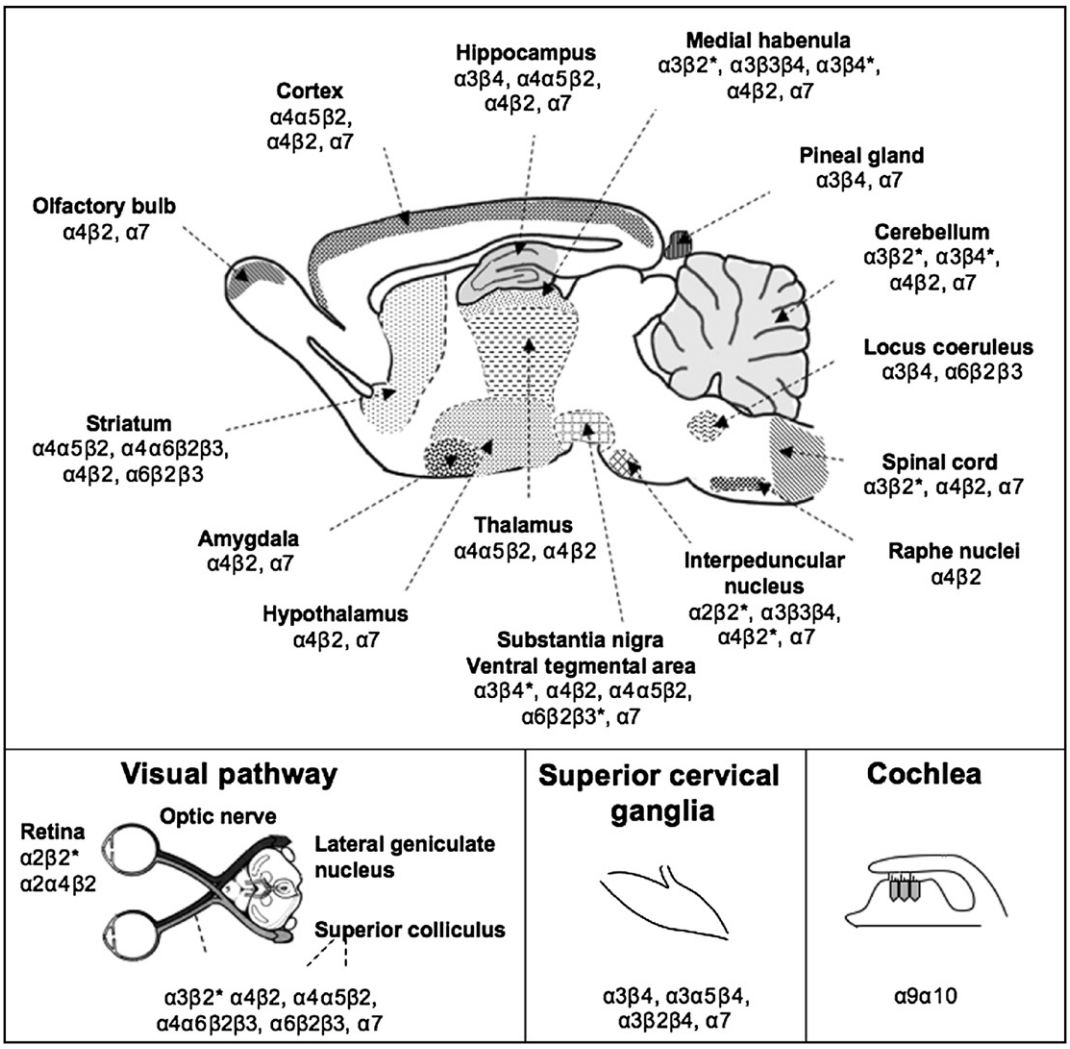

Fig. 2. Distribution of nAChR subtypes. Sagittal brain section showing nAChR subtypes expressed in different areas of the rodent central nervous system (upper panel). In addition, nAChRs expressed in the visual pathway, superior cervical ganglia and cochlea are illustrated (lower panel). The nAChR subtypes shown in the cortex, cerebellum, hippocampus, interpeduncular nucleus, medial habenula, pineal gland, superior cervical ganglia (see Table 2 for the references) have been deducted from binding, immunoprecipitation and/or immunopurification assays in tissues from rat and/or from wild-type/knock-out mice. The nAChR subtypes shown in the amygdala, hypothalamus, locus coeruleus, olfactory bulb, raphe nuclei, spinal cord, substantia nigra-ventral tegmental area, thalamus and cochlea have been deduced on the basis of the results of *in situ* hybridisation, single cell PCR, binding studies or functional assay of tissues obtained from rat and/or wild-type/knock-out mice (as reviewed in Picciotto et al., 2001; Drago et al., 2003; Gotti and Clementi, 2004; Jensen et al., 2005).

