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Review

Structural and functional diversity of native brain neuronal nicotinic receptors

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are a family of ligand-gated ion channels present in the central and peripheral nervous systems, that are permeable to mono- and divalent cations. They share a common basic structure but their pharmacological and functional properties arise from the wide range of different subunit combinations making up distinctive subtypes.

nAChRs are involved in many physiological functions in the central and peripheral nervous systems, and are the targets of the widely used drug of abuse nicotine. In addition to tobacco dependence, changes in their number and/or function are associated with neuropsychiatric disorders, ranging from epilepsy to dementia.

Although some of the neural circuits involved in the acute and chronic effects of nicotine have been identified, much less is known about which native nAChR subtypes are involved in specific physiological functions and pathophysiological conditions.

We briefly review some recent findings concerning the structure and function of native nAChRs, focusing on the subtypes identified in the mesostriatal and habenulo-interpeduncular pathways, two systems involved in nicotine reinforcement and withdrawal. We also discuss recent findings concerning the effect of chronic nicotine on the expression of native subtypes.

Contents

1. Introduction

Nicotine is a major component of tobacco smoke whose behavioural effects are due to its interactions with a family of acetylcholine (ACh)-gated channels (nicotinic ACh receptors, nAChRs) present in the central and peripheral nervous systems [1–5]. These share a common basic structure but have specific

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pharmacological and functional properties that are due to the very different subunit combinations that make distinctive subtypes. nAChRs are not only permeable to monovalent Na^+ and K^+ ions, but also to Ca^{2+} ions, and their ability to alter intracellular [Ca^{2+}] [6] by activating different downstream intracellular pathways plays a pivotal role in neuron signalling (reviewed in [7]).

Brain nAChRs have a very widespread and non-uniform distribution. The majority have a presynaptic and/or preterminal localisation where they modulate the release of almost all neurotransmitters, but some also have a somatodendritic post-synaptic localisation [3,4]. The activation of nAChRs can have opposite modulatory effects in the same circuit depending on where they are expressed (for instance on excitatory or inhibitory neurons) [3,8]. nAChRs are involved in a wide range of physiological functions in the central and peripheral nervous system, and changes in their number and/or function are associated with a number of pathophysiological conditions.

The recent development of genetically engineered mice with the targeted deletion of specific subunits (knock-out mice, Ko) or mutations in critical receptor domains (knock-in mice, Kin), as well as Ko mice made to re-express nAChR subunits in selected brain regions by means of lentiviral vectors, has led to the *in vivo* identification of complex subtypes and allowed the study of individual subtypes in specific cells and complex neurobiological systems (reviewed in [1,9–12]).

As a number of comprehensive reviews have described the structure and function of nAChRs [3–5,13,14], the aim of this article is to provide a short overview of some aspects that have been the object of recent studies: the composition and function of native nAChR subtypes, particularly those present in the mesostriatal and habenulo-interpeduncular pathways, and how they are modulated by chronic exposure to nicotine.

2. Structure of the nAChR

nAChRs form a heterogeneous family of subtypes consisting of five subunits arranged around a central pore whose variety is mainly due to the diversity of the possible combinations of the known nine α (α2–α10) and three β (β2–β4) subunits [15]. Unlike the β subunits, all nine α subunits have adjacent cysteines, analogous to cysteines 192–193 of the α subunit of muscle-type nAChRs [16]. The subunits have been cloned from neuron-like cells or cDNA libraries obtained from vertebrate brain, but it has recently been shown that some of them are expressed by non-neuronal cell types throughout the body and have various functions. However, the nAChRs expressed in non-neuronal tissues are beyond the scope of this review and interested readers are referred elsewhere [17–21].

Neuronal nAChRs belong to a large superfamily of homologous receptors, the so-called Cys-loop ion channel receptors, which also include muscle-type nAChRs and GABAA, GABAC, glycine and serotonin $5HT_3$ receptors (reviewed in [16]). Recent crystallisation and structural determinations of ACh binding proteins (homopentameric soluble proteins whose affinity spectrum resembles that of homomeric α7 receptors) from *Lymnaea stagnalis* and *Bulinus truncates* snails [22,23], and the saltwater mollusc *Aplysia californica* [24], have helped define the molecular details of the receptor binding sites. Moreover, the crystallisation of prokaryotic ligand-gated cation channels from the bacterium *Erwinia chrysanthemi* (ELIC) [25,26] and the proton-opened pentameric ligand-gated ion channel homologue from the bacterium *Gloeobacter violaceus* (GLIC) have further helped to define the molecular details of the receptor structure [27].

Like all of the other members of this superfamily of ligand-gated ion channels, nAChR subunits have a relatively hydrophilic extracellular amino terminal portion that carries the ACh binding site and faces the synaptic cleft, followed by three hydrophobic transmembrane domains (M1–M3), a large intracellular loop, and then a fourth hydrophobic transmembrane domain (M4) (reviewed in [28]). The M1–M4 transmembrane domains are arranged in concentric layers around the central aqueous pore: the M2 domain lines the pore membrane, M1 and M3 shield M2 from the surrounding lipid bilayer, and M4 is the most exposed to lipids.

Upon agonist binding to pentameric nAChRs, the domains of each of the five subunits are rearranged in such a way as to open the central pore and allow ion flux through the channel for a few milliseconds, after which the receptor closes to a non-conducting state [29]. Chronic exposure to a low nicotine concentration (such as that present in the blood of smokers) leads to consistent receptor desensitisation which stabilises the receptor in a closed state that is unresponsive to agonists (reviewed in [30]).

Binding studies using radioactive ligands have allowed the identification of two principal classes of nAChRs in the central nervous system: one class binds nicotine and other nicotinic agonists with nM affinity but not αBungarotoxin (αBgtx); the other binds nicotine and nicotinic agonists with μM affinity, and αBgtx with nM affinity [2]. The pharmacological heterogeneity of the nAChRs revealed by these studies was since been confirmed and extended by means of the molecular cloning of a large family of genes coding multiple nAChR subunits, and studies of their expression in heterologous systems. The αBgtx-sensitive receptors can be homomeric or heteromeric, and are made up of the α7, α8, α9, α7–α8, or α9–α10 subunits whereas the αBgtx-insensitive receptors are heteromeric and consist of combinations of α (α2–α6) and β (β2–β4) subunits [31].

Each heteromeric nAChR is arranged as a pinwheel and has two agonist binding sites, i.e. hydrophobic pockets formed at the interface between two adjacent subunits that respectively contribute a primary and a complementary face [32]. Each subunit is asymmetric, and carries the primary and complementary face on opposite sides. The primary face is carried by the α2, α3, α4, α6, α7, α8 or α9 subunits (which have two adjacent cysteines), and contributes three loops from discontinuous sections of the primary sequence; the complementary face is carried by the β2, β4, α7, α8, α9, or α10 subunits and also contributes three discontinuous loops. The α10 subunit has been classified as an α-type subunit, but it cannot act as a primary subunit at the agonist binding site, and only works when it is associated with the α9 subunit [32,33]. Similarly, α5 is not a true α subunit (see below). In general, the identical nature of hydrophobic residues of the primary component determines ligand binding affinity, whereas the residues contributed by the complementary component determine ligand selectivity [4].

Homomeric α7, α8 or α9 receptors have five binding sites to which the same subunit contributes both the primary and complementary components present on opposite sites of the same subunit [31].

3. Role of non-ligand-binding (accessory) subunits in pentameric receptors

In heteromeric αBgtx-insensitive receptors, the accessory subunits are those that do not directly participate in forming the binding site. Heterologous expression studies have shown that the α5 and β3 subunits only form functional channels when they are co-expressed with a principal and a complementary subunit [34,35], thus indicating that they can only function as accessory subunits, whereas the α3 or α4 and β2 or β4 subunits can form ligand binding sites or assemble in the accessory position to produce receptors with different stoichiometries [4,31,36]. The

role of accessory subunit has been investigated in the α4β2* [1] subtypes in which the presence of different accessory subunits (α5, β3, α4, β2) changes their pharmacological and biophysical properties, their sensitivity to allosteric modulators and their sensitivity to up-regulation [37–40]. The $(\alpha4\beta2)_2\alpha5$ subtype has the highest Ca^{2+} permeability, whereas the $(\alpha4\beta2)_2\beta2$ subtype has the greatest affinity for ACh and nicotine activation, and is also the most sensitive to nicotine desensitisation [37]. Moreover, the presence of the α5 subunit in the α4β2* subtype confers sensitivity to the allosteric modulator galantamine [37]. The inclusion of the α5 subunit also affects the pharmacological and functional properties of other subtypes: for example in the α3β2* and α3β4* subtypes, it increases desensitisation and Ca^{2+} permeability, and alters agonist-stimulated responses [39].

It has been shown that the β3 subunit co-assembles with several nAChR subunit combinations but, in all cases other than α3β3β4, it appears to have a dominant negative effect that leads to the absence of the functional expression of the assembled β3* receptor complex [41,42]. However our ex-vivo studies [31] indicate the great propensity of β3 to assemble with α6 subunit, and α6* receptor expression in β3 knock-out mice is decreased in the cell bodies and nerve terminals of dopaminergic neurons. This decrease suggests that the β3 subunit is important for the formation of the majority of α6β2* or α4α6β2* receptors, and that its loss causes defects in nAChR assembly, degradation and/or trafficking. The exclusive role of the β3 subunit as an accessory subunit has been confirmed using fluorescently labelled α6 and β3 subunits and the FRET technique, which has shown that only a single β3 subunit is incorporated in pentameric α6β2* receptors [43].

4. Subunit stoichiometry

As nAChRs are pentameric, they can show considerable molecular diversity. In addition to the differences in subunit composition, some receptor subtypes may have the same subunit composition but different subunit stoichiometries.

Two different methodological approaches have shown that heterologously expressed α4β2 subtypes have an $(\alpha4)_2(\beta2)_3$ stoichiometry that is highly sensitive to activation by ACh [44,45]. Subsequently it has been found that the α4β2 subtype has biphasic ACh concentration–effect curves in heterologous expression systems [38,46,47]. Changes in the ratio of α4 to β2 subunits alter agonist sensitivity. The cells receiving excess α4 subunits are less sensitive to ACh (EC50 ≈ 100 μM). whereas those receiving excess β2 subunits are more sensitive (EC50 ≈ 1 μM). The injection of mRNAs encoding linked α4β2 subunits with mRNA encoding β2 or α4 generates ACh concentration–effect curves indicating respectively higher or lower ACh sensitivity [48]. These results strongly support the hypothesis that variations in α/β stoichiometry generate molecular subtypes with distinctive physiological and pharmacological characteristics. Furthermore, these different stoichiometries also have different Ca^{2+} permeability [39].

It has been shown that a number of brain regions contain a substantial number of low affinity β2* epibatidine binding sites that may represent native $(\alpha4)_3(\beta2)_2$ receptors [49]. In addition, recent functional and biochemical studies have shown that cortical and thalamic nAChRs in heterozygous $\alpha4^{+/-}$ and $\beta2^{+/-}$ mice have different relative expressions of α4 and β2 subunits, and that this correlates with differences in the functional properties of native nAChRs [50]. Overall, these findings support the conclusion that α4β2 nAChRs with different stoichiometries are expressed in native tissue.

[1] The native subtypes are identified by their known subunits; if these are followed by an asterisk, it means that other unidentified subunits may also be present.

Finally, different receptor stoichiometries may play a relevant role in pathophysiological states. Studies of transfected cells have shown that chronic exposure to nicotine up-regulates the expression of $(\alpha4)_2(\beta2)_3$ stoichiometry and normalises the intracellular subunit stoichiometry of nAChRs carrying mutations linked to autosomal dominant nocturnal frontal lobe epilepsy [51].

5. nAChR subtype assembly

Expression studies using heterologous systems have shown that nAChR assembly is a tightly regulated and ordered process, which requires appropriate subunit–subunit interactions and perhaps other proteins (chaperones) that can assist receptor assembly [52,53]. Vertebrate nAChR subunits may co-assemble in many possible combinations, and many more subtypes have been heterologously expressed than those identified *in vivo*. It seems that native nAChRs are assembled into functional pentamers with a relatively restricted number of subunit combinations [14].

In neurons, the first limitation on subunit assembly is, the cell-specific expression of subunits, but other factors play important roles, such as the influence of chaperone molecules, relative subunit concentrations, and intrinsic affinities between pairs of subunits. For example, the α7 subtype is mainly a homomeric receptor in neurons that co-express other subunits, but can form heteromeric receptors made up of the α7 subunit with the β3, α5 and β3 subunits in heterologous expression systems [41,54,55].

An additional level of complexity when comparing native and heterologously expressed subtypes is that nAChRs behave differently in different cell contexts [56]. It seems that there are receptor-specific assembly folding factors for different steps in the assembly of the different subtypes present in some mammalian cell lines [57]. Different cell lines infected with adenoviruses encoding the α7, α4 and β2 subunits produce the appropriate mRNA, but have very different levels of α7 and α4β2 subtype expression, furthermore, the ratio between surface and intracellular receptors may be very different in the same subtype. On the other hand, nAChRs made up of four different subunits have been detected in some brain regions but this complex subunit composition is difficult to obtain in heterologous systems, possibly because they lack the appropriate factors necessary for correct assembly.

6. Native nAChR subtypes

Important contributions to the identification of native nAChRs in the brains of rats and wild-type, Ko and Kin mice have been made using biochemical, immunoprecipitation and immunopurification techniques. It is now well established that the most abundant nAChR subtypes in the nervous system are homomeric α7 receptors and heteromeric receptors containing only one type of α and one type of β subunit [2,4]. The α4β2* receptors account for 90% of the high affinity neuronal nAChRs in mammalian brain whereas the α3β4*subtype is predominant in the autonomic ganglia and adrenal medulla, as well as in subsets of neurons in the medial habenula, nucleus interpeduncularis, dorsal medulla, pineal gland and retina, although a certain proportion of α3β4* receptors in these tissues also contain accessory subunits.

In agreement with data obtained from studies using recombinant nAChRs, *ex vivo* studies have shown that brain nAChRs can contain more than two types of α subunit: for example, approximately 20% of α4β2* nAChRs also contain the α5 subunit, which is widespread in the brain [58]. Deletion of the α5 subunit reduces the high affinity agonist activation of presynaptic nAChRs in the striatum and thalamus without altering their number, and these results indicate that the primary effect of α5 incorporation is to increase nAChR function without affecting nAChR expression,

which may explain why α5 Ko mice are less sensitive to the acute effects of nicotine administration [59,60].

Besides the widespread α4β2* and more restricted α3β4* subtypes, other native subtypes have been recently identified in specific brain regions.

In situ hybridisation studies have shown that the α2β2* subtype is highly expressed in many regions of primate brain [61,62], whereas its expression in rodents is limited to retina and interpeduncular nucleus (IPn) [63,64].

6.1. α6β3* subtypes in mesostriatal and visual pathways

Early *in situ* hybridisation studies showed that mRNAs for the α6 and β3 subunits co-localise in the soma of dopaminergic cells of the mesostriatal pathway and retina, whereas the medial habenula (Hb) contains only β3 mRNA in large amounts [65,66]. Biochemical, ligand binding and functional assays, and immunopurification procedures using subunit specific antibodies, have shown that the striatum (a region that receives nerve terminals from midbrain dopaminergic cells) and the superior colliculus and lateral geniculate nucleus (two retina target regions) have α6β2β3* receptors that consist of the α6β2β3 and α4α6β2β3 subtypes [13,67].

The two ACh binding sites are identical in the α6β2β3 subtype, but different in the α4α6β2β3 subtype, which has both an α6β2 and an α4β2 interface [67]. Purely α6β2β3 or mixed α4α6β2β3 receptors have different pharmacological and physiological properties, and their cell biology may also be different. The α4α6β2β3 subtype has higher affinity for nicotinic agonists than α6β2β3 [68], and chronic nicotine [69] has opposite effects on the expression of these subtypes in the striatum.

The α6β2* receptors are localised presynaptically in both visual and mesostriatal pathways and, together with the α4β2* subtype, modulate the release of dopamine from dopaminergic terminals in the striatum. Both receptor populations have indistinguishable binding affinities for various classical nicotinic agonists and antagonists, but different binding affinities and sensitivities for αconotoxin MII, which recognizes only α6β2 interfaces with nM affinity [67].

The mesostriatal dopamine (DA) pathway is a major brain target for nicotinic agonists and has two principal components: the ventral mesolimbic pathway, which has cell bodies in the ventral tegmental area (VTA), and terminals in the nucleus accumbens (nAc) and tuberculum olfactorium (TO); and the dorsal nigrostriatal pathway, which has cell bodies in the substantia nigra (SN) and terminals in the caudate-putamen (CPu). nAChRs in the dopaminergic neurons of the mesostriatal pathway play an important role in controlling locomotion and the development of some long-lasting adaptations associated with nicotine abuse. Behavioural and functional studies of rats, nicotinic subunit β2 Ko mice, and β2 Ko mice selectively re-expressing the β2 and/or α6 subunits in the ventral midbrain [70] [71] have shown that nAChRs in the dopaminergic neurons of the VTA are necessary for the rewarding effects of nicotine. Moreover, locomotion studies of β2 Ko mice have shown that an imbalance in DA neurotransmission makes them hyperactive in the open field [72], and that selective re-expression of the β2 subunit in the SN rescues this effect. Other studies of mice expressing hypersensitive α6* receptors have shown that their activation in DA neurons finely tunes DA release and is sufficient to cause hyperactivity [73].

Unpublished data from our laboratories show that the subunit composition of α6* receptors in the different subsystems of mesostriatal DA neurons are partially heterogeneous. The DA terminals of the nigrostriatal pathway exclusively express α4α6β2β3 receptors, whereas those of the mesolimbic pathway express a majority of α6β2β3 receptors. In addition, a minor population of α4β2β3 receptors is only expressed in the CPu. The fact that most of the receptors with two α6β2 interfaces are found in the ventral striatum, and that the dorsal striatum expresses only receptors with mixed α4β2 and α6β2 interfaces may partially explain recent evidence indicating the dominance of purely α6* sensitive responses in the nAc, and predominantly α4 responses in the CPu [74]. Furthermore, binding studies in striatal tissue have shown that α4α6β2β3 is the subtype that is preferentially vulnerable to nigrostriatal damage as it is lost in the striatum of animal models of Parkinson's disease and human patients [75].

6.2. nAChRs in the habenulo-interpeduncular pathway

As mentioned above, the addictive properties of nicotine are mainly due to the interaction of nicotine with the β2* receptors present in the VTA and the induction of increased DA levels in the NAc. Recent studies have shown that the somatic manifestations of nicotine withdrawal are due to nAChRs containing the β4 or α5 or α2 subunits in the habenulo-interpeduncular pathway (Hb-IPn) [76,77]. The Hb is a diencephalic structure that receives substantial input from multiple parts of the limbic system, and communicates with the IPn by means of the fasciculus retroflexus. Components of the Hb-IPn system are involved in the physiology and pathophysiology of reward phenomena [78,79], and subserve a variety of behaviours such as learning and memory, nociception, stress, sleeping and eating.

Both Hb and IPn express variable levels of all known heteromeric nAChR subunit mRNAs, and the highest level of nAChRs in the CNS. Biochemical and immunoprecipitation studies have confirmed the heterogeneity of the nAChR subtypes expressed in the Hb-IPn pathway, and found that rat and mouse Hb and IPn contain two major and distinct populations of β2* and β4* receptors [64]. The β2* population in the Hb contains the α4β2* and α3β2*subtypes, some of which also contain the accessory α5 or β3 subunits. In the IPn, β2* nAChRs exist as three populations of approximately equal size: α2β2*, α3β2* and α4β2*[64].

In agreement with binding studies [80–82], our immunoprecipitation studies found that the β4* nAChR population in both regions is mainly associated with the α3 subunit, and a significant fraction of the α3β4* nAChRs contain accessory, mostly β3 subunits. The β3 subunits are associated with α6β2* nAChR in the mesostriatal dopaminergic and visual pathways [67,83] but the presence of β3 subunits not associated with α6 subunits in the Hb-IPn pathway is in line with the findings of *in situ* hybridisation studies showing high levels of β3 but not α6 subunit mRNA in the Hb [65,66,84]. This is a novel subtype because ganglionic α3β4*nAChRs contain α5 as an accessory subunit [85]. β3 subunit mRNA is not expressed in the IPn [66] and so nAChRs containing β3 cannot be synthesised in the IPn.

Among the subtypes present in the Hb-IPn pathway, only the α3β4 and α3β3β4 subtypes mediate [^{3}H]-ACh release in mouse IPn. The deletion of the β3 subunit gene does not affect the level of expressed subtypes in the Hb, but decreases the number of α3 and β4 subunits in the IPn by ∼50%, and similarly decreases the [^{3}H]-ACh release in mouse IPn with no obvious change in EC_{50}. This underlines the fact that, as has been suggested in the case of the α6β2β3* receptors in the visual and mesostriatal pathways, the β3 subunit may play a targeting role in the Hb [13,37].

The role of the other subtypes in the Hb-IPn system is difficult to establish, but studies of Rb^+ efflux from IPn synaptosomes indicate that β2* nAChRs are functional [64]. These receptors are not involved in ACh release, and may modulate the release of other neurotransmitters in non-cholinergic inputs to the IPn [86,87].

In Hb synaptosomes, Rb^+ efflux indicates that β2* nAChRs may be the only functional presynaptic subtype [64] and may mediate

the release of several neurotransmitters as dopaminergic [88], GABA, glutamatergic [89] and noradrenergic terminals [90] have all been identified in this nucleus.

The IPn contains α2β2*, α3β2* and α4β2* nAChRs. The α2β2* nAChRs are expressed by intrinsic IPn neurons because α2 mRNA is only expressed by the IPn [91]. The α3β2*nAChRs may only be located presynaptically as α3 mRNA is not expressed by intrinsic neurons, whereas α4β2* nAChRs may be expressed on intrinsic IPn neurons and/or afferents to the IPn, and may therefore have both pre- and postsynaptic locations (see lower part Fig. 1).

In conclusion the essential role of α3β4 and α3β4β3 in mediating ACh release has been established (see Fig. 1) but many more studies using different approaches are necessary to define the cellular and subcellular localisation and function of all of the subtypes identified in the Hb-IPn pathway.

6.3. Homomeric and heteromeric α7 receptors

Homomeric α7 receptors are the most widely expressed αBgtx-binding receptors in mammalian brain, particularly in the cortex, hippocampus and subcortical limbic regions, and (at low levels) in the thalamic regions and basal ganglia. These α7 receptors may have a presynaptic localisation where they are involved in the direct release of glutamate in the hippocampus and VTA, and of excitatory amino acid in the prefrontal cortex [92], or the indirect release of DA from striatum and prefrontal cortex, and noradrenaline from the hippocampus [93–95]) or a postsynaptic or somatic localisation, where their high Ca^{2+} permeability can have long-term effects on metabolic pathways and gene expression.

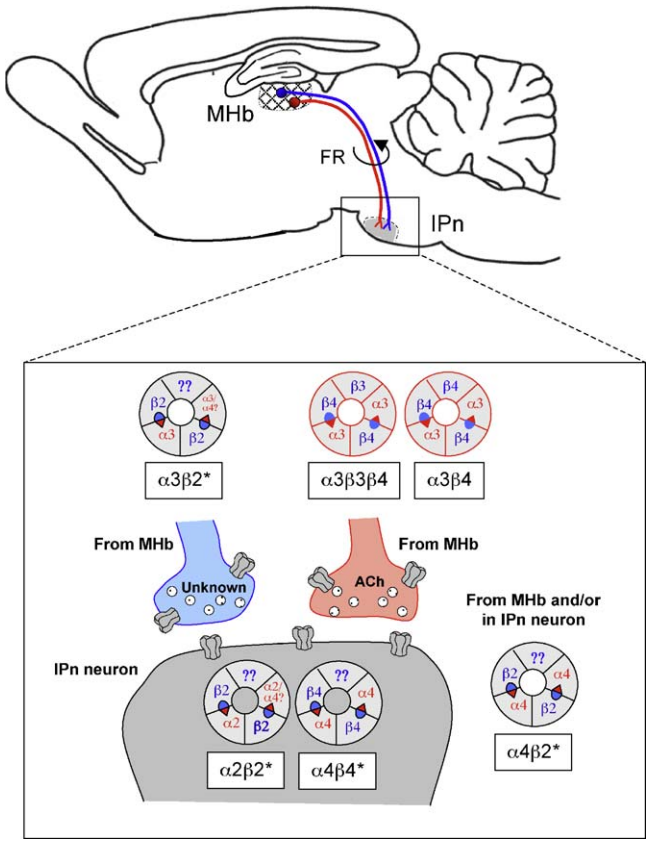

Fig. 1. nAChR subtypes in the habenula-interpeduncular pathway.
Upper panel: A simplified illustration of the habenulo-interpeduncular pathway highlighