

Neuronal nicotinic receptors: from structure to pathology

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Abstract

Neuronal nicotinic receptors (NACHRs) form a heterogeneous family of ion channels that are differently expressed in many regions of the central nervous system (CNS) and peripheral nervous system. These different receptor subtypes, which have characteristic pharmacological and biophysical properties, have a pentameric structure consisting of the homomeric or heteromeric combination of 12 different subunits ($\alpha 2$ – $\alpha 10$, $\beta 2$ – $\beta 4$).

By responding to the endogenous neurotransmitter acetylcholine, NACHRs contribute to a wide range of brain activities and influence a number of physiological functions. Furthermore, it is becoming evident that the perturbation of cholinergic nicotinic neurotransmission can lead to various diseases involving nAChR dysfunction during development, adulthood and ageing. In recent years, it has been discovered that NACHRs are present in a number of non-neuronal cells where they play a significant functional role and are the pathogenetic targets in several diseases. NACHRs are also the target of natural ligands and toxins including nicotine (Nic), the most widespread drug of abuse.

This review will attempt to survey the major achievements reached in the study of the structure and function of NACHRs by examining their regional and cellular localisation and the molecular basis of their functional diversity mainly in pharmacological and biochemical terms. The recent availability of mice with the genetic ablation of single or double nicotinic subunits or point mutations have shed light on the role of nAChRs in major physiological functions, and we will here discuss recent data relating to their behavioural phenotypes. Finally, the role of NACHRs in disease will be considered in some details.

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Abbreviations: Abs, polyclonal antibodies; β -AP, β -amyloid protein; α Bgtx, α Bungarotoxin; α Bgtx-nAChRs, α Bgtx-sensitive neuronal nicotinic receptors; 6OHDA, 6 hydroxydopamine; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; ADHD, attention deficit hyperactivity disorder; ADNFLE, autosomal dominant frontal lobe epilepsy; CNS, central nervous system; α CntxMII, α conotoxin MII; DA, dopamine; dLGL, dorso lateral geniculate nucleus; Epi, epibatidine; Kin, knock in; Ko, knock out; LPS, endotoxin polysaccharide; MLA, methyllycaconitine; NA, noradrenaline; nAChRs, non α Bgtx-sensitive neuronal nicotinic receptors; NACHRs, neuronal nicotinic receptors; Nic, nicotine; PD, Parkinson's disease; PET, positron emission tomography; SCG, superior cervical ganglion; SCLC, small-cell lung carcinoma; SHR, spontaneously hypertensive rats; VOCCS, voltage operated calcium channels; VTA, ventral tegmental area; WT, wildtype

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1. Introduction

The cholinergic system is one of the most important and filogenetically oldest nervous pathways. Acetylcholine (ACh) is the neurotransmitter that is synthesised, stored and released by cholinergic neurons, and the key molecules that transduce the ACh message are the cholinergic muscarinic and neuronal nicotinic acetylcholine receptors (NACHRs). NACHRs are widely expressed in the nervous system, where they transduce cholinergic transmission at the synapses in the peripheral ganglia and in various brain areas. In the central nervous system (CNS), the cholinergic innervation acting via NACHRs regulates processes such as transmitter release, cell excitability and neuronal integration, which are crucial for network operations and influence physiological functions such as arousal, sleep, fatigue, anxiety, the central processing of pain, food intake and a number of cognitive functions (Changeux and Edelstein, 2001; Gotti et al., 1997a; Hogg et al., 2003; Lindstrom, 1997; McGehee and Role, 1995; Role and Berg, 1996). Furthermore, it is becoming evident that the perturbation of cholinergic nicotinic neurotransmission can lead to various diseases involving NACHR dysfunction during development, adulthood and aging (Changeux and Edelstein, 2001; Gotti et al., 1997a; Hogg et al., 2003; Lindstrom, 1997).

This review will attempt to survey the major achievements reached in the study of the structure and function of NACHRs by examining their regional localisation and the molecular basis of their functional diversity mainly in pharmacological and biochemical terms. The recent availability of mice with the genetic ablation of single or double nicotinic subunits (knock out, Ko) or a single gene mutation (knock in, Kin), have shed light on the role of NACHRs in major physiological functions and we will here discuss recent data relating to their phenotypes. We will draw the attention of the reader on the relatively new discovery of NACHRs in non-neuronal cells and we will discuss their relevance in physiology and pathology of the tissues where they are present. Finally, the role of nAChRs in pathology will also be considered. For the specific aspects of nAChR physiology, cell biology, pharmacology and pathology that are not covered by this review, we would like to draw the attention of readers to various excellent reviews (Arneric and Holladay, 2000; Changeux and Edelstein, 2001; Corringer et al., 2000; Dajas-Bailador and Wonnacott, 2004; Decker et al., 2004; Dwoskin and Crooks, 2001; Hogg et al., 2003; Lester et al., 2003; Picciotto, 2003; Picciotto et al., 2001).

1.1. Molecular structure of NACHRs

NACHRs are a family of cationic channels consisting of different subtypes, each of which has a specific pharmacology, physiology and anatomical distribution in brain and ganglia. They belong to the gene superfamily of ligand-gated ion channels (of which muscle AChRs are the

prototype), which also includes gamma aminobutyric acid (GABA_A and GABA_C), glycine and 5-hydroxytryptamine (5-HT₃) receptors (reviewed in Changeux and Edelstein, 1998; Karlin, 2002; Le Novere and Changeux, 1995).

1.1.1. Two classes of NACHRs

Earlier studies designed to characterise NACHRs were based on binding assays with nicotinic radioligands in different brain areas reviewed in (Lukas and Bencherif, 1992). These demonstrated that at least two distinct classes of putative NACHRs exist in the nervous system: one consisting of receptor molecules that bind ³H-agonists with nM affinity but not α Bungarotoxin (α Bgtx) (from now on called nAChRs), and the other that bind the agonists with μ M affinity and α Bgtx with nM affinity (from now on called α Bgtx-nAChRs).

The pharmacological heterogeneity of NACHRs revealed by these ligand studies was later confirmed and extended by means of the molecular cloning of a family of genes encoding various subunits. Twelve genes coding for NACHR subunits have so far been cloned and, like all of the other members of the ligand-gated ion channel superfamily, they encode for peptides that all have a relatively hydrophilic extracellular amino terminal portion, followed by three hydrophobic transmembrane domains (M1–M3), a large intracellular loop, and then a fourth hydrophobic transmembrane domain (M4) (reviewed in Hogg et al. (2003); Sargent (1993)). These subunits have a common ancestor, have been highly conserved during evolution, and the same subunit has more than 80% amino acid identity across vertebrate species Fig. 1A (Le Novere and Changeux, 1995).

The genes that have been cloned so far are divided into two subfamilies of nine neuronal α subunits (α 2– α 10) and three β subunits (β 2– β 4) (Le Novere and Changeux, 1995; Lindstrom, 2000). The α subunits have two adjacent cysteines that are homologous to those present at positions 192 and 193 of the α 1 subunit of muscle-type AChRs whereas the β subunits (β 1– β 4) lack the pair of adjacent cysteines (reviewed in Le Novere and Changeux, 1995; Changeux and Edelstein, 1998). Both α and β subunits contribute towards the pharmacological specificity of NACHR subtypes (Luetje and Patrick, 1991).

On the basis of their different phylogenetic, functional and pharmacological properties, the heterogeneous family of NACHR subtypes have been divided into two main classes: the α Bgtx-nAChRs, which may be homomeric (made up of α 7– α 9 subunit homo-pentamers) or heteromeric (made up of α 7, α 8 or α 9, α 10 subunit heteropentamers), and the nAChRs, which contain the α 2– α 6 and β 2– β 4 subunits, and only form heteromeric receptors that bind agonists with high affinity (reviewed in Lindstrom, 2000).

1.1.2. The ligand binding sites

It is presumed that both homomeric and heteromeric nAChRs have a pentameric structure with the subunits

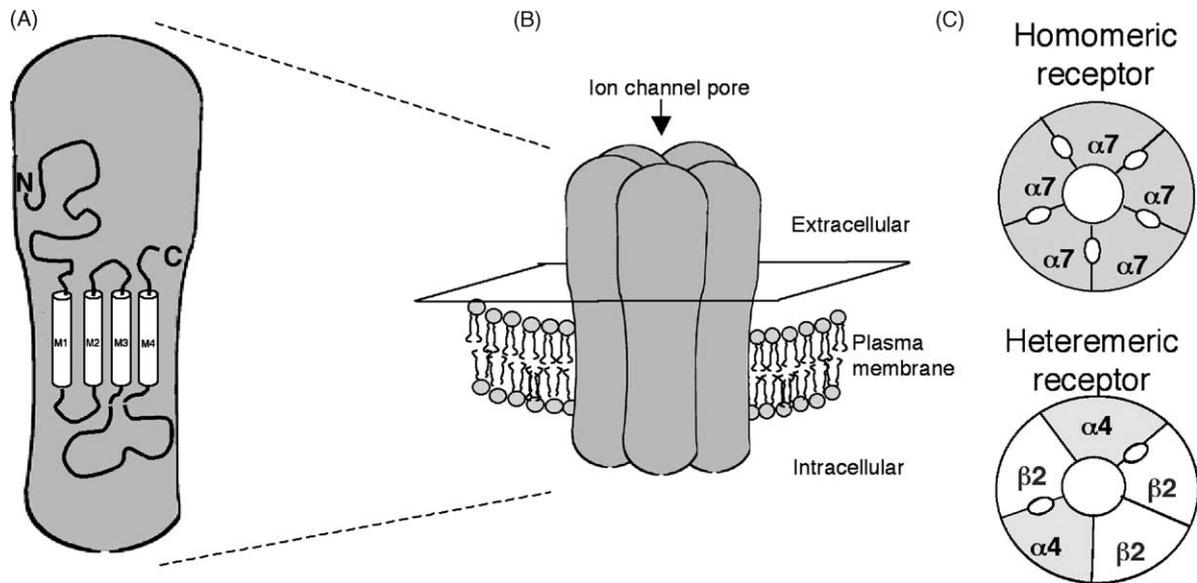


Fig. 1. (A–C) Organisation and structure of NACHRs. (A) Schematic representation of the putative transmembrane topology of NACHR subunits. The model shows the extracellular amino terminal portion, followed by three hydrophobic transmembrane domains (M1–M3), a large intracellular loop, and then a fourth hydrophobic transmembrane domain (M4). (B) Pentameric arrangement of nAChR subunits in an assembled receptor. (C) Subunit arrangement in the homomeric $\alpha 7$ and heteromeric $\alpha 4\beta 2$ subtypes, and localisation of the ACh binding site.

organised around a central channel: the homo-oligomeric receptors have five identical ACh-binding sites per receptor molecule (one on each subunit) located at the interface between two adjacent subunits, whereas the hetero-oligomeric receptors have two α subunits and three β subunits and therefore two binding sites per receptor molecule located at the interface between the α and β subunits see (Fig. 1B and C). The ACh binding site has a principal and a complementary component. In heteromeric nAChRs, the principal component is carried by the $\alpha 2$ – $\alpha 4$ and $\alpha 6$ subunits with the complementary site carried by the $\beta 2$ or $\beta 4$ subunits, whereas each subunit in the homomeric receptors contributes to both the principal and complementary components of the binding site (Changeux and Edelstein, 1998; Corringer et al., 2000) (see Fig. 1C). Notwithstanding their initial classification in the α and β subunit list, respectively, $\alpha 5$ and $\beta 3$ subunits carry neither the principal nor the complementary component of ACh binding site and are therefore considered auxiliary subunits (see below).

A significant contribution to the identification of the ligand binding site in NACHRs was made by the crystal structure of the acetylcholine binding protein from the fresh water snail *Lymnaea stagnalis*. This homopentameric soluble protein is 210 residues long, binds ACh, is secreted by snail glial cells into cholinergic synapses (Brejc et al., 2001; Smit et al., 2001) and is analogous to the extracellular ligand binding domain of the NACHRs. Structural data of the crystallised acetylcholine binding protein have revealed that the topology of the binding sites is very similar to that predicted by mutations and computer modelling.

1.1.3. NACHR transition states

Functionally, the different NACHR subtypes can exist in four distinct conformations: resting, open, and two ‘desensitised’ closed channel states (I or D) that are refractory to activation on a timescale of milliseconds (I) or minutes (D), but have a high affinity (pM–nM) for agonists. The binding of ligands to the receptors at the neurotransmitter binding site or in any of the allosteric sites can modify the equilibrium between the different conformational states of the receptors. Moreover, the transition between the different receptor states can also be regulated by receptor phosphorylation, as has been shown in the case of muscle-type receptors (reviewed in Changeux and Edelstein, 1998).

1.2. α Bgtx-nAChRs

In heterologous systems, the expression of the $\alpha 7$ – $\alpha 9$ subunits alone produces homomeric receptor channels activated by ACh and blocked by nanomolar concentrations of α Bgtx with high Ca^{2+} permeability and a rapid desensitisation rate. The $\alpha 7$ -containing subtypes account for most of the high affinity α Bgtx binding sites in the central and peripheral nervous systems of different species. The $\alpha 8$ -containing receptors are only present in the chick nervous system, where they not only form homomeric receptors, but also heteromeric $\alpha 7$ – $\alpha 8$ receptors (Gotti et al., 1994; Keyser et al., 1993). The $\alpha 9$ -containing receptors are expressed extraneuronally and have an unusually mixed nicotinic-muscarinic pharmacological profile (Elgoyhen et al., 1994). $\alpha 7$ -Containing receptors have been found in many brain regions and are especially concentrated in the hippocampus, where they can presynaptically facilitate the

release of transmitters such as glutamate or GABA (Alkondon et al., 1996; MacDermott et al., 1999), or exert a direct postsynaptic action by mediating fast synaptic transmission (Jones et al., 1999; MacDermott et al., 1999). The $\alpha 7$ -containing receptors are also found in perisynaptic locations, where they modulate other inputs to neurons and activate a variety of downstream signalling pathways (Berg and Conroy, 2002; Shoop et al., 1999). The $\alpha 7$ -containing receptors on autonomic ganglia are involved in fast synaptic transmission despite their perisynaptic localisation (Dajas-Bailador and Wonnacott, 2004; MacDermott et al., 1999).

The $\alpha 10$ subunit is similar to the $\alpha 9$ subunit by amino acid sequence (Elgoyhen et al., 2001; Sgard et al., 2002), but oocyte injections of the mRNA encoding the $\alpha 10$ subunit alone or in combination with mRNA encoding for the $\alpha 2$ – $\alpha 6$ subunits or $\beta 2$ – $\beta 4$ subunits give no detectable currents. A new current that is distinct from that of the homomeric $\alpha 9$ receptors was detected only when mRNAs coding for the $\alpha 9$ and $\alpha 10$ subunits were coinjected. This new current has functional and pharmacological properties that are indistinguishable from those of the endogenous cholinergic receptors present in cochlear hair cells, which have transcripts for both the $\alpha 9$ and $\alpha 10$ subunit genes (Elgoyhen et al., 2001; Sgard et al., 2002).

1.2.1. Homomeric or heteromeric receptors?

Recent studies have shown that the $\alpha 7$ subunit can also form functional channels with the subunits of nAChRs. This has been shown in oocytes in which a mutated form of the chick $\alpha 7$ subunit ($^{L247T}\alpha 7$) co-assembles with the $\beta 3$ subunit (Palma et al., 1999), and a rat $\alpha 7$ subunit co-assembles with the $\beta 2$ subunit when expressed in heterologous systems (Khiroug et al., 2002). Heteromeric $^{L247T}\alpha 7\beta 3$ receptors have less ACh affinity and a faster desensitisation rate than $^{L247T}\alpha 7$ receptors, whereas the heteromeric $\alpha 7\beta 2$ receptors form channels with higher ACh affinity, a slower desensitisation rate, and pharmacological properties that are different from those of the $\alpha 7$ homomeric channel (Khiroug et al., 2002; Palma et al., 1999).

No biochemical evidence of the presence of $\alpha 7$ heteromeric receptors *in vivo* is yet available, but multiple functional $\alpha 7$ -containing subtypes (some of which have a slower desensitisation rate and reversibly bind α Bgtx) have been described in rat hippocampal interneurons, the intracardiac ganglion, the superior cervical ganglion (SCG) and chick sympathetic neurons (Alkondon et al., 1997; Cuevas and Berg, 1998; Cuevas et al., 2000; Yu and Role, 1998b) thus suggesting that these tissues may contain heteromeric $\alpha 7$ receptors or alternatively transcribed $\alpha 7$ subunit.

1.2.2. Calcium permeability

Studies of native $\alpha 7$ receptors have confirmed that they are as highly permeable to calcium as NMDA receptors but, unlike the latter, do not require depolarisation of the plasma membrane to promote calcium influx. It is likely that the high degree of Ca^{2+} permeability underlies most of their

functions: Ca^{2+} influx can facilitate transmitter release when presynaptic $\alpha 7$ receptors are activated, depolarises postsynaptic cells and acts as a second messenger to initiate many cell processes, including those promoting neuronal survival (Messi et al., 1997; Role and Berg, 1996). The effects of the Ca^{2+} entering through $\alpha 7$ receptors are limited by a rapid receptor desensitisation, that prevents the excitotoxicity of an excessive influx, which is mainly due to a 247 leucine residue located in the second transmembrane region. The substitution of the leucine residue responsible for desensitisation with threonine greatly changes the functional and pharmacological properties of the $\alpha 7$ subtype ($L247T$), leading to a receptor with higher ACh affinity, a reduced desensitisation rate, and no ionic current rectification (Revah et al., 1991). $\alpha 7$ receptor functions are also modulated by divalent cations (including Ca^{2+} , Zn^{2+} , Mg^{2+} , Pb^{2+} , Cd^{2+}) interacting with a site located in the 160–174 region at the N terminal of the $\alpha 7$ subunit of homomeric receptors, potentiates the ACh-induced response (Hogg et al., 2003; McGehee and Role, 1995), and extracellular Ca^{2+} modulates both the activation and deactivation of $\alpha 7$ receptors in cultured hippocampal neurons (Bonfante-Cabarcas et al., 1996).

1.3. nAChRs

Although the functional and pharmacological properties of the subtypes expressed in heterologous systems may be influenced by the type of cells in which they are expressed (Lewis et al., 1997) much of our knowledge concerning the electrophysiological and pharmacological properties of nAChR subtypes comes from these systems. Various functional nAChR subtypes can be generated by injecting neuronal mRNAs or cDNAs encoding $\alpha 2$ – $\alpha 4$ or $\alpha 6$ subunits in pairwise combinations with $\beta 2$ or $\beta 4$ subunits. These different subtypes (i.e. $\alpha 2\beta 2$, $\alpha 3\beta 2$, $\alpha 4\beta 2$, $\alpha 6\beta 2$, $\alpha 2\beta 4$, $\alpha 3\beta 4$, $\alpha 4\beta 4$ and $\alpha 6\beta 4$) have different biophysical and pharmacological properties, some of which may match those of native nAChRs (Gotti et al., 1997a). Both the α and β subunits determine the pharmacological and functional properties of the expressed subtype: when expressed with the $\beta 2$ subunit the $\alpha 2$ – $\alpha 4$ and $\alpha 6$ subunits all form channels that vary in their average open times, single channel conductance, agonist and antagonist sensitivity. β Subunits appear to regulate the rate at which agonists and antagonists bind and dissociate from the subtypes, and the pharmacological sensitivity of nAChRs (Papke, 1993).

1.3.1. Receptor stoichiometry

nAChRs are pentamers, but the stoichiometry of many nAChRs remains to be fully elucidated. Biochemical and electrophysiological approaches have shown that both chick $\alpha 4\beta 2$ (Anand et al., 1991; Cooper et al., 1991) and human $\alpha 3\beta 4$ subtypes (Boorman et al., 2000) have a stoichiometry of 2 α and 3 β when expressed in oocytes injected with cRNAs or cDNAs in a ratio of 1/1 (α/β). However, more

recent studies have shown that different classes of functional $\alpha 4\beta 2$ subtypes are formed in oocytes when the rat $\alpha 4/\beta 2$ subunit ratio is varied. When the ratio of $\alpha 4/\beta 2$ is 1:9, the subtypes generated are more sensitive to activation and desensitise more slowly but, when the ratio is 1:1 or 9:1, the $\alpha 4\beta 2$ subtypes are less sensitive to activation and desensitise more rapidly (Zwart and Vijverberg, 1998).

HEK cells stably transfected with the $\alpha 4\beta 2$ subtype have a large majority of receptors with low ACh affinity and slow desensitisation but, when the cells are transiently transfected with the $\beta 2$ subunit, exposed overnight to nicotine (Nic) or kept at a low temperature (29 °C), there is an increase in the number of receptors that are more sensitive to activation (Nelson et al., 2003). Metabolic labelling of these cells with ^{35}S methionine has shown that the receptors have a stoichiometry of ($\alpha 4$)₃ and ($\beta 2$)₂, but long-term exposure to Nic or to low temperature increases the number of receptors with a high affinity for Nic and with a stoichiometry of ($\alpha 4$)₂ and ($\beta 2$)₃.

The results of all these studies clearly indicate that the stoichiometry of heterologous subtypes is not only dictated by their cDNA, but also by their relative ratio and possible pharmacological treatments. However, it is not yet known whether this plasticity also exists in neurons in vivo and plays a role in mammalian brain, or whether more stringent rules govern the assembly of the subtypes in native neurons.

1.3.2. The role of $\alpha 5$ and $\beta 3$ subunits

A further complexity of the structure of nAChRs is demonstrated by the subtypes containing the $\alpha 5$ and $\beta 3$ subunits. Neither the $\alpha 5$ nor the $\beta 3$ subunits can form functional channels when co-expressed with another α or β subunit, which is why they were long referred to as “orphan subunits”. They only form functional channels when are co-expressed with both α and β subunits (Lindstrom, 2000). The chick $\alpha 5$ subunit forms a functional $\alpha 4\beta 2\alpha 5$ subtype when co-expressed with the $\alpha 4$ and $\beta 2$ subunits, and this subtype (in which the $\alpha 5$ subunit participates directly in the lining of the channel) has properties distinct from those of the $\alpha 4\beta 2$ subtype, with a higher Nic-gated conductance, open probability desensitisation rate and Ca^{2+} permeability, and a higher half-maximal effector concentration (EC_{50}) for nicotinic agonists (Ramirez-Latorre et al., 1996). When expressed with the $\alpha 3$ and $\beta 2$ subunits, $\alpha 5$ increases sensitivity to ACh, but this effect is not seen when the $\beta 2$ subunit is replaced by $\beta 4$ (Wang et al., 1996). Conversely, the presence of the $\alpha 5$ subunit increases Ca^{2+} permeability and the rate of desensitisation in both $\alpha 3\beta 2$ and $\alpha 3\beta 4$ subtypes. In chick sympathetic neurons, the deletion of the $\alpha 5$ subunit alters the sensitivity of native receptors to both agonist and antagonists (Yu and Role, 1998a).

When co-expressed with the human $\alpha 3$ and $\beta 4$ subunits, a mutated form of the human $\beta 3$ subunit ($\beta 3^{\text{V273T}}$) forms functional channels in oocytes whose pharmacological and biophysical properties are different from those of the $\alpha 3\beta 4$ combination (Groot-Kormelink et al., 1998). These recep-

tors have a subunit stoichiometry of 2($\alpha 3$), 2($\beta 4$), and 1($\beta 3$) when the injected cRNA have a ratio of 1:1:20.

All these studies together indicate that both the $\alpha 5$ and $\beta 3$ subunits (known as auxiliary subunits) do not directly participate in the formation of the ligand binding site at the interface of α and β subunits, and may occupy a position comparable to that of the muscle $\beta 1$ subunit in assembled receptors. They may have a role in controlling ion permeability and perhaps receptor localization.

1.3.3. nAChR and calcium homeostasis

NACHR expression studies have also demonstrated that both heteromeric and homomeric receptors have two important properties: (a) they are not only permeable to monovalent cations but also to Ca^{2+} ; and (b) they are functionally modulated by changes in extracellular Ca^{2+} regardless of any increase in intracellular Ca^{2+} . In neurons, NACHRs activation can play a relevant role in Ca^{2+} homeostasis and signalling not only because of the Ca^{2+} entry through different NACHR subtypes, but also because NACHR depolarisation of the plasma membrane can activate voltage operated calcium channels (VOCCs) and increase intracellular Ca^{2+} , and this may induce Ca^{2+} mobilisation from intracellular stores. The absolute quantity and strategic localisation of Ca^{2+} entry through NACHRs is likely to be relevant for the regulation of calcium-mediated events such as transmitter release, cell excitability, gene expression, cell differentiation and survival (reviewed in Bonfante-Cabarcas et al., 1996; Dajas-Bailador and Wonnacott, 2004; McGehee and Role, 1995). The quantity of Ca^{2+} in the different neuron microdomains depends on the receptor subtypes and their Ca^{2+} permeability that varies and changes depending on the different subunit combinations. The $\text{Ca}^{2+}:\text{Na}^{+}$ permeability ratio of the heteromeric subtypes obtained using different α/β combinations is in the range of 0.1:1.6, but close to 10:20 for the homomeric $\alpha 7$ or $\alpha 9$ receptors (Fucile et al., 2003).

Moreover, a recent technique relying on the simultaneous recording of fluorescent Ca^{2+} signals and transmembrane currents has given a more direct estimate of the Ca^{2+} current (in this case referred to as fractional current, Pf) through NACHRs. These studies have confirmed that homomeric $\alpha 7$ receptors have a higher Ca^{2+} current (a Pf of 6–12% depending on the species) than nAChRs (Pf of 2–5%) (Fucile et al., 2003). This technique has also confirmed that the incorporation of additional subunits in heteromeric receptors can change Ca^{2+} permeability, as in the case of the $\alpha 5$ subunit in the $\alpha 3\beta 4$ subtype, whose presence greatly increases the calcium permeability of the subtype (reviewed in Fucile et al. (2003)).

2. NACHR localisation

2.1. Brain cholinergic system

In order to clarify the functions of NACHRs especially in brain, it is useful to summarise the most important

cholinergic pathways in which NACHRs act as transducer molecules. The brain cholinergic system is made up of a series of closely connected subsystems consisting of eight major and largely overlapping groups of cells, with the dendrites of one cell contacting those of many others; furthermore, gap junctions and dendrodendritic synapses are relatively common. However, each cell innervates a discrete area and tends to establish its own discrete connections. Although individual cholinergic neurons receive only a small number of innervating fibers, the fact that these come from different areas of the brain means that each cholinergic subsystem receives a large and complete set of sensory-based information. It can therefore be postulated that this pattern of extensive interconnections may lead to the coordinated firing of a group of contiguous neurons, and hence to the activation of different cholinergic subsystems (see for a review Mesulam and Geula (1988); Mesulam et al. (1989); Woolf (1991)).

The major cholinergic subsystems are:

Magnocellular basal complex: This represents the most significant group of cholinergic neurons, which provide the greatest cortical and hippocampal input. They were identified by Meynert as large neuronal cells (30–50 μm) that constitute 5–10% of all of the neurons in the sub-system.

Pedunculopontine-laterodorsal tegmental complex: This is the second most important cholinergic complex in brain. The cholinergic neurons are concentrated in the pedunculopontine tegmental nucleus and project to the thalamic nuclei and midbrain dopamine (DA) neurons.

Striatum: This is densely innervated by cholinergic fibers that originate from the intrinsic cholinergic neurons constituting approximately 1% of all striatal neurons and do not project beyond the borders of the striatum.

Lower brain stem: Cholinergic neurons are present in the brainstem reticular formation and spinal intermediate grey matter that innervate the superior colliculus, cerebellar nuclei and cortex.

Habenula–interpeduncular system: This system consists of cholinergic neurons mainly located in the medial habenula that project to the interpeduncular nucleus through the habenula–interpeduncular tract or fasciculus retroflexus. The habenula receives inputs from the thalamus via the stria terminalis and therefore is an important station through which the limbic system can influence the brainstem reticular formation.

Autonomic nervous system: The preganglionic neurons in both the sympathetic and parasympathetic systems are cholinergic. The parasympathetic preganglionic cells are located in a number of nuclei in the encephalic trunk and segments S2–S4 of the spinal cord; they project long neurites to the parasympathetic ganglia located in or near the target organs. The preganglionic neurons of the sympathetic system are located in a column in the intermediolateral grey matter of the spinal cord extending from T1 to L3. They

project to the paravertebral sympathetic ganglia and each fibre can innervate different ganglia.

NACHRs are present in neural and non-neural cells in brain and other organs, thus indicating their pleiotropic role in physiology and pathology.

2.2. *Methods of study*

The distribution of NACHRs in brain and other tissues has been hampered by the difficulty of identifying them, particularly their subtypes or subunits. The distribution of subunit mRNA can be studied by means of in situ hybridisation with selective subunit-specific probes. In the case of NACHR protein distribution, the tools are less selective:

- 1) Labelled nicotinic agents can be used to study NACHR distribution in homogenates of discrete brain areas (Fels et al., 1982) or histological brain sections (Adem et al., 1989); this method is very sensitive, but its specificity for NACHR subtypes is not very high because no subtype-specific ligands are yet available: $^3\text{H-Nic}$ and $^3\text{H-Epi}$ label all NACHRs (Gerzanich et al., 1995; Gotti et al., 1997a); $^3\text{H-Cyt}$ labels the receptors containing the $\alpha 3$, $\alpha 4$ and $\beta 2$ or $\beta 4$ subunits (Anderson and Arneric, 1994); $^3\text{H-}\alpha\text{conotoxin}$ (αCntx)-MII labels $\alpha 3$ and $\alpha 6$ containing receptors (Nicke et al., 2004); and $^{125}\text{I-}\alpha\text{Bgtx}$ labels $\alpha 7$ – $\alpha 9/10$ receptors (reviewed in (Gotti et al., 1997a).
- 2) Immunohistochemistry or immunopurification from selected brain areas with subunit-specific antibodies (Abs) give good results in terms of specificity and sensitivity, but the available Abs should be used with caution as not all of them have been carefully characterised for receptor selectivity and many of them work in a specific way only in immunoprecipitation assays, Western blots or immunolocalisation. In order to study the structure of native NACHRs, we have recently prepared a series of polyclonal Abs for each receptor subunit that specifically recognise all of the known subunits in chick, rodent and man. The specificity of these Abs has been tested qualitatively in Western blots and quantitatively in immunoprecipitation experiments using receptors labelled by nicotinic ligands present in transfected cells or tissue obtained from Ko mice. These Abs, together with radiolabelled nicotinic ligands and tissues obtained from the different areas of the nervous system have been used to quantify the expressed receptors and/or immunopurify different subtypes (see Section 2.3).
- 3) In vivo mapping using PET and specifically prepared nicotinic ligands is the only non-invasive method that can be used in humans; however, it needs to be further improved in terms of ligand specificity, and time and space resolution (Paterson and Nordberg, 2000).

2.3. Brain localisation of nAChRs

Despite the limitations indicated above, it is possible to draw a tentative map of brain nAChR distribution.

2.3.1. Rodent distribution

Nicotinic receptor distribution in rodent brain has been well known for some years on the basis of data obtained from binding studies using radioactive nicotinic drugs, in situ hybridisation and immunohistochemistry. Only recently has the availability of subunit-specific Abs allowed us to map the receptor subunit localisation in different brain areas see (Fig. 2). The most important findings emerging from these studies and careful binding studies (Ferreira et al., 2001; Lena et al., 1999; Perry et al., 2002) are that the most diffuse receptor subtype in the brain is $\alpha 4\beta 2$, which can also contain $\alpha 5$ and the $\alpha 3\beta 4$ receptors, with or without $\alpha 5$ are present not only in autonomic ganglia but also in pineal gland, the anteroventral nucleus of the thalamus, the subiculum of the hippocampus, the medial habenula and interpeduncular nucleus, spinal cord and retina. The $\alpha 6$ containing receptors, very often in conjunction with the $\beta 3$ subunit, are present in the optic pathway, the locus coeruleus and dopaminergic neurons of the mesostriatal pathways, where they control dopamine release. The $\alpha 7$ -containing receptor is also a rather diffuse receptor subtype particularly

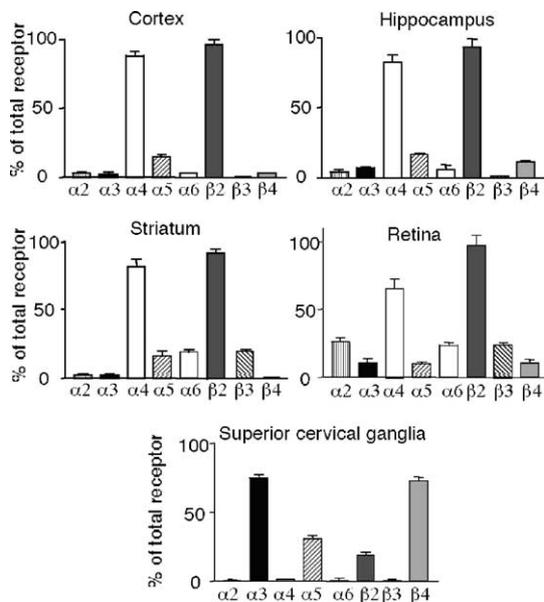


Fig. 2. Analysis of nAChR subunit content in different nervous system areas. Immunoprecipitation analysis of ^3H -Epi labelled nAChRs expressed in rat cortex, hippocampus, striatum, retina and superior cervical ganglia. The Triton X-100 tissue extracts and immunoprecipitation were carried out as previously described (Zoli et al., 2002) using saturating concentrations of subunit-specific antibodies. Two Abs were used for each subunit (except $\alpha 2$): one directed against a cytoplasmic peptide and the other against a COOH peptide. The level of Ab immunoprecipitation is expressed as the percentage of ^3H -Epi labelled receptors immunoprecipitated by the indicated antibodies, taking the amount of receptors present in the Triton X-100 extract solution before immunoprecipitation as 100%.

in the hippocampus, hypothalamus, cortex and motor nucleus of the vagus nerve whereas the $\alpha 9/\alpha 10$ -containing receptors are present extraneuronally in limited areas such as the pituitary pars tuberalis, the olfactory epithelium and the cochlea.

The detailed subcellular distribution of receptor subtypes is not easy to discover as the majority of Abs are not suitable for immunolocalisation studies. However, in some areas (such as the mesostriatal pathways and retina), the combination of immunoprecipitation, functional and degeneration techniques has made it possible to construct a putative but reliable map of the localisation of receptor subtypes in the soma and synaptic boutons of the different neurons present in the area (Champtiaux and Changeux, 2002; Champtiaux et al., 2003; Klink et al., 2001; Salminen et al., 2004; Zoli et al., 2002). Below, we describe in detail two studies as an example of the possibilities offered by combining immunochemical methods with biochemical and pharmacological analyses.

2.3.2. nAChRs in retina

During embryonic development, chick retina expresses a high level of both $\alpha\text{Bgtx-nAChRs}$ and nAChRs labelled by ^3H -Epibatidine (Epi) and their number increases respectively ten- and six-fold from embryonic day 7 (E7) to postnatal day 1 (P1). Retinal $\alpha\text{Bgtx-nAChRs}$ mainly contain $\alpha 7$ at E7, but there is a subsequent increase in the number of $\alpha 8$ -containing receptors, which are present in both homomeric and heteromeric $\alpha 7$ – $\alpha 8$ subtypes on P1 (Gotti et al., 1994, 1997b; Keyser et al., 1993).

Like other brain regions (i.e. optic lobe and forebrain–cerebellum), the nAChRs expressed in E7 retina are those containing the $\alpha 4\beta 2$ subunits but, by E11, there is an increase in $\alpha 3$ -, $\beta 3$ - and $\beta 4$ -containing receptors. Affinity purification of E11 $\beta 2$ - and $\beta 4$ -containing retinal receptors showed that both populations contain the $\alpha 3$ and $\alpha 4$ subunits, but the $\alpha 4$ subunit is mainly associated with the $\beta 2$ subunit and $\alpha 3$ with the $\beta 4$ subunit; the $\beta 3$ subunit is present in a similar fraction of both types of receptors. After E14, there is a considerable increase in the receptors containing the $\alpha 6$ and $\alpha 2$ subunits, which reached a peak by P1 when both the $\beta 2$ - and $\beta 4$ -containing subtypes are heterogeneously associated with the $\alpha 2$, $\alpha 3$, $\alpha 4$ and $\alpha 6$ subunits (Vailati et al., 1999, 2000, 2003).

Immunopurification studies of P1 $\alpha 6$ -containing receptors show that they have a complex subunit composition (with the $\alpha 6$ subunit being associated with the $\beta 4$, $\beta 3$, $\alpha 3$ and $\beta 2$ subunits) and a particular pharmacology insofar as they are specifically labelled with high affinity by $\alpha\text{CntxMII}$ and methyllycaconitine (MLA). The $\alpha 6$ -containing receptors are only present in the retina but not in its target tissue optic tectum (Balestra et al., 2000b), which expresses the $\alpha 4\beta 2$ subtype and a developmentally regulated $\alpha 2\alpha 5\beta 2$ subtype, whereas forebrain and cerebellum coexpress the $\alpha 4\alpha 5\beta 2$ and $\alpha 4\beta 2$ subtypes as also previously reported by Conroy and Berg (1998).

Similar studies of rat retina have shown the presence of a high level of nAChRs and α Bgtx-nAChRs at birth. However, during the post-natal period, the increase in nAChRs is much higher and they become predominant during postnatal development and adulthood. This increase in rat retinal nAChRs is due to selective increases in the receptors containing the α 2, α 4, α 6, β 2 and β 3 subunits.

Immunopurification experiments on P21 rat retina have shown that it contains a relatively wide range of different nAChR subtypes grouped into three populations: α 6*, α 4 (non- α 6)* and (non- α 4–non- α 6)*, which respectively represent 26, 60 and 14% of the total P21 nAChRs (Moretti et al., 2004). We do not know the localisation of these subtypes, but in situ hybridisation experiments have shown that nAChR subunit mRNAs have highly heterogeneous distribution patterns throughout the retinal layers.

The large majority of nAChRs in developing and adult rat retina contain the β 2 subunit; this prevalence of β 2-containing receptors appears to be mammal-specific as Keyser et al. (2000) also found them in adult rabbit retina.

These studies suggest that, although the temporal pattern of expression and principal subtypes expressed are species-specific, vertebrate retina shows increased nAChR heterogeneity and complexity during development that is also maintained in adulthood. Moreover, α CntxMII is the only available tool capable of discriminating between the α 6 nAChR subtypes expressed in both species.

2.3.3. nAChRs in striatum

In agreement with other pharmacological and functional studies using α 3, α 4, α 6 or β 2 Ko mice (Marubio and Changeux, 2000; Zoli et al., 1998; Champtiaux and Changeux, 2002; Champtiaux et al., 2003; Salminen et al., 2004; Whiteaker et al., 2002) (see below), we have biochemically and pharmacologically identified two principal nAChR populations in rat and mouse striatum: one contains the α 4 and β 2 subunits, but not the α 6 subunits (α 4 β 2*), and accounts for approximately 70% of the nAChRs; the other contains the α 6 and β 2 subunits, and accounts for approximately 20% of the striatal receptors. These two populations can only be distinguished pharmacologically by α CntxMII and MLA which have a low (μ M) affinity for the α 4 β 2*, but both low (μ M) and high (nM) affinity for the α 6 β 2* receptors.

In addition to dopaminergic terminals, the striatum also contains a number of non-dopaminergic cell structures. We used denervation with the neurotoxin 6-hydroxydopamine (6OHDA), which is selective for dopaminergic neurons, to distinguish the nAChR subtypes expressed by the different structures. On the basis of the changes in subunit content observed in 6OHDA denervated striatum, we concluded that α 4 β 2 nAChRs are expressed by both dopaminergic and non-dopaminergic cell types, whereas α 6 β 2 β 3, α 4 α 6 β 2 β 3 and α 4 α 5 β 2 nAChRs are only expressed by dopaminergic terminals, and α 2 α 4 β 2 nAChRs only by non-dopaminergic cell types.

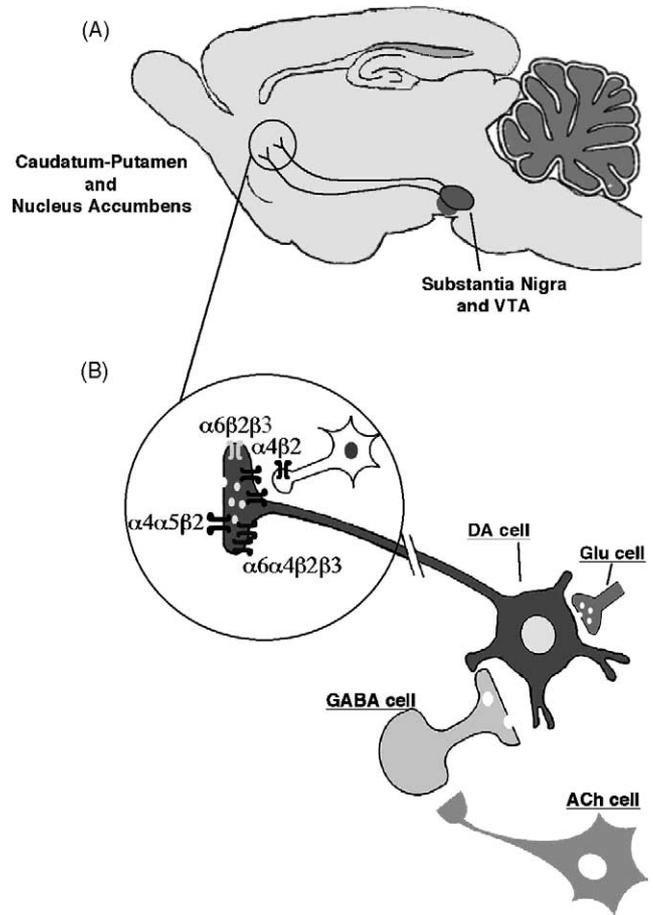


Fig. 3. nAChR subtypes in the striatum. (A) Scheme of the mesostriatal dopaminergic pathway. (B) Subunit composition of the functional nAChRs expressed by dopaminergic nerve terminals (α 6 β 2 β 3, α 6 α 4 β 2 β 3, α 4 β 2, α 4 α 5 β 2).

The subtypes identified in striatum are summarised in (Fig. 3).

While the functional data obtained from oocytes show that simple two-subunit nAChRs would be sufficient to assure a nicotinic response to a target cell, the studies of retina and striatum show that often native nAChRs contain more than one type of α or β subunit and are therefore structurally homologous to muscle AChRs (reviewed in Changeux and Edelman (1998); Corringer et al. (2000)). These data showing that native subtypes can consist of up to four different subunits demonstrate that the number of biologically relevant receptor subtypes is larger than it was previously thought. This fact has important functional implications because the presence of a certain subunit can modify the localisation and/or pharmacological and/or functional properties of native receptors.

2.3.4. Primate distribution

Two classes of 3 H-Nic binding sites have so far been described: a high affinity site with a K_d of 5.5 nM (range 2–18 nM), and a low affinity site with a K_d of 80 nM. nAChRs are present in a variety of brain structures, especially in the

Table 1
Distribution of nicotinic receptors in primate brain (human “+”, monkey “0”)

	³ H-Nicotine		³ H-Epiibatidine		¹²⁵ I- α Bgtx	
	+	0	+	0	+	0
Cortex						
Occipital	+	0	+	0	+	0 0 0
Fronto-parietal	++	0	++	0		0 0 0
Forebrain						
Putamen-caudate	+++	0	++	0 0	++	
Hippocampus	++		+		+++	
CA pyramid lay			+			0 0
Subiculum	++	0 0	++	0 0		0
Presubiculum		0		0 0 0		0 0
Thalamus	+++		+++		+	
Lateral geniculatus	+++	0 0 0		0 0 0	+	0 0 0
Anterior		0 0 0 0				0 0 0
Dorsal		0 0 0		0 0 0		0 0
Reticular		0 0		0 0	++	0 0 0
Hypothalamus		0		0	+	0 0
Pineal gland						0 0 0
Medial habenula						0 0 0 0
Mesencephalon						
SN	+++	0		0 0	+++	0
VTA		0		0		
Superior colliculus		0 0		0 0 0		0 0
Interpeduncular nucleus		0		0 0 0 0		
Cerebellum			++		+	
Molecular lay	+		++		++	
Granular lay	++		+		+	
Dentate nucleus	+					

thalamic, neocortical and striatal regions. The number of receptors in these areas varies from 80 to 4 fmol/mg of protein (with a mean value of 27 fmol/mg), or from 7 to 0.8 fmol/g of tissue.

Tables 1–3 show the most relevant areas in which NACHRs are located as described in the literature, taking into account the results obtained by binding of radioactive ligands and by immunolocalisation using subunit-selective antibodies. The description of subunit distribution is still at the beginning for the reasons mentioned above. In general, the described regional distribution agrees well with the results of in vivo PET studies (Nordberg, 2000).

Tables 2 and 3 show the distribution of subunit mRNA in human brain (Breese et al., 1997; Graham et al., 2003; Paterson and Nordberg, 2000). Receptor distribution is very similar among primates (Han et al., 2000, 2003), although there are important differences that are difficult to analyse in detail because the number of subjects studied is limited, the techniques used are not always comparable, and in situ studies with immunohistochemistry or immunoprecipitation have to be completed using well-controlled Abs.

On the basis of the results of in situ hybridisation studies, it seems that the most important and diffused receptor

subtype in human brain is the α 4 β 2, but the α 4 β 4/ β 2 subtype is also present in important parts such as the striatum, hippocampus habenula and cortex; unlike rodent brain, primate brain has a substantial presence of α 2 β 2, which may be an important subtype in particular brain regions (Han et al., 2000). There is also a selective but important distribution of the subtype containing α 6 β 3 subunits in the mesencephalic nuclei, particularly in the monoaminergic neurons.

α 7 Receptors are present in the autonomic ganglia, hippocampus and thalamic nuclei (reticular, geniculate), and moderately dense in cerebellum, the pituitary and pineal glands, and cortex. The α 9/10 subtypes are mainly expressed in the cochlea, as in other animal species. Analysis of the tables in which selected receptor subunit distribution is reported as protein or mRNA shows that there is a discrepancy between the relative distribution of protein and mRNA. It is important to recognise a discrepancy between protein and mRNA expression as several times distribution in subunit mRNA is taken as subunit distribution. This discrepancy could be due to the fact that subunits may be synthesised in the neuronal soma in one brain area and transported to other locations in presynaptic structures, or that the different subunits have a different turnover and assembly rate, or that mRNA for the different subunits have a different stability or transcription efficiency.

2.4. Pre- or postsynaptic distribution?

Anatomical and functional evidence suggests that NACHRs are preferentially located at the presynaptic boutons regulating neurotransmitter secretion in several parts of the brain (see Wonnacott, 1997; Wonnacott et al., 1995). In particular, presynaptic NACHRs have been implicated in the release of ACh (Wilkie et al., 1993), noradrenaline (NA) (Clarke and Reuben, 1996), DA (Grady et al., 1992; Rapier et al., 1990; Wonnacott et al., 1990), glutamate (McGehee and Role, 1995; Alkondon et al., 1997) and GABA (Yang et al., 1996). Very recently, a method has been developed to detect presynaptic NACHRs in neurons in vitro (Girod et al., 2003). In rodents and monkeys, some attempts have been made to determine the subtypes present in presynaptic boutons and it has been found that their subunit composition varies in different brain areas.

In addition to controlling and modulating the release of various neurotransmitters, presynaptic NACHRs can play other roles under particular conditions (i.e. denervation and development), such as path finding and neuritogenesis (Role and Berg, 1996; Zheng et al., 1994).

There is evidence that high affinity Nic binding NACHRs are located on postsynaptic membranes in the somatodendritic regions of various brain areas (Clarke, 1993), and that Nic can elicit a cell response through postsynaptic neuronal receptors (de la Garza et al., 1987). The fast synaptic current in autonomic ganglia is mediated through α 3 β 4/ α 5

Table 2
Nicotinic receptor subunit mRNA distribution in human brain

	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 7$	$\beta 2$	$\beta 3$	$\beta 4$
Cortex			[1]			[1]	[1]
Frontal	[2, 3]	[2–7]		[2, 5, 6, 8, 9]	[10]		
Occipital		[4]		[8, 9]			
Temporal	[2, 11, 12]	[2, 4, 5, 13]		[2, 5, 8, 9, 12]	[11]		
Parietal	[2]	[2, 4]		[8, 9, 11]			
Insular	[13]	[4]			[13]		
Thalamus	[1, 12]	[1, 12]	[1]	[1, 12]	[1]	[1]	[1]
Anterior				[8]			
Lateral nuclei	[13, 14]			[8, 13]	[13]		
Dorsomedial	[13, 14]			[8, 9, 13]	[13]		
Lateral geniculate				[8]			
Reticular	[13, 14]			[8, 9]			
Midbrain	[1, 12]	[1, 12]	[1]	[1, 12]	[1]	[1]	[1]
Brainstem				[8]			
Hippocampus	[2, 11–13]	[2, 11–13]	[1]	[2, 11–13]	[1, 10, 11, 13]	[1]	[1]
Entorhinal Cx	[14]	[4, 13]		[9, 13]	[13]		
Subiculum	[13, 14]	[4]		[13]	[13]		
Dentate gyrus	[13, 14]	[4]		[9, 13]	[13]		
Basal ganglia	[1, 12]	[1, 12]	[1]	[1, 12]	[1]	[1]	[1]
Caudate	[11, 12]	[11]		[11, 13]*	[11, 13]		
Putamen	[13]	[4, 14]		[8, 13]	[13, 15]		
Globus pallidus		[13]		*			
Cerebellum	[2, 6]	[2, 4, 6]	[1]	[1, 2, 6]*		[1]	[1]

This table is updated from Graham, A.J., et al., 2002. *Curr. Drug. Targets CNS Neurol Disord* 1, 387–397. In situ hybridisation and RT-PCR studies are included (tissue from prenatal to elderly individuals). Where no specific sub-region is defined, either the whole region was homogenised for RT-PCR analysis or no area was specified. Asterisk (*) indicates that $\alpha 7$ mRNA was observed in deep cerebellar nuclei but not in cerebellar cortex, globus pallidus or caudate [8]. References in table are as follows:

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- Wevers, A., et al., 1994. *Brain Res. Mol. Brain Res.* 25, 122.
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- Martin Ruiz, C.M., et al., 2000. *Neuropharmacology* 39, 2830.
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- Martin-Ruiz, C., et al., 2002. *Neurosci. Lett.* 335, 134.
- Graham, A., et al., 2002. *Neuroscience* 113, 493.

Table 3
Nicotinic receptor subunit protein expression in human brain*

	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 6$	$\alpha 7$	$\beta 2$	$\beta 3$	$\beta 4$
Cortex								
Frontal		[7, 14]			[16, 17]			
Temporal	[11]	[11, 16, 18–23]			[16, 18, 20–24]	[11, 19]		
Parietal		[25]				[25]		
Midbrain								
		[19]				[19]		
Hippocampus								
Entorhinal Cx	[11, 26]	[11, 26]			[11, 26]	[11, 26]		
Pyramidal cells	[28]	[28]			[28]	[28]		[28]
Dentate gyrus	[28]	[28]			[28]	[28]		[28]
Basal ganglia								
Putamen	[27, 29]	[27, 29]	[29]	[29]	[27, 29]	[27, 29]	[29]	
Caudate	[11]	[11]			[11]	[11]		
Cerebellum								
	[30]	[30]		[30]	[30]	[30]		[30]

This table is updated from Graham, A.J., et al., 2002. *Curr. Drug. Targets CNS Neurol Disord* 1, 387–397. For references in table, please see legend of Table 2.

* From immunohistochemical (describing neuronal immunoreactivity) and Western blot studies.

subtypes. In agreement with a postsynaptic localisation of AChRs are the data of Del Signore et al. (2002, 2004) concerning the rodent cervical superior ganglion (SCG), and Horch and Sargent (Horch and Sargent, 1995) in chick ciliary ganglion, who found that mAb35-AChRs are located in both synaptic and perisynaptic sites on the surface of ciliary ganglion neurons, and that their activity is blocked by neuronal bungarotoxin (NBT) (Chiappinelli, 1983). Similarly, using a monoclonal antibody that recognises neuronal AChRs (WF6), Schroder et al. (1989) found that NACHRs in the cortex are located in the postsynaptic thickening. The location of α Bgtx binding sites corresponding to the human $\alpha 7$ subtype has been studied in man only at cellular level (Cimino et al., 1992), but the results of studies of rat and frog SCG (Smolen, 1983; Marshall, 1981), and chick ciliary ganglion (Horch and Sargent, 1995; Zhang et al., 1996) make it possible to suggest that they are located in the somata and dendrites; in rat CNS (Dominguez del Toro et al., 1994) they have been found at both synaptic (Marshall, 1981; Smolen, 1983) and non-synaptic sites, where they can transduce synaptic and/or regulatory signals. The α Bgtx-AChRs on chick ciliary ganglion are mainly located in patches around the synaptic sites (Horch and Sargent, 1995; Zhang et al., 1996). Even in this non-canonical location, α Bgtx-AChRs are capable of generating postsynaptic currents, thus indicating that the membrane domain regulating membrane potential is broader than previously thought. Moreover, it is possible to speculate that, in addition to controlling rapid communications between pre- and postsynaptic neurons, these receptors may regulate other cell functions by increasing Ca^{2+} influx (for example, neuroprotection).

The predominantly presynaptic localisation of NACHRs on nerve terminals containing different neurotransmitters, and the presence on the same boutons of different NACHR subtypes, is the basis of the pleiotropic effects of nicotinic drugs that modulate several pathways. This aspect is

therefore crucial in the development of selective nicotinic drugs for pharmacological and therapeutical purposes.

2.5. Specificity of primate nAChR distribution

Although the distribution of receptors in primates is still not completely known (particularly in the case of human receptors and the distribution of receptor subtypes), the available data suggest that, overall, it is not greatly different from that in rodents. However, there are some discrepancies: e.g. $\alpha 2$ mRNA is more widely distributed in primate than rodent brain and the binding of nicotinic drugs correlates well with this subunit distribution, whereas it is known that the main nicotinic binding site in rodents is due to $\alpha 4\beta 2$ receptors; $\beta 2$ is the most widely expressed subunit in rat (Wada et al., 1989; Zoli et al., 1995), whereas $\beta 2$ distribution in human brain is less ubiquitous; α Bgtx binding and $\alpha 7$ distribution is more diffuse in monkey than in rodent brain, thus suggesting that this receptor subtype plays an important role in monkey brain function. It is conceivable that future studies in this field will reveal further species-related differences. Knowledge of receptor subtype distribution is important in order to allow the correct correlations to be made between receptor subtypes and brain functions or pathologies, which would assist in creating valid animal models of human brain pathologies and finding subtype-specific therapeutic agents.

3. Changes during development and aging

NACHRs change considerably during development and aging in all animal species. The earliest detection of nAChRs, as mRNA or as ligand binding, is on E7 in chick (Vailati et al., 2003), E11 in rat (Zoli, 2000a,b) and after 5–7 weeks of gestational age in human brain (Zoli, 2000b). Studies of ^3H -Nic and ^{125}I - α Bgtx binding sites and mRNA

expression for the individual subunits during development have shown that they have different temporal and spatial behaviours.

3.1. nAChRs

In rat, the mRNA for the $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 6$, and the $\beta 2$ and $\beta 4$ subunits, are present from the early moments. Their brain distribution is not dissimilar from that of adult animals, with high intensity in the diencephalon, brainstem and spinal cord, and lower intensity in the telencephalic structures; however, the temporal expression of the individual subunits may vary because, for example, $\beta 4$ precedes $\alpha 3$ by two days, but they then colocalise. The postnatal behaviour of the subunits is particular in the sense that many of them (notably $\alpha 3$ and $\alpha 4$, $\beta 2$) are suppressed early in development and increase later in postnatal life (Cimino et al., 1994; Shacka and Robinson, 1998; Zoli, 2000b; Zoli et al., 1995). This temporal behaviour depends on the different brain structures: for example, in cortex and hippocampus, $\beta 2$ is low on P7 and increases by P14, whereas the expression of $\alpha 4$ is already high at P7.

In humans, high-affinity ^3H -Nic binding increases steadily during gestation (from 12 to 27 weeks), reaching higher levels than at any other time during life (Cairns and Wonnacott, 1988; Court and Clementi, 1995; Court et al., 1995). The highest concentrations have been observed in the nucleus basalis of Meynert and the tegmental nuclei, followed by the globus pallidus, the putamen, the cerebellar-relay nuclei, the parietal and cerebellar cortex, the thalamus and the spinal cord; the lowest level is in the medulla (Cairns and Wonnacott, 1988; Court et al., 1995; Kinney et al., 1993). During the perinatal period and early infancy, the concentration of NACHRs in the different brain areas decreases considerably, with the exception of the major cerebellar nuclei in which the concentration of receptors remains unchanged.

There is general agreement that Nic binding and the expression of subunit mRNA slightly decrease in rat during aging, although with some regional specificity. For example, $\alpha 4$ mRNA decreases in the thalamus but not in cortex, and the decrease in $\alpha 3$ is more marked than that of $\alpha 4$ and present in nearly all of the brain zones assayed except for the medial habenula (Cimino et al., 1994). The finding that there is marked neurodegeneration in $\beta 2$ Ko mice during aging indicates that $\beta 2$ -containing receptors are important for neuronal survival during aging (Zoli, 2000b).

There is general consensus that the concentration of Nic binding sites steadily decreases throughout life in nearly all of the studied human brain regions. The reported discrepancies are probably due to difficulties in comparing different clinical populations. One exception could be the thalamus, in which the binding sites remain constant or increase (Court and Clementi, 1995; Flynn and Mash, 1986; Nordberg et al., 1992). In the case of subunit distribution, there is a general agreement that the $\beta 2$ subunit decreases

with age in all brain structures, but $\alpha 4$ and $\alpha 3$ do not decrease in the hippocampus and putamen.

3.2. $\alpha\text{Bgtx-nAChRs}$

In rat fetal brain, ^{125}I - αBgtx binding sites and mRNA for $\alpha 7$ are present from E13 to E14 and increase until birth, after which, they decrease in the first days to adult expression levels. They are restricted to specific areas, such as the hippocampus and the dorsal motor nucleus of the vagus nerve (Tribollet et al., 2004). In human fetal brain, ^{125}I - αBgtx binding sites are present as early as 5–7 weeks of gestational age and subsequently increase steadily. They are high in the pons, medulla oblongata, mesencephalon, cerebellum and spinal cord (Falk et al., 2002).

After birth and throughout life (between 60 and 90 years of age), their concentration slowly decreases in the hippocampal CA1 region and the entorhinal cortex (Court and Clementi, 1995), the thalamus (Nordberg and Winblad, 1986), and the striatum (Schulz et al., 1993) but remains constant in the frontal cortex and cerebellum (Falk et al., 2002).

3.3. Relevance of NACHRs during ageing

These findings indicate that high affinity Nic and αBgtx binding sites are independently regulated among species during the development and aging of different brain areas. The different transcriptional regulation of NACHR subunits is probably due to gene promoter structure and different transcription factors, which may be different among subunit genes and brain areas (Gotti et al., 1997a). The described changes during development and aging are specific to NACHRs because, for example, the changes in muscarinic and glutamatergic receptors, as well as in choline acetyltransferase activity, follow a different pattern (see Court and Clementi (1995) for more details). The pattern of nAChR expression over time suggests that these receptors play a particular role during brain development because their concentration is high during the stage of synapse formation. In vitro experiments suggest that NACHRs (particularly $\alpha 7$) may control the development of neuronal architecture, stabilise synapse formation, and orient and control neurite outgrowth (Lipton and Kater, 1989; Pugh and Berg, 1994; Quik, 1995; Role and Berg, 1996; Zheng et al., 1994).

These data indicate that NACHRs are of great relevance in two critical periods for brain life: early pre- and perinatal circuit formation, and cell degeneration during aging. The use of nicotinic drugs in these two periods could therefore be very important. The exposure of fetal brain to Nic, as result of active or passive smoking of the mother or via breast milk, may greatly modify brain circuitry as it is known that the administration of nicotinic drugs at this stage can also increase the number of NACHRs (Narayanan et al., 2002) and particularly of some receptor subtypes, and that NACHRs are important in establishing synaptic connections

(Role and Berg, 1996). Furthermore, the use of nicotinic drugs to prevent or decrease the brain degeneration and consequent intellectual decline that occurs during aging is an extremely important and positive aim to pursue. However, the experimental and human data are still not complete and do not allow a rational intervention. Is it better to stimulate all of the receptor subtypes, or only the homomeric $\alpha 7$ (hippocampus) or those that are more decreased (the $\beta 2$ -containing receptors) or those that are expressed in particular regions ($\alpha 6\beta 3$ in dopaminergic regions)? These therapeutic dilemmas will be easier to approach when more information is available especially in human beings.

4. Non-neuronal localisations of NACHRs

ACh is probably the oldest signalling molecule, and appeared very early in evolution before nervous cells. It is present in bacteria, algae, protozoa and plants, and so it is not surprising that it is still involved in cell-to-cell communications in various non-neuronal tissues and controls important cell functions such as proliferation, adhesion, migration, secretion, survival and apoptosis in an autocrine, justacrine and paracrine manner (Grando et al., 1993; Sastry et al., 1979). Many of these functions are mediated through NACHRs on non-neuronal cells, and it is clear that they can play an important role.

4.1. Muscle

mRNAs coding for the $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 4$ and $\beta 2$ subunits have been found in vertebrate adult muscle (Corriveau et al., 1995; Maelicke et al., 2000; Sala et al., 1996), in a human muscle-like cell line (TE 671), and in neuron-free cultures of rat muscle. $\alpha 7$ is highly expressed in mammalian muscle during development and the perinatal period, and decreases later on in adult life. It is upregulated by denervation (Maelicke et al., 2000). The function of NACHRs in muscle is not known, but it is possible that they may control various metabolic and trophic functions, and perhaps gene expression in areas that will receive the incoming nerve fibres, by increasing Ca^{2+} influx. The innervating fibres contain presynaptic $\alpha 3\beta 2$ subtype receptors whose activation facilitates ACh release (Faria et al., 2003).

4.2. Lymphoid tissue

Nic binding sites are present in B-lymphocytes (Nordberg et al., 1990), and in circulating and thymic T lymphocytes (Paldi-Haris et al., 1990); and their number increases during aging (Nordberg et al., 1990). Ligand binding, RT-PCR, or Southern or Western blotting studies have shown that human T lymphocytes, and lymphocyte cell lines such as Jurkat, Molt4 and H9, as well as hypertrophic human thymi (Mihovilovic et al., 1993), express the $\alpha 3$, $\alpha 4$, $\alpha 7$, $\beta 2$ and $\beta 4$ receptor subunits (Kuo et al., 2002; Sharma and Vijayar-

aghavan, 2002); the receptor subtypes likely to be expressed are $\alpha 3\beta 4$, $\alpha 4\beta 2$ and $\alpha 7$. The thymus receptors are located in T cells and epithelial cells (Kuo et al., 2002); and, although their function in human lymphoid tissue is unknown, their location and modulatory effects on the proliferation of thymic and circulating T lymphocytes (Middlebrook et al., 2002; Richman and Arnason, 1979; Rinner et al., 1994) suggest that they may affect T cell proliferation, as well as thymic differentiation and selection processes. Functional NACHRs containing either $\alpha 4$ or $\alpha 7$ subunits are also present on B cells, where they stimulate growth and decrease antibody production (Skok et al., 2003). Lymphocytes possess cholinergic acetyltransferase, acetylcholinesterase (AChE) and vesicular acetylcholine transporter (Kawashima and Fujii, 2003), and so it is possible that ACh may activate nACHRs via an autocrine pathway. The presence of NACHRs in peripheral blood cells have spurred great interest in the possibility of investigating the response of brain NACHRs to diseases and drug treatments (Benhammou et al., 2000; Perl et al., 2003). The NACHRs of peripheral blood cells respond to drugs and are modified in some diseases, but too little is known about the mechanisms regulating the expression of central and peripheral receptors to be able to propose peripheral receptors as a reliable marker of brain receptors: for example, the promoter of the $\alpha 3$ human gene is differently regulated in neurons and lymphocytes (Battaglioli et al., 1998).

4.3. Macrophages

It has long been known that circulating phagocytic cells have NACHRs (Davies et al., 1982), but it has only recently become evident that human macrophages have $\alpha 7$ NACHRs, and that their activation by Nic reduces the release of $\text{TNF}\alpha$ and interleukins 1 and 6 induced by the endotoxin polysaccharide (LPS) (Wang et al., 2003a). It is also known that vagal stimulation inhibits the secretion of pro-inflammatory cytokines and reduces the inflammatory process, and that this does not occur in animals deficient of $\alpha 7$ receptors (Borovikova et al., 2000). Mice alveolar macrophages express the $\alpha 4\beta 2$, but not the $\alpha 7$ subtype and, also in this case, activation by Nic down-regulates the production of interleukins 6, 12 and $\text{TNF}\alpha$, thus allowing the proliferation of infecting bacteria (Matsunaga et al., 2001).

It has very recently also been shown that brain microglia have $\alpha 7$ receptors and that Nic inhibits LPS-induced $\text{TNF}\alpha$ release in microglial cells in vitro (Shytle et al., 2004). The presence of a cholinergic anti-inflammatory pathway in different tissues makes it easier to understand: (a) the paradoxical protective effect of smoking on immune-mediated lung diseases (e.g. immune alveolitis) and its negative effect on bacterial infection that leads to its positive association with active tuberculosis (Floto and Smith, 2003); (b) the anti-inflammatory activity of Nic in inflammatory bowel disease; and (c) the neuroprotective effect of Nic in degenerative diseases of the CNS associated with chronic

inflammation. These findings suggest a possible new therapeutic approach to a variety of inflammatory conditions.

4.4. Skin

Human epidermal keratinocytes express nAChRs that have the biophysical and pharmacological properties of an $\alpha 3$ subtype (Grando et al., 1995). Furthermore, the presence of $\alpha 3$, $\alpha 7$, $\alpha 9$, $\beta 2$ and $\beta 4$ subunits in these cells has been shown by means of antibody binding, RT-PCR experiments and Ca^{2+} influx (Grando, 2001; Zia et al., 2000). The $\alpha 3$ subunit is more expressed in small cells, localised in membranes forming cell junctions, and the $\alpha 7$ in large differentiated cells (Zia et al., 2000). NACHRs mediate several skin functions, particularly keratinocyte proliferation, apoptosis, differentiation, adhesion and motility, mainly by regulating Ca^{2+} influx in keratinocytes (Grando et al., 1995; Arredondo et al., 2002; Grando, 2001; Nguyen et al., 2003; Zia et al., 2000). As ACh is synthesised and released by human keratinocytes (Grando et al., 1993), and skin is also innervated by cholinergic fibers, ACh can activate NACHRs via paracrine-autocrine pathways. All of these nicotinic actions are important in controlling skin physiology and development, particularly the formation of an efficient skin barrier (Burkhart and Burkhart, 2001).

$\alpha 9$ is the target of pemphigus Abs (Grando, 2001) and it is known that smoking has a positive effect on pemphigus flaring (Wolf et al., 1998); furthermore, Nic can have positive effects on other dermatological diseases and wound healing.

4.5. Lung cells

ACh is synthesised, released and destroyed in various cells throughout human airways, as well as in effector cells of the lung immune system, and its non-neuronal cholinergic effects are mediated by muscarinic and NACHR (Wessler and Kirkpatrick, 2001). $\alpha 3$, $\alpha 5$ and $\alpha 7$ NACHR subunits are present in bronchial epithelial cells, $\alpha 4$ in alveolar epithelial cells (Zia et al., 1997) and $\alpha 4$, $\alpha 7$ and $\beta 2$ in neuroepithelial bodies (Fu et al., 2003). Various nicotinic receptor subunits ($\alpha 3$, $\alpha 5$, $\alpha 7$, $\beta 2$, $\beta 4$), α Bgtx binding sites and the capacity to synthesise, release and degrade ACh (Song et al., 2003) are also present in pulmonary neuroendocrine cells and in the human small-cell carcinoma cell lines derived from them (Chini et al., 1992; Cunningham et al., 1985; Maneckjee and Minna, 1990; Quik et al., 1994; Schuller et al., 2003; Song et al., 2003; Tarroni et al., 1992). Nicotinic stimulation in these cells induces the secretion of neurotransmitters and increases cell proliferation (Cattaneo et al., 1993; Klapproth et al., 1998; Quik et al., 1994; Schuller et al., 2003; Song et al., 2003). The presence of nAChRs in lung is important because the Nic in cigarette smoke reaches lung cells at high concentrations and may play a role in stimulating the growth of small-cell lung carcinoma, an extremely aggressive

tumour associated with tobacco abuse (Weiss, 1991). In a fetus exposed to maternal or passive smoking, Nic can alter lung development and cause lung hypoplasia with a reduction in the complexity of the gas-exchange surface (Sekhon et al., 1999). On the other hand, nAChRs may be a relevant pharmacological target in the case of proliferative, immunological and developmental diseases of the lung.

4.6. Vascular tissue

Nic is a potent stimulus of angiogenesis and increases proliferation in endothelial cells acting through NACHRs (Heeschen et al., 2001; Villablanca, 1998). The vascular system contains a number of nicotinic subunits in endothelial cells ($\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 10$, $\beta 2$, β and $\beta 4$) and vascular smooth muscle ($\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\alpha 10$) (Bruggmann et al., 2003; Macklin et al., 1998; Wang et al., 2001). Smooth muscle cells selectively express NACHR subtypes depending on the tissue localisation of the vessels. $\alpha 3$ and $\alpha 5$ are widely distributed among arteries but are not present in intrapulmonary or kidney vessels; $\alpha 4$ is not present in muscle, kidney or lung small arteries; $\alpha 7$ is widespread but lacking in the renal circulation. NACHRs are present in arteries devoid of cholinergic innervation, but it has been reported that endothelial cells can synthesise, release and degrade ACh (Kawashima et al., 1990). Although the *in vivo* function of arterial NACHRs is not yet known, it is possible to postulate that they may play an important role in controlling angiogenesis and smooth cell proliferation, thus suggesting the obvious therapeutic application of nicotinic drugs in arteriosclerosis, tumour growth, and revascularisation after ischemic insults. If the specific complement of NACHRs in individual arteries is confirmed, we can expect to exert possible selective interventions in particular organ vessels.

Brain endothelial cells, an important component of the blood–brain barrier, express the $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 3$ nicotinic subunits (Abbruscato et al., 2002). It is known that Nic alters the permeability of the blood–brain barrier, which could be mediated by a decrease in the expression of $\alpha 7$ and $\beta 2$ subunits, and of the tight junctional protein ZO-1 (Abbruscato et al., 2002). These findings suggest that NACHRs are involved in controlling this important function and should be borne in mind when the neuronal effects of smoking are considered, when drugs acting on the CNS are given to smokers, and when it is convenient to modify pharmacologically the permeability of the blood–brain barrier.

4.7. Astrocytes

The presence of NACHRs in astrocytes was first described many years ago (Hosli et al., 1988), but it has only recently been confirmed and studied in more detail. $\alpha 3$, $\alpha 4$, $\alpha 7$, $\beta 3$ and $\beta 4$ subunits have been detected in hippocampal astrocytes (Graham et al., 2003; Sharma and Vijayaraghavan, 2002), and $\alpha 7$ seems to be the most important. In mouse

strains, there is a correlation between the level of nAChRs in hippocampal astrocytes and the susceptibility of mice to Nic seizures (Gahring et al., 2004b). Some data indicate that $\alpha 4\beta 2$ receptors exist in glial processes, and $\alpha 3\beta 4$ receptors in glial soma (Graham et al., 2003). Spinal cord astrocytes also have a complement of $\alpha 3$, $\alpha 5$ and $\beta 2$ subunits (Khan et al., 2003). The real role, relevance and functions of these receptors in astrocyte life or astrocyte–neuron relationships are not known, but the presence of nAChRs in astrocytes is in line with an integrated view of synaptic activity as being not only confined to neuron–neuron interactions, but also consistently modulated by perisynaptic astrocytes. This view opens the way to the exploration of new pharmacological interventions aimed at influencing synaptic activity and modifying astrocyte function in pathologies with associated gliosis.

5. Studies of knock out and knock in mice

The use of genetically engineered Ko or Kin in which one or more genes of interest are silenced or mutated provides a unique opportunity to analyse the pharmacology and functional role of nAChRs in complex neurobiological systems.

The use of Ko mice may have some drawbacks because their lack of the subunit of interest may lead to some forms of adaptation during development, with the up- or down-regulation of some receptor subtypes that may confound the interpretation of behavioural changes. Only future studies of animals with the conditional or inducible Ko of individual subunits will help us to draw final conclusions concerning the role of individual subunits in behaviour.

The following nAChR subunits have so far been knocked out: $\alpha 3$ – $\alpha 7$, $\alpha 9$, $\beta 2$ – $\beta 4$. Only the $\alpha 3$ subunit appears to be necessary for survival as the mice lacking the other subunits are all viable and appear grossly normal. We here mainly review the recent results obtained in Ko and Kin mice (for more exhaustive review, see (Champtiaux et al., 2003; Cordero-Erausquin et al., 2000; Drago et al., 2003; Lester et al., 2003; Marubio and Changeux, 2000; Picciotto et al., 2000, 2001; Picciotto and Corrigan, 2002; Wang et al., 2002b).

5.1. Phenotype of $\alpha 3$, $\alpha 5$, $\beta 4$ Ko and $\beta 2$ – $\beta 4$ double Ko mice

The $\alpha 3$ subunit is highly expressed in the autonomic ganglia, but is also found in subsets of neurons in the medial habenula, dorsal medulla and retina. $\alpha 3$ Ko animals usually die in the first week of life due to multiorgan dysfunction, with impaired growth and increased mortality before weaning and a phenotype very similar to that of the double $\beta 2$ – $\beta 4$ Ko mice (Xu et al., 1999a,b). Both $\alpha 3$ Ko and double $\beta 2$ – $\beta 4$ Ko mice have an enlarged bladder and develop bladder infection, dribbling urination and urinary stones.

In the SCG, both the $\beta 2$ and $\beta 4$ subunits are associated with $\alpha 3$ subunits (Del Signore et al., 2002, 2004) and, in heterologous systems, both form functional channels with the $\alpha 3$ subunit (McGehee and Role, 1995). Nic-induced whole-cell current is abolished in the SCG neurons of $\alpha 3$ Ko and double $\beta 2$ – $\beta 4$ Ko mice but, although reduced, it is still present in the SCG neurons of $\beta 4$ Ko mice (Xu et al., 1999a,b). These results suggest that the $\alpha 3$ subunit may combine with the $\beta 2$ subunit in $\beta 4$ Ko mice, and this may be sufficient for functional compensation in the SCG.

Studies of $\beta 4$ Ko mice have shown that the deficiency affects ganglionic transmission and leads to an attenuated bradycardic response to high-frequency vagal stimulation, and significantly reduced ileal and bladder contractile responses to nicotinic agonists (Wang et al., 2001, 2003b). These and the SCG results together strongly support the hypothesis that the lack of the $\beta 4$ subunit impairs nicotinic conductance in both sympathetic and parasympathetic ganglia, and that ganglionic nAChR is formed at least by $\alpha 3$ and $\beta 4$ subunits.

Quantitative autoradiography studies of the brains of $\alpha 3$ Ko mice (Whiteaker et al., 2002) have shown that, unlike what was thought on the basis of the results of expression studies in heterologous systems (Cartier et al., 1996), the large majority of the high affinity α CntxMII binding sites is unchanged and there is only a reduction in the habenula interpeduncular tract, thus suggesting that this is the major site where the $\alpha 3\beta 2$ subtype is expressed and contributes to α CntxMII binding. These results, together with those relating to $\alpha 6$ Ko mice indicate that the large majority of the α CntxMII binding sites in rodent brain are $\alpha 6$ -containing receptors.

Studies of $\alpha 5$ Ko mice have revealed impaired cardiac parasympathetic ganglionic transmission and increased sensitivity to hexamethonium (Wang et al., 2002a). Moreover, although they have no visible phenotype with normal baseline behaviours, they are less sensitive to Nic-induced seizures and behaviours related to locomotor activity than WT mice (Salas et al., 2003). $\beta 4$ Ko mice are even more resistant to Nic-induced seizures than $\alpha 5$ Ko mice, and double $\alpha 5/\beta 4$ Ko mice are more resistant than either single Ko animal (Kedmi et al., 2004). Furthermore, both $\alpha 5$ and $\alpha 5/\beta 4$ Ko mice showed a significantly shorter latency time to seizures than WT mice.

5.2. Phenotype of $\alpha 4$ Ko and Kin mice

$\alpha 4$ Ko mice show dramatically reduced antinociceptive effects of Nic in hot-plate test (primarily brain-mediated pain response) and a slightly reduced analgesic response in tail-flick tests (primarily spinal cord-mediated pain response) (Marubio et al., 1999).

The same pattern is seen in $\beta 2$ Ko mice, although higher doses of Nic have a residual analgesic effect during both tests. Binding and electrophysiological studies of the thalamus and raphe magnus of $\alpha 4$ and $\beta 2$ Ko mice have

revealed the disappearance of high affinity agonist binding and the loss of Nic-evoked currents. On the contrary, the sensory neurons of the dorsal horn of the spinal cord showed a Nic-dependent increase in the current frequency, probably due to the presence of an $\alpha 3\beta 4$ subtype.

By acting through different nAChR subtypes, nicotine can decrease locomotor activity in a novel environment and increase locomotion via activation of dopaminergic pathways in a familiar environment. Marubio et al (1999, 2003) found no difference in basal locomotor activity between $\alpha 4$ wildtype (WT) and Ko mice in either environment, but an independent line of $\alpha 4$ Ko mice showed increased locomotion, sniffing and total rearing, and an increase in the basal level of anxiety (Ross et al., 2000).

This last effect is not surprising as rodent studies based on behavioural models of anxiety and depression have shown that Nic can have anxiolytic and antidepressant effects (Picciotto et al., 2002). However, as the large majority of $\alpha 4$ -containing receptors in the brain are associated with the $\beta 2$ subunit, the fact that no difference in anxiety was found in $\beta 2$ Ko mice is surprising, and can perhaps only be explained by differences in the genetic background of the $\alpha 4$ and $\beta 2$ Ko mice, or by the fact that a non- $\alpha 4\beta 2$ nAChR, present in $\alpha 4$ Ko but absent in $\beta 2$ Ko has ansiogenic effects (Picciotto et al., 1995).

Epidemiological evidence suggests that smoking can protect against Parkinson's disease (PD) (Fratiglioni and Wang, 2000), a hypothesis that has also been tested using $\alpha 4$ Ko mice in an animal model in which acute Nic pretreatment significantly inhibited methamphetamine-induced nigrostriatal neurodegeneration in WT but not $\alpha 4$ Ko mice (Ryan et al., 2001). The role of $\alpha 4$ -containing receptor in the neuroprotection of the dopaminergic system was also examined using $\alpha 4 9'S$ Kin mice (Labarca et al., 2001), which express nAChRs with a mutation in the transmembrane M2 domain of the $\alpha 4$ subunit that makes them more agonist sensitive. Even in the hemizygous state, the Kin mutation led to a late embryonic loss of mid-brain dopaminergic neurons. If the expression of this mutated subunit is decreased by the inclusion of a neomycin-resistant cassette in an intron, the mice survive, and electrophysiological recordings from mid-brain neuroprogenitor cells are hypersensitive to choline. This cell death was possibly due to persistent activation of mutated receptors induced by choline which, at low concentrations, is also an agonist of mutated ($\alpha 4 9'S$) $\beta 2$ receptors (Fonck et al., 2003). In brief, these studies indicate that the activation of $\alpha 4$ -containing receptors can be neuroprotective, but their hyperactivation can lead to neurodegeneration.

5.3. Phenotype of $\beta 2$ Ko mice

$\beta 2$ subunit was the first of the nAChRs subunit to be knocked down. The $\beta 2$ Ko mice have lost the vast majority of high affinity nAChRs and Nic-elicited currents in neurons from various brain areas, but no change in the level of

expression of $\alpha Bgtx$ -nAChRs (Picciotto et al., 1995). A large number of studies have used these mutated animals to examine the role of $\beta 2$ -containing nAChRs in learning and memory, neurodegeneration, drug reinforcement, nociception, the development of the visual system and the organisation of sleep.

5.3.1. Learning, memory and neuroprotection

Nicotinic transmission participates in many cognitive processes. Nic improves performances in various tasks involving spatial and associative learning, working memory and attention, whereas mecamylamine (Mec) (a general nicotinic antagonist) impairs memory performance (Levin, 2002). Behavioural tests have shown that $\beta 2$ Ko mice do not have the Nic-induced enhancement of passive avoidance performance that is thought to model learning and memory (Picciotto et al., 1995). Additionally, young $\beta 2$ Ko mice perform identically to WT mice in the Morris water maze test, a cognitive test that assesses the acquisition of spatial information, but 22–24 month old $\beta 2$ Ko mice show a significant deficit that indicates a major impairment in spatial memory (Zoli et al., 1999). Histological analyses of the brain of these aged $\beta 2$ Ko mice show region-specific cerebral cortex alterations with neocortical hypotrophy, a loss of hippocampal neurons, and astro- and micro-gliosis. $\beta 2$ Ko mice also have increased cortical susceptibility to ibotenic acid lesions, and primary cortical neurons obtained from them no longer have Nic-induced neuroprotection (Laudenbach et al., 2002).

These studies suggest that $\beta 2$ -containing receptors contribute to neuronal survival and the maintenance of cognitive performance during aging.

5.3.2. Drug reinforcement

It is believed that the reinforcement properties of Nic are due to its capacity to modulate the dopaminergic system. Like many drugs of abuse, Nic exerts its additive properties by increasing DA release in the ventral part of the mesostriatal dopaminergic pathway. This effect is partially due to the direct activation of nAChRs on the dopaminergic neurons of the VTA, because the direct infusion of nicotinic antagonists into the VTA prevents Nic-elicited DA release in the nucleus accumbens, and blocks systemic Nic self-administration in rats (Corrigall et al., 1994; Picciotto and Corrigall, 2002). nAChRs that release DA are also present in the terminal field of mesolimbic neurons, but the direct administration of nicotinic antagonists on the nucleus accumbens does not block the self-administration of Nic. Experiments performed in $\beta 2$ Ko mice have shown that somatic $\beta 2$ -containing receptors are required for the effects of Nic on the firing rate of DA neurons in vitro as well as for the release of DA from striatal synaptosomes (Champtiaux et al., 2003; Picciotto et al., 1998). Furthermore, $\beta 2$ Ko mice do not learn to self-administer Nic even if they have learnt to self-administer cocaine and WT mice self-administer both drugs (Picciotto et al., 1998). Overall, these studies reveal

that $\beta 2$ -containing nAChRs located in dopaminergic neurons are important for the regulation of DA and, by doing so, they are also important for mediating the addictive properties of Nic (see also Section 2.3).

5.3.3. Development of the nervous system

nAChRs are expressed very early in the nervous system, where they are not only finely regulated during CNS development but probably actively contribute to it. In the visual system of mammals, the refinement of the formation of eye-specific layers at thalamic level depends on retinal waves of spontaneous activity that rely on nAChR activation (Rossi et al., 2001; Champtiaux and Changeux, 2002). Between P1 and P10, spontaneous retinal waves are mediated by nAChRs and are sensitive to nicotinic antagonist blockade but, at about P10, the waves become insensitive to nicotinic antagonists and sensitive to glutamatergic antagonists (Bansal et al., 2000). $\beta 2$ Ko mice have retinal waves with altered spatiotemporal properties and retinofugal projections in the dorsolateral geniculate nucleus (dLGL) and superior colliculus that do not segregate into eye-specific areas (Rossi et al., 2001). Furthermore, recent anatomical and functional studies of dLGL $\beta 2$ Ko mice have revealed normal gross retinotopy but disrupted fine mapping, a loss of retinotopicity in the nasoventral visual axis, and a gain in on/off cell organisation (Grubb et al., 2003). $\beta 2$ Ko mice also have reduced visual acuity and functional expansion of the binocular subfield of the primary visual cortex (Rossi et al., 2001).

$\beta 2$ -containing receptors are necessary for the normal development of the visual system, but the α subunit that coassembles with the $\beta 2$ subunit to mediate these effects is unknown: the pattern of retinothalamic projection is normal in $\alpha 4$ and $\alpha 6$ Ko mice (two subunits highly expressed in the visual system) and, in $\alpha 3$ Ko mice, retinal waves are not abolished although have altered spatiotemporal characteristics (reviewed in Champtiaux and Changeux, 2002).

These visual pathway studies suggest that adequate nAChR activation in other brain areas during development may be essential for the anatomical and functional maturation of cerebral neuronal circuits.

5.3.4. Organisation of sleep

There is evidence in humans that mutations in $\alpha 4$ and $\beta 2$ subunits are correlated with seizures occurring during slow-wave sleep. The use of $\beta 2$ Ko mice has made it possible to demonstrate that nAChRs containing the $\beta 2$ subunit can influence the REM sleep, controlling the duration and onset of REM sleep episodes and the REM sleep-promoting effects of stress (Cohen et al., 2002; Lena et al., 2004). These findings, and the fact that $\beta 2$ -containing receptors are important in controlling the rhythms of breathing and arousal during sleep, should be kept in mind especially in newborns, who are at risk of sudden infant death syndrome

and in whom correct REM sleep can influence the forming neuronal circuits. This is a further stimulus to avoid the exposure of newborns to passive smoking.

5.4. Phenotype of $\alpha 6$ and $\beta 3$ Ko mice

In the CNS, the $\alpha 6$ and $\beta 3$ subunits colocalise in dopaminergic neurons and retina (Champtiaux et al., 2002; Moretti et al., 2004). Deletion of the $\alpha 6$ subunit does not change the level of mRNA for the $\alpha 3$ – $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ subunits in the different brain regions (Champtiaux et al., 2002) and, in $\beta 3$ Ko mice, no changes in the mRNA level for these and the $\alpha 6$ subunits have been found in the substantia nigra (SN) and ventral tegmental area (VTA) (Cui et al., 2003). In both $\alpha 6$ and $\beta 3$ Ko mice, the major finding is a global loss of high affinity α CtxMII binding in striatal nerve terminals and, in $\alpha 6$ Ko mice, there is also the disappearance of α CtxMII binding sites from retina, optic nerve and the retina-target tissues of the superior colliculus and dLGL (Champtiaux and Changeux, 2002). The absence of $\alpha 6$ -containing receptors in $\alpha 6$ Ko mice does not alter the anatomy of the dopaminergic (Champtiaux and Changeux, 2002; Cui et al., 2003) and visual pathways, and the organisation of the retinothalamic projections is preserved.

Biochemical and pharmacological studies of striatal extracts from WT and $\alpha 6$ Ko mice have identified two major population of nAChRs: $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ (all of which are involved in DA release from synaptosomes, Champtiaux et al., 2003). A subset of about 40% of the $\alpha 6\beta 2^*$ nAChRs in rat and WT mouse striatum (Champtiaux et al., 2003) also contain the $\alpha 4$ subunit, and competition binding experiments on immunomobilised $\alpha 6$ subtypes using α CtxMII have shown that the toxin biphasically displaced Epi with both high and low affinity (Fig. 4).

Identical experiments on $\alpha 6$ -immunomobilised nAChRs obtained from $\alpha 4^{-/-}$ striatum (which mainly contains the $\alpha 6$ and $\beta 2$ subunits) have shown that α CtxMII displacement of Epi binding is only monophasic with a single high-affinity site (Fig. 4). These pharmacological results clearly indicate that, in a fraction of WT striatal $\alpha 6^*$ receptors, one of the two Epi binding sites (located at the interface between the α and β subunits) is made up of an $\alpha 4\beta 2$ interface with a low affinity for α CtxMII. This binding site is absent from the $\alpha 6$ receptors present in the striatum of $\alpha 4$ Ko mice, which therefore have both binding sites with high affinity for the toxin (Fig. 4).

Electrophysiological studies of DA neurons of WT, $\alpha 4$, $\alpha 6$, and double $\alpha 4/\alpha 6$ Ko mice indicate that the major heteromeric functional subtype expressed in dopaminergic soma is $\alpha 4\beta 2$, which is probably the subtype that contributes to Nic reinforcement (Champtiaux et al., 2003).

The immunoprecipitation studies of WT striatal $\alpha 6$ -purified receptor, and the lack of α CtxMII binding sites and sensitivity in the striatum of $\beta 3$ Ko mice, indicate that

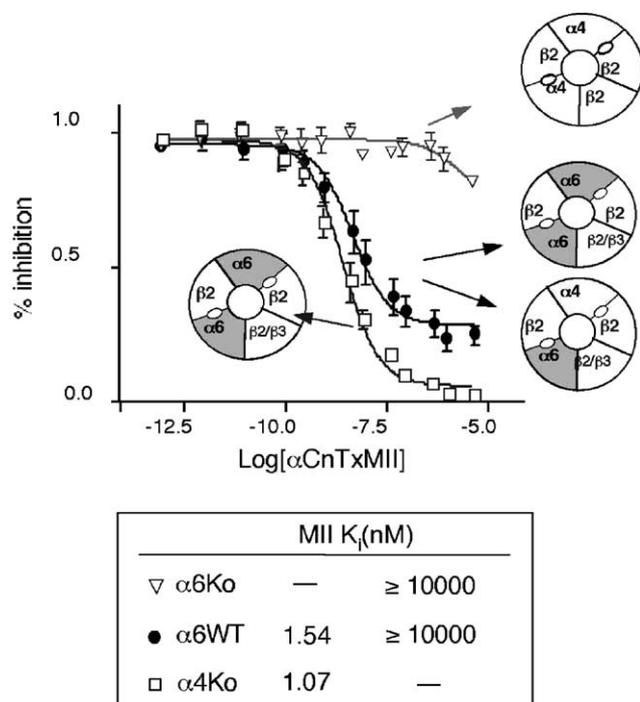


Fig. 4. Displacement of ^{125}I -Epibatine binding by $\alpha\text{CnTxMII}$ in immunobilised subtypes. The $\alpha 4\beta 2$ subtype from $\alpha 6$ KO (▽), the $\alpha 6\beta 2\beta 3$ and $\alpha 6\alpha 4\beta 2\beta 3$ subtypes from WT tissue (●) and the $\alpha 6\beta 2\beta 3$ subtype from $\alpha 4$ Ko mice (□) were incubated with increasing concentrations of $\alpha\text{CnTxMII}$. The inhibition curve of $\alpha\text{CnTxMII}$ is biphasic on the WT $\alpha 6$ subtype (K_i 1.54 nM and >10000 nM) and monophasic on the $\alpha 4$ Ko $\alpha 6$ subtype (K_i 1.07 nM). These pharmacological results indicate that in a fraction of WT striatal $\alpha 6^+$ receptors, one of the two Epi binding sites (located at the interface between the α and β subunits) is made up of an $\alpha 4\beta 2$ interface with low affinity for $\alpha\text{CnTxMII}$. This binding site is absent from the $\alpha 6$ receptors present in the striatum of $\alpha 4$ Ko mice, which therefore have both binding sites with high affinity for the toxins. From these data and the data from immunoprecipitation experiments, we believe that the possible arrangement of the subunit in the different subtypes are those indicated by the scheme.

both the $\alpha 6$ and $\beta 3$ subunits coexist in the same receptor in the large majority of $\alpha\text{CnTxMII}$ -sensitive receptors that modulate DA release from striatal nerve terminals.

$\alpha 6$ -containing receptors do not seem to be involved in the DA release induced by systemic Nic because *in vivo* microdialysis studies of freely moving mice have shown that there is no difference in DA levels in the ventral striatum of WT and Ko mice under basal condition or after systemic Nic injection. However, as binding studies with ^3H -Epi have revealed an increase in $\alpha 4\beta 2$ receptors in the SN-VTA, of $\alpha 6$ Ko mice, a functional compensation by these receptors cannot be excluded (Champtiaux et al., 2003).

$\alpha 6$ Ko mice do not show a change in locomotor activity, but $\beta 3$ Ko mice also display increased locomotor activity in the open field area and reduced prepulse inhibition of the acoustic startle response, two behaviours that are partially controlled by nigrostriatal and mesolimbic dopaminergic activity (Cui et al., 2003). These results suggest that nAChRs

containing the $\beta 3$ subunit may also modulate the dopaminergic pathways that control these two important behaviours.

5.5. Phenotype of $\alpha 7$ Ko mice

$\alpha 7$ Ko mice are viable and their brain anatomy is apparently normal, but a more detailed search for specific phenotypes is under way. The hallmark of these mice is the loss of αBgtx receptors and the lack of Nic-evoked fast desensitising currents in neurons (Orr-Urtreger et al., 1997).

At high doses, Nic causes seizures and previous studies have suggested that sensitivity to Nic-induced seizures may be related to the density of αBgtx nAChRs but, surprisingly, there is no difference in the dose of Nic necessary to induce seizures in $\alpha 7$ Ko mice (Franceschini et al., 2002). However, findings in another mouse model expressing the “knock in” L250T mutation, a leucine to threonine mutation in the transmembrane M2 region, that results in a slow desensitizing receptor, suggest that the $\alpha 7$ receptor may play a role in Nic-induced seizures (Broide et al., 2002). Mice homozygous for the mutation have a lethal phenotype, whereas heterozygous animals survive but have altered $\alpha 7$ -type currents with increased amplitudes and slower desensitisation. In comparison with control mice, the heterozygous mice experienced a significantly greater number of generalised tonic clonic seizures in response to Nic. Homozygous L250T mutant mice show increased apoptosis in the somatosensory cortex, probably due to increased Ca^{2+} influx through the non-desensitising L250T $\alpha 7$ receptors. These data suggest that αBgtx -nAChR activation may play a role in cell death and that, by stimulating apoptosis, they may also influence development. This is also suggested by the finding that the antagonist αBgtx rescues ciliary ganglion neurons that physiologically undergo apoptosis (Renshaw et al., 1993). Moreover, in an experimental model of anoxia based on ibotenate-induced cortical lesions, newborn $\alpha 7$ Ko mice have smaller lesions than WT controls (Laudenbach et al., 2002), thus suggesting that $\alpha 7$ nAChRs may be important in regulating neuronal survival (see 1.2.2).

The role of $\alpha 7$ -containing receptors in the peripheral nervous system has also been studied. *In vivo* and *in vitro* studies have shown that $\alpha 7$ receptors are not important for parasympathetic-mediated responses as both the negative inotropic effect of Nic and the baroreflex-mediated parasympathetic responses to vasoconstriction are unchanged in Ko mice (Franceschini et al., 2000). On the contrary, $\alpha 7$ receptors contribute to the sympathetic response: the increased heart rate, following baroreflex-mediated activation of the sympathetic nerve, is consistently reduced in $\alpha 7$ Ko mice, and this is not due to impaired availability of NA in sympathetic nerve terminals (Franceschini et al., 2000).

5.6. Phenotype of $\alpha 9$ Ko mice

The $\alpha 9$ subunit is not expressed in the brain, but is expressed in cochlear hair cells innervated by cholinergic efferent fibres originating from the brainstem/superior olivary complex. $\alpha 9$ Ko mice show abnormal development of the synaptic connections in cochlear outer hair cells and abnormal cochlear responses after efferent fibre activation, thus suggesting that $\alpha 9$ -containing receptors play a role in this cholinergic system (Elgoyhen et al., 1994).

5.7. Conclusions

Ko mice experiments have shown that brain NACHRs are not essential for survival or the execution of basic behaviours: for example, $\beta 2$ and $\alpha 4$ Ko mice, which completely lack the most abundant high-affinity nAChRs, can accomplish routine behaviour. However, NACHRs are important for the fine control of a number of more sophisticated and complex behaviours that can be evaluated only by means of appropriate tests or in particularly labile situations such as the aged brain.

These findings put NACHRs in a different and perhaps more important perspective in terms of their involvement in brain pathologies and as drug targets. Many pathological situations involve a lack of fine control and tuning rather than complete loss of a particular function, and the pharmacological restoration of appropriate tuning may have a very important clinical effect.

6. NACHRs in pathology

Neuronal NACHRs are involved in a wide variety of diseases affecting the nervous system and non-neuronal tissues. We here review the diseases in which NACHR involvement has been experimentally validated.

6.1. Diseases affecting the nervous system

6.1.1. Age dependent disorders

6.1.1.1. Tourette syndrome. Is a chronic, familial neuropsychiatric disorder involving persistent extrapyramidal movement disturbances, inappropriate vocalisations and cacolalia. It is commonly treated with neuroleptics, but these are not always effective and can have toxic effects. The administration of Nic in the form of chewing gum or a transdermal patch significantly improves the motor disorder (tics) and other symptoms, and also improves the response to neuroleptics (Dursun et al., 1994; McConville et al., 1991, 1992; Sanberg et al., 1997; Silver et al., 1995, 2001; Honson et al., 2004). Although there is as yet no evidence of the direct involvement of NACHRs in this syndrome, the positive effect of Nic administration suggests that they may play a role in symptom manifestation, perhaps by modulating DA release from striatal and limbic cortical areas.

6.1.1.2. Autism. Is a severe developmental disorder that becomes apparent in earliest childhood and is characterised by severely impaired social relations and communication, planning and attention, and by restrictive, odd and stereotyped behaviour. There is no drug that specifically targets autism. A number of genetic and brain biochemical, neurochemical and morphological features have been reported in these patients, but there is no conclusive evidence concerning the etiopathogenesis of the disease. Abnormalities in the brain cholinergic system have recently been reported (Perry et al., 2001), including the fact that there is a decreased number of $\alpha 4\beta 2$ nAChRs in the parietal cortex and cerebellum and that $\alpha 7$ levels remain normal in cortex but increase in the cerebellum (Lee et al., 2002; Martin-Ruiz et al., 2004). Moreover studies on $\beta 2$ Ko mice have shown that these animals have altered social behaviour resembling those present in autism and attention deficit hyperactivity disorder (Granon et al., 2003). These findings can explain some of the neurological dysfunctions in these patients (e.g. cerebellar abnormalities) and provide some insights into the cellular and molecular mechanisms of the abnormal brain development, thus opening up possible new therapeutic approaches towards controlling at least some of the nicotine-controlled brain functions.

6.1.1.3. Attention-deficit hyperactivity disorder (ADHD). Is an inheritable multigenic psychiatric disorder of childhood characterised by difficulties in attending to tasks, hyperactivity and hyperactivity-impulsive symptoms. Several findings suggest that NACHRs are involved in this disease: ADHD is associated with early smoking, maternal smoking is a risk factor (Leonard et al., 2001), nicotinic drugs have positive effects on experimental ADHD, and Nic increases DA release (it is known that an important drug treatment for the disease inhibits the DA transporter and increases dopaminergic activity). However, no genetic evidence of NACHR involvement has been reported, and a search for an association between ADHD and polymorphisms in human $\alpha 4$ and $\alpha 7$ genes was unsuccessful (Kent et al., 2001; Todd et al., 2003).

6.1.1.4. Schizophrenia. The possible involvement of NACHRs in schizophrenia is suggested by: (1) the high prevalence of smoking among schizophrenic patients (90% compared to 33% in the general population (Lohr and Flynn, 1992; Perry et al., 2001; Poirier et al., 2002)); (2) the fact that neuroleptic neuronal side effects are fewer among smokers (Poirier et al., 2002); and (3) the fact that there is a positive correlation between smoking and negative (but not positive) symptoms (Patkar et al., 2002). Recent brain autopsy data indicate that schizophrenic patients have altered NACHRs (Court et al., 2000; Martin-Ruiz et al., 2003). The involvement of heteromeric receptors is doubtful. Unlike preliminary studies, recent investigations evaluating the confounding effects of smoking using more appropriate techniques have failed to show any differences between

controls and schizophrenic patients in terms of $^3\text{H-Nic}$ and $^3\text{H-Epi}$ binding (Breese et al., 2000) and the levels of $\alpha 4$, $\alpha 3$ or $\beta 2$ subunits in the hippocampus, thalamus, cortex and caudate (Martin-Ruiz et al., 2003). All of these data suggest that the most diffuse heteromeric receptors in brain are not modified in schizophrenia, although it is possible that heavy smoking (whose effects on the patients' brains are not completely known) may mask some small modifications.

On the other hand, the involvement of homomeric $\alpha 7$ receptors is much more relevant. Schizophrenic patients have a small, but significant and reproducible decrease in $^{125}\text{I-}\alpha\text{Bgtx}$ binding sites and $\alpha 7$ immunoreactivity in the hippocampus (Freedman et al., 1995; Leonard et al., 1996), the reticular nucleus of the thalamus, and the cingulate and frontal cortex (Martin-Ruiz et al., 2003). Genetic linkage analysis has also provided evidence of the involvement of $\alpha 7$ receptors. The P50 auditory evoked potential gating deficit that is observed in some schizophrenic patient families maps to a region of chromosome 15 q13–14 (Leonard et al., 1996) that includes the $\alpha 7$ gene (Chini et al., 1994; Freedman et al., 2001). The human gene is partially duplicated in schizophrenic families (Freedman et al., 2001; Xu et al., 2001) and genetic studies suggest that this chromosomal location is involved in the genetic transmission of schizophrenia (Freedman et al., 2001). Polymorphisms in the core promoter of the human $\alpha 7$ gene are associated with schizophrenia and with diminished inhibition of the P50 response (Freedman et al., 2003; Leonard et al., 2002). The finding that Nic transiently reverses P50 deficit is consistent with the fact that $\alpha 7$ receptors may control auditory sensory gating (see Leonard et al. (1996)).

Altogether, these data indicate that schizophrenics may have an $\alpha 7$ receptor deficit that they attempt to overcome by smoking. More clinical, genetic and experimental investigations are clearly needed but, in the meantime, nicotinic drug treatment in schizophrenia could be worth exploring.

6.1.2. Age-independent disorders

6.1.2.1. Epilepsy and febrile convulsions. Recent genetic studies have demonstrated the central role of ion channels in the pathophysiology of idiopathic epilepsies (Scheffer and Berkovic, 2003). Autosomal dominant nocturnal frontal lobe epilepsy (a partial epilepsy that causes clusters of brief, frequent and violent seizures during sleep) is a genetic disease with abnormalities located in chromosome 20q13.2–q13.3, which contains the gene encoding the $\alpha 4$ nicotinic subunit, the most commonly expressed subunit in human brain (Phillips et al., 1995). Mutations in the $\alpha 4$ gene have been described in families from many countries and they are located in hotspots along the transmembrane domain that forms the ion channel (Hirose et al., 1999; McLellan et al., 2003; Saenz et al., 1999; Steinlein et al., 1995, 1997). Mutations in the gene *CHRNA2*, which encodes the $\beta 2$ nicotinic subunit, have also been described and are again localised in regions that form the ion channel (De Fusco et al., 2000; Phillips et al., 2001). The mutations lead to

increased sensitivity to ACh and perhaps a change in Ca^{2+} permeability, thus facilitating the synchronisation of the spontaneous oscillations in the thalamo-cortical circuits and generating seizures (see Raggenbass and Bertrand (2002)). Further evidence of the relevance of $\alpha 4$ -containing receptors in epilepsy comes from the finding that mice lacking $\alpha 4$ subunits are more sensitive to the proconvulsant effects of GABA antagonists (McCull et al., 2003) and the fact that antinicotinic drugs have antiepileptic activity (Loscher et al., 2003). However, the mutations in NACHRs do not cover all of the symptoms of ADNFLE and probably represent only one of the many factors leading to the clinical manifestations of the disease (Sutor and Zolles, 2001).

It has been reported that there is an association between the *CHRNA4* gene and febrile convulsions in children (Sahakian et al., 1989), but not with any $\beta 2$ subunit gene polymorphism (Peng et al., 2004). In the case of febrile convulsions, $\alpha 4$ receptor abnormalities could modify not only neuronal circuitry, but also the permeability of the blood–brain barrier that is already affected by the high temperature.

There are experimental indications that $\alpha 7$ receptors are also involved in seizure control: one strain of mice with a large number of brain $^{125}\text{I-}\alpha\text{Bgtx}$ binding sites is more prone to develop seizures in response to Nic (Marks et al., 1989), and a correlation exists between the level of NACHRs in hippocampal astrocytes and the susceptibility of mice strains to Nic seizures (Gahring et al., 2004a) [see also 5.5]. Although nicotinic abnormalities have been reported in a very small minority of epilepsies, these data confirm that both heteromeric and homomeric nicotinic receptor subtypes are important in the control of brain excitability, and that appropriate nicotinic agents could be of value in seizure control or prevention in at least some forms of epilepsy.

6.1.2.2. Depression and anxiety. Although to a lesser extent than schizophrenic patients, there is evidence that the prevalence of tobacco smoking is higher in depressed individuals than in the normal population (Poirier et al., 2002), smoking cessation is associated with depression in individuals with a history of depression, Nic has been reported to be antidepressive and mood stabiliser in humans (Shytle et al., 2002a,b), and a number of antidepressants are antinicotinic agents (Fryer and Lukas, 1999a,b). NACHRs have been involved in both the anxiolytic and anxiogenic effects of Nic in experimental animals (see review by Picciotto et al. (2002)). Studies suggest that NACHRs can modulate the nervous pathways related to depression and anxiety, but the results are too preliminary to enable us to understand whether they have real relevance in the control of mood disorders.

6.1.3. Age-related degenerative diseases of the brain

Alzheimer's disease (AD) and Parkinson's disease are psychiatric or neurological degenerative disorders in which

cholinergic pathways are consistently affected. The nucleus basalis of Meynert undergoes varying degrees of degeneration and cortical choline acetyltransferase activity is consistently decreased (see Clementi et al. (2000) for a review). Early evidence that NACHRs are involved in these diseases came from epidemiological data correlating smoking and AD and PD. Careful epidemiological studies confirmed that tobacco smoking reduces the risk of developing PD and that this relationship is not due to any obvious confounding factors (Clementi et al., 2000). On the other hand, the protective effect of tobacco smoking in AD remains highly controversial (Kukull, 2001). A number of reasons may explain the differences in the protective effects of tobacco smoking between PD and AD, one of which may be that there is a vascular component in AD (but less in PD), and it is known that smoking adversely affects cardiovascular and cerebrovascular function.

6.1.3.1. Parkinson's disease. In addition to the involvement of the nicotinic cholinergic system (Burghaus et al., 2003) the relevant pathology of this disease is the loss of dopaminergic neurons in the nigro-striatal pathway. NACHRs are very important in promoting the release of DA in this pathway, and both the $\alpha 6\beta 3\beta 2$ and the $\alpha 4\beta 2$ subtype in striatum are responsible for the release of DA from nerve endings (Champtiaux et al., 2003; Quik and Kulak, 2002). Other neuronal systems that contribute to the proper function of the nigro-striatal pathway contain NACHRs (for example, $\alpha 7$ on glutamatergic nerve endings, $\alpha 4\beta 2$ on cholinergic and gabaergic interneurons, $\alpha 6\alpha 4\beta 2$ on GABAergic neurons of the SN) (see Section 2.3).

In experimental rodent and monkey models of PD, there is a selective decrease in the number of $\alpha 6$ -, $\alpha 4$ - and $\beta 2$ -containing receptors as detected by means of specific ligand binding or immunoprecipitation experiments (Zoli et al., 2002a,b; Champtiaux et al., 2003; Kulak et al., 2002; Lai et al., 2004; Quik et al., 2003, 2004; Quik and Kulak, 2002). There is a general consensus that $^3\text{H-Nic}$ and $^3\text{H-Epi}$ binding is decreased in the striatum of PD patients (Court et al., 2000; Guan et al., 2002; Martin-Ruiz et al., 2000; Quik and Kulak, 2002), and we have recently seen a consistent decrease in the $\alpha 6$ subunit in both human and experimental models of PD (Gotti et al., unpublished data). The importance of $\alpha 6$ -containing receptors in PD is also supported by the experiments of Lai et al (Lai et al., 2004). This group reports that in an experimental PD monkeys, a selective reversal of the $\alpha 6$ -containing receptor decrease is observed when monkeys recover from the induced lesion. A decrease in nACHRs similar to that reported in AD has also been found in the cerebral cortex of PD patients, and is mainly due to a decrease in $\alpha 4$ - and $\alpha 7$ -containing receptors (Burghaus et al., 2003). These latter findings are in agreement with the impaired cognitive functions of some PD patients. All of the data briefly reported strongly support the involvement of NACHRs in this

disease and particularly the $\alpha 6$ - and $\alpha 4$ -containing receptors in DA release from the nigrostriatal pathway, and the $\alpha 4\beta 2$ subtype in the cognitive cortical aspects.

However, clinical trials of Nic treatment in PD have led to controversial results, with little or no improvement in cognitive and motor symptoms (Kelton et al., 2000). These results are not unexpected as a treatment that affects all NACHRs, and not a selective subtype, unpredictably disrupts the complex cholinergic network of the mesostriatal dopaminergic pathway, and its positive effects can be masked or counterbalanced by the activation of other receptor subtypes.

6.1.3.2. Alzheimer's disease. There is general consensus that the number of brain NACHRs is decreased in this disease, in the absence of a general decrease in the number of neurons (Clementi et al., 2000; Court et al., 2001; Nordberg, 1992, 1995; Nordberg et al., 1990; Schroder et al., 1991). The most affected areas are the neocortical areas, hippocampus, presubiculum and various thalamic nuclei. It has more recently been shown that $\alpha 4$ nicotinic subunits are decreased in cortical areas and in the hippocampus (Court et al., 2000) which suggests that the loss of high affinity agonist binding is due to the loss of $\alpha 4$ -containing receptors. The observation that the $\beta 2$ subunit (which, together with $\alpha 4$, forms the most abundant nicotinic receptor in brain) is not affected (Court et al., 2000; Engidawork et al., 2001) is puzzling and difficult to explain (also see the data on aging in $\beta 2$ Ko mice (Picciotto and Zoli, 2002). A relationship between NACHRs and AD also emerges from genetic studies that show the presence of genetic polymorphisms of the neuronal $\alpha 4$ and $\beta 2$ genes in some AD patients (Cook et al., 2004; Kawamata and Shimohama, 2002).

The data concerning $\alpha 7$ receptors are more contradictory and less convincing, and probably reflect unrecognised differences among subgroups of patients or an imprecise analysis of the cell distribution of the receptors (Court et al., 2000; Wevers et al., 2000).

Another factor relating AD and NACHRs is β -amyloid protein (β -AP), whose deposition is a typical feature of AD. There is general consensus that β -AP plaques are related to the neurodegeneration; β -AP is severely neurotoxic in vitro and in vivo; it disrupts cholinergic neurotransmission even at low concentrations; in a mouse model of human AD, there is an up-regulation of NACHRs, probably due to compensatory mechanisms in response to β -AP burden (Bednar et al., 2002) although the mechanisms of this effect are not clear (see reviews by Clementi et al. (2000); Zamani and Allen (2001)).

β -AP interacts with NACHRs at nM concentration with the $\alpha 4\beta 2$ subtype and at pM with the $\alpha 7$ receptors (Wang et al., 2000a,b) and inhibits in a non-competitive way the $\alpha 4\beta 2$ receptor function (Wu et al., 2004). The effect of β -AP on $\alpha 7$ receptor is controversial since both the inhibition and activation of wildtype and heterologously expressed $\alpha 7$

receptors (Grassi et al., 2003; Liu et al., 2001; Pettit et al., 2001) (Dineley et al., 2002) has been reported. The effects of β -AP on both classes of NACHRs could contribute to the impairments of memory and cognition in AD patients. As it has also been shown that both the β 2 and α 7-containing receptors contribute to neuronal survival during aging (Laudenbach et al., 2002; Zoli et al., 1999), it is possible that the effects of β -AP on these receptors may be responsible of a premature neuronal death.

The important involvement of NACHRs in AD suggests that nicotinic drugs may have a positive effect on the disease. We can envisage two objectives for nicotinic therapy: the slowdown of disease development and symptomatic relief. The marked regional variations in nicotinic receptor densities, as well as their specific reductions in the absence of a general decrease in the number of neurons ((Schroder et al., 1991), also see above), suggest that receptor abnormalities may occur at an early stage of the pathological process before irreversible neuronal degeneration takes place, and that they may be due to a neurotoxic effect of β -AP. At later stages, microvascular abnormalities may account for the severe neuronal loss and precipitate the clinical symptoms. Early treatment with nicotinic agonists or AChE inhibitors could therefore be positive, and the epidemiological data relating heavy smoking in AD patients and the delayed appearance of AD in smoking subjects agree with this suggestion. However, it is not yet clear whether it is better to intervene with a drug affecting all NACHRs (nicotine or AChE inhibitors) or a subtype-selective drug, in which case it would be necessary to identify the most appropriate receptor subtype. Preliminary clinical data indicate that a nicotinic approach may be feasible, although the therapeutic effects of cholinergic agents are not dramatic. In AD patients, Nic improves perceptual and visual attention deficits (Jones et al., 1992) attention performances (Rezvani and Levin, 2001) and semantic memory performance and these effects persist over time. Of the cholinesterase inhibitors, tacrine (Knopman et al., 1996) and galanthamine (which are also channel modulators) have beneficial effects in some group of patients, Nootropic drugs, which have some place in AD treatment, potentiate α 4 β 2 activity (Zhao et al., 2001).

6.2. Pathologies in non-neuronal tissues and cells

6.2.1. Lung cells

NACHRs have been implicated in a number of diseases characterised by cell proliferation on the basis of the results of epidemiological studies linking tobacco smoking to lung carcinoma.

6.2.1.1. Small-cell lung carcinoma. Lung cancer is one of the major causes of death and is associated with exposure to tobacco smoke (Am. Cancer Soc., 1994). Small-cell lung carcinomas (SCLC) are particularly linked to smoking (Weiss and Benarde, 1983). Among the more than 2000

smoke constituents, Nic and related alkaloids and a nitrosamine 4(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which can bind and stimulate nAChRs (Crooks and Dwoskin, 1997), may contribute to this effect.

SCLC cell lines have binding sites for α Bgtx (Cattaneo et al., 1993; Maneckjee and Minna, 1990; Quik et al., 1994; Schuller and Orloff, 1998), for α -CntxMII (Codignola et al., 1996) and also express mRNA for the α 2– α 5, α 7, β 2 and β 4 subunits (Chini et al., 1992; Tarroni et al., 1992; West et al., 2003). These cell lines synthesise and release ACh (Song et al., 2003), thus suggesting that there is an autocrine or paracrine cholinergic pathway in lung tumours. Acute and long-term treatment of SCLC cell lines in vitro with Nic and NNK stimulate proliferation (Cattaneo et al., 1993; Fucile et al., 1997), an effect that depends on Ca^{2+} influx and is suppressed by α Bgtx (Codignola et al., 1994; Maneckjee and Minna, 1990). These data suggest that NACHRs control the rate of proliferation of these cell lines, probably via a Ca^{2+} influx that activates MAP and Akt kinases (Codignola et al., 1994; Cattaneo et al., 1997; West et al., 2003; Schuller et al., 2000). Which receptor subtype is involved is not yet clear, but experiments with selective nicotinic antagonists suggest that Akt kinase activation is via an α 4 β 2 receptor, whereas MAP kinase activation is probably via an α 7 receptor (Minna, 2003; West et al., 2003).

6.2.1.2. Other lung cells. NACHRs have been found in human and murine bronchial epithelial cells. These cells express α 3, α 5, β 2, β 4 and α 7 receptor subunits that form functional ion channels that are highly permeable to Ca^{2+} (Maus et al., 1998; Zia et al., 1997). Stimulation by Nic induces the cell release of granulocyte-macrophage colony stimulating factor (Klapproth et al., 1998). Smoking induces an increased expression of NACHRs in these cells in vivo and in vitro, and a long-lasting increase in intracellular Ca^{2+} concentration. The high levels of Nic in lung can activate α 7 receptors in lung macrophages, thus inhibiting the release of TNF α and proinflammatory cytokines. Therefore, NACHRs probably play an important role in controlling hormone and mucus secretion in the bronchi, in cell-to-cell communication, adhesion and tactility, and in ciliary motion. The data indicate that long exposure to high Nic concentrations, as in chronic smokers, plays an important role in lung cancers, bronchial toxicity and the control of local immune responses.

6.3. Vascular smooth muscle and endothelial cells

Tobacco smoking increases the risk of vascular occlusion after bypass grafting and angioplasty, and the failure of vascular grafts due to intima hyperplasia caused by smooth muscle cell proliferation (Crooks and Dwoskin, 1997). Very low Nic concentrations (10^{-8} M, similar to those found in the blood of smokers) can enhance DNA synthesis and stimulate endothelial cell proliferation in vitro (Carty et al., 1997; Villablanca, 1998). Long-term exposure to Nic

accelerates intimal hyperplasia after endothelial lesion (Hamasaki et al., 1997). Furthermore, together with high cholesterol levels, smoking is a risk factor in atherosclerosis, and chronic Nic associated with a cholesterol diet increases vascular plaque formation in rabbits (Strohschneider et al., 1994). Nic may induce smooth muscle cell proliferation by activating the MAP kinase pathways, releasing mitogenic factors such as the basic fibroblast growth factor, and stimulating the metalloproteases that are important in cell migration (Carty et al., 1996).

6.4. *Dysautonomia and blood pressure control*

The control of blood pressure by peripheral autonomic ganglia is a well-established physiological fact. Peripheral autonomic neuropathies lead to neurogenic orthostatic hypotension and other signs of ganglionic failure. In a consistent number of patients, autonomic neuropathy can be due to autoimmune phenomena as their blood contains Abs against ganglionic receptors (Balestra et al., 2000a; Goldstein et al., 2002; Klein et al., 2003; Sandroni et al., 2004; Vernino et al., 2000). Immunisation with $\alpha 3$ ganglionic subunit in rabbits induces an experimental autoimmune autonomic disease with symptoms similar to those present in patients with autonomic neuropathy (Lennon et al., 2003; Vernino et al., 2004). Furthermore some sera of patients with autoimmune autonomic neuropathy can induce a mild form of this syndrome in mice (Vernino et al., 2004). Anti-neuronal Abs are also present in patients with SCLC, and account for various paraneoplastic symptoms and, in particular, autoimmune autonomic neuropathy (Sher et al., 1991; Gotti et al., 2001). These Abs are useful markers for diagnostic purposes, and can partially explain the pathophysiology of the disease, in a way similar to that found in myasthenia gravis (Drachman, 2003) suggesting that a selective removal of $\alpha 3$ Abs could be beneficial in this situation.

In addition to the peripheral ganglionic control of blood pressure, it is known that the circulation is also under central cholinergic control. NACHRs are present in key areas regulating blood pressure, such as the nucleus of the solitary tract (Wada et al., 1989; Clarke, 1993), and evidence suggests that nAChRs are involved in the regulation of blood pressure and hypertension (see Buccafusco (1996)). The most impressive evidence in favour of a nicotinic component of blood pressure control relates to spontaneously hypertensive rats (SHR). These animals spontaneously develop hypertension in adulthood and have fewer nAChRs ($\alpha 3$ and $\alpha 4$ subunits) than age-matched controls in the cerebral cortex, thalamus, midbrain, medulla oblongata, and spinal cord (Hernandez et al., 2003; Khan et al., 1996; Ueno et al., 2002). The injection of Nic or cytosine intrathecally or in the area postrema in adult SHR rats increases blood pressure, tachycardia and nociception behaviour. Antihypertensive pharmacological treatment does not modify the number of nAChRs, thus indicating that the altered number of receptors

is not due to a homeostatic response (Khan et al., 1994, 1996). Interestingly, these animals have a cognitive impairment typical of NACHR loss, and could be a genetic model for the study of dementia and ADHD (Gattu et al., 1997a,b; Ueno et al., 2002).

6.5. *Intestinal epithelium*

Overwhelming epidemiological and clinical evidence suggests that NACHRs are involved in inflammatory bowel disease (Baron, 1996; Richardson et al., 2003). There is a close negative association between smoking and ulcerative colitis, but smokers are at increased risk of developing Crohn's disease (reviewed in (Thomas et al., 1998)). Smoking or Nic treatment have an adverse effect on the clinical course of Crohn's disease (Bonapace and Mays, 1997) whereas treatment with transdermal Nic patches significantly improves ulcerative colitis in the active phase, a benefit similar to that obtained with corticosteroids (Guslandi and Tittobello, 1996; Pullan et al., 1994). However, a careful evaluation of long-term Nic treatment is not available (Kennedy, 1996; Thomas et al., 1998).

The mechanisms by which Nic have such opposite effects in these bowel pathologies are not known, but recent findings concerning the decreased secretion of TNF α and proinflammatory cytotoxins by macrophages via $\alpha 7$ activation, and the divergent effects of Nic on cytokine levels in colonic mucosa (Eliakim and Karmeli, 2003), can add further light on this matter (see also 4.3).

7. Conclusions and perspectives

Over the last few years, a number of important technological advances have increased our understanding of the functioning of NACHRs. In particular, the application of new molecular and cellular techniques, immunological assays with subunit-specific Abs for NACHR localisation and purification, in vivo localisation using non-invasive imaging techniques, new selective ligands, and especially the availability of Ko and Kin animals for the individual subunits, have made it possible to correlate the subunit composition of NACHRs with specific nicotine-elicited behaviours and better define some of the in vivo physiological functions of the NACHR subtypes.

The most relevant new findings emerging from these studies are the widespread expression of nAChRs, their specific and complex organisation, and their relevance to normal brain function. Moreover, the combination of clinical research with the above approaches has better defined the involvement of NACHRs in a growing number of nervous pathologies other than degenerative diseases, such as autism, ADHD, anxiety and schizophrenia, and led to the discovery of NACHRs in non-neuronal cells and their role in non-nervous diseases such as inflammatory reaction,

pemphigus, pathological angiogenesis, tumour growth, pulmonary pathologies, autonomic dysfunctions, and immunological reactions.

Unfortunately, except for the large family of toxins, the compounds so far available are largely not subtype selective and some can have agonist action on one subtype coupled with antagonistic actions on others. Furthermore, although several nicotinic drugs have reached clinical application for pain, AD, PD, nicotine addiction, anxiety and La Tourette syndrome, they never reached the final goal because of their poor efficacy and/or toxic effects.

A number of reasons may explain this failure. First of all, the widespread localisation of NACHRs that do not control a single specific nervous pathway, but modulate several at a time; secondly, the lack of reliable experimental models for testing the possible usefulness of new drugs because of the complexity of human brain diseases and the specific localisations of animal and human NACHRs; thirdly, we still do not know if it is preferable to use specific drug bullets or drugs capable of interfering with different receptor subtypes and, furthermore, in some pathological conditions (a typical example is La Tourette syndrome in which agonists and antagonists are both positive), it is not clear whether the receptors are decreased or desensitised or if it is better to intervene with agonists, antagonists or channel modifiers; fourthly, the most appropriate kinetics with which the treatment should be applied is unknown. Nicotine, the only successfully used nicotinic drug, has some effects on the CNS after short intense pulses whereas it acts in heart even it is administered with a larger spectrum of drug delivery. Given the different targets of nicotinic treatment, appropriate kinetics could be relevant to increasing selectivity.

All of these uncertainties make it difficult to design clinical trials but, without them, it is difficult to explore the possible therapeutic applications of new compounds. However, we strongly think that basic and preclinical research on NACHRs should be strengthened because the clinical application of this enormous pack of knowledge will certainly produce benefits for understanding and therapeutically managing a discrete number of diseases of nervous and non-nervous tissues.

The goal of current and future NACHR research is therefore to continue combining our increasing knowledge of native receptor structure and function, nicotinic ligand docking sites and the genetic background of the behavioural responses to nicotine in order to be able to design new ligands specifically targeted against the subtypes and/or mutated receptors at the origin of the disease states.

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