$\alpha3\beta2^*$ ($\alpha3\alpha5\beta2^*$ and $\alpha3\beta2\beta3^*$) subtypes expressed in the visual pathway (Moretti et al., 2004; Gotti et al., 2005b; Marritt et al., 2005). The $\alpha3\beta4$ subtype is expressed in the pineal gland (Hernandez et al., 2004), cerebellum and retina (Marritt et al., 2005; Turner and Kellar, 2005), whereas $\alpha3\beta4^*$ subtypes are present in the cerebellum and retina (as $\alpha3\beta2\beta4^*$ and $\alpha3\alpha5\beta4^*$) (Moretti et al., 2004; Marritt et al., 2005; Turner and Kellar, 2005) and in the habenulo-interpeduncular pathway (as $\alpha3\beta3\beta4$) (Gotti et al., 2006b). As mentioned above, $\alpha3\beta4^*$ receptors, either with the subunit composition $\alpha3\beta4$, or associated with the $\alpha5$ and/or $\beta2$ subunits, are the major heteromeric subtypes expressed in autonomic and sensory ganglia (Vernallis et al., 1993; Conroy and Berg, 1995; Mao et al., 2006).

The expression of $\alpha2^*$-containing receptors is very limited in rodents, being restricted primarily to the retina and interpeduncular nucleus (Drago et al., 2003), but is more widespread in primates, where the $\alpha2$ subunit has been detected (in the $\alpha2\alpha5\beta2$ subtype) in the monkey (Quik et al., 2005) and human cortex (Gotti et al., 2006a). An $\alpha2\alpha4\beta2$ subtype is expressed at high level in the chick optic lobe and is strongly developmentally regulated (Balestra et al., 2000).

Binding studies with α-bungarotoxin have enabled the localization and purification of the $\alpha7$-containing receptors from the brain of various vertebrate species, confirming that these receptors are homopentameric complexes in rat (Drisdel and Green, 2000). In avian species there is evidence that $\alpha7$ can co-assemble the $\alpha8$ subunit (Gotti et al., 1997; Lindstrom, 2000). Note, $\alpha8$-containing receptors have been identified only in the chick nervous system, where they form both homomeric and heteromeric $\alpha7\alpha8$ receptors (Anand et al., 1993; Keyser et al., 1993; Gotti et al., 1997). As was mentioned earlier, there is evidence that the $\alpha7$ subunit can form functional heteromeric nAChRs in heterologous expression systems when co-assembled with the $\beta2$ subunit (Khiroug et al., 2002) and there are some initial reports from studies in rat brain which indicate that similar $\alpha7$-containing heteromeric nAChRs may exist *in vivo* (Liu et al., 2007). Interestingly, an $\alpha7$ gene incorporating a unique 87-base pair cassette exon has recently been identified and immunolocalisation studies suggest that this subtype may constitute a distinct subset of $\alpha7$ receptors (Severance et al., 2004).

The $\alpha9$ and $\alpha10$ subunits are expressed in cochlea hair cells (Elgoyhen et al., 1994, 2001), where they have a role in auditory processing, and are probably co-assembled into heteromeric $\alpha9\alpha10$

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nAChRs (Elgoyhen et al., 2001; Katz et al., 2004; Vetter et al., 2007). There is also evidence that α9α10 nAChRs are involved in processing of inflammatory pain, a finding which may be related to evidence for the expression of this receptor subtype in tissues such as dorsal root ganglia and lymphocytes (Vincler et al., 2006). In addition, there is evidence that α7 nAChRs play an important role in inflammatory responses (Wang et al., 2003; Ulloa, 2005). Indeed, there is increasing evidence that several 'neuronal' nAChR subunits are expressed in non-excitable cells (as has been reviewed by Sharma and Vijayaraghavan (2002), Grando (2008) and Wessler and Kirkpatrick (2008)). There is evidence, for example, of nAChR expression in epithelial cells (Summers et al., 2003), microvascular endothelial cells (Saeed et al., 2005), lung fibroblasts (Roman et al., 2004), macrophages (Matsunaga et al., 2001), T lymphocytes (Kawashima and Fujii, 2000) and B lymphocytes (Skok et al., 2003).

In general, the distribution of nAChR subtypes appears to be relatively conserved amongst different vertebrate species, although some differences are apparent. For example, as was discussed earlier, α2 subunit mRNA is more widely distributed in primate brain than it is in rodents, suggesting that $\alpha2\beta2^*$ nAChRs may be more abundant subtype in primates (Han et al., 2000). A potentially powerful approach to identifying species-specific differences in the distribution of nAChR subtypes is immunolocalization. Unfortunately, however, difficulties associated with non-specific binding of some currently available antibodies have restricted the extent to which such techniques have been able to provide this information (Jones and Wonnacott, 2005; Moser et al., 2007).

8. Rules governing nAChR assembly and subunit stoichiometry

Functional studies have shown that heteromeric neuronal receptors consisting of a simple pair-wise $\alpha + \beta$ subunit combination are capable of generating functional nAChRs, but studies of native subtypes have shown that more complex receptors (with up to four different subunits) are present *in vivo*. Understanding such diversity is important since the presence or absence of particular subunits can influence the biophysical, pharmacological and functional properties as well as receptor assembly and/or trafficking.

The stoichiometry and subunit composition of neuronal nAChRs may depend critically on the transcriptional or translational regulation of subunit expression. A conclusion which is supported by comparing the results obtained in heterologous systems and native tissue. It has been shown, for example, that the α4β2β3 and α4α5β2 subtypes are formed efficiently in heterologous expression systems (Kuryatov et al., 2008) whereas the former has never been determined *in vivo* and the latter present only in limited amount. It is also possible that observations such as this may be explained by some subunits exerting a dominant-negative effect on assembly, as has been proposed for the β3 subunit (Broadbent et al., 2006).

The α4, α5, α6, β2, and β3 subunits are all expressed in mesostriatal dopaminergic cells (Klink et al., 2001), but only the α4α5β2, α4α6β2β3, α4β2, α6β2 and α6β2β3 subtypes have been biochemically and functionally identified (Zoli et al., 2002; Salminen et al., 2004, 2007). The apparent absence of, for example, the α4β2β3 and α5α6β2 subtypes *in vivo* may be due to the limited amount of the β3 subunit, the greater propensity of the β3 subunit to assemble with α6 than α4 (and of α5 to assemble with α4). A greater propensity of β3 to assemble with α6 is also suggested by recent findings with β3 knock-out mice in which $\alpha6^*$ receptor expression was decreased in the dopaminergic cell bodies in the midbrain and more markedly in their terminal field in the striatum (Gotti et al., 2005a). This decrease suggests that the β3 subunit is important for the formation of the majority of $\alpha6\beta2^*$ or $\alpha4\alpha6\beta2^*$ receptors and that its loss causes defects in nAChR assembly, degradation and/or trafficking.

As discussed above, studies of dopaminergic cells within striatum suggest that there may be as many as five different nAChR subtypes (α4α5β2, α4α6β2β3, α4β2, α6β2 and α6β2β3) expressed in a single cell. It is unclear what the advantage might be (if any) of such nAChR diversity. These different subtypes may, perhaps, allow very fine control of dopamine release, or their presence in different nerve terminals or cellular subdomains may favour their association with other receptors or ion channels that modulate the release of dopamine (Quarta et al., 2007).

An additional level of complexity in studying the function of heteromeric nAChRs is the recent finding that both subunit composition and stoichiometry can generate subtypes with a distinct physiology and pharmacology (Zwart and Vijverberg, 1998; Nelson et al., 2003; Moroni et al., 2006; Tapia et al., 2007). Recent studies have shown that cortical and thalamic nAChRs in heterozygous ($\alpha4^{+/-}$ and $\beta2^{+/-}$) mice have different relative expressions of α4 and β2 subunits, and that this correlates with differences observed in the functional properties of native nAChRs (Gotti et al., 2008), a finding which supports the conclusion that α4β2 nAChRs with different stoichiometries are expressed in native tissue.

9. Conclusion

Despite the considerable diversity of receptor subtypes within the family of nAChRs, a picture is now emerging of the extent of this diversity and of the influence of subunit composition upon physiological and pharmacological properties. Important goals for the future will be to understand in greater detail the rules governing receptor assembly and the roles played by distinct nAChR subtypes in cellular signalling and in disease.

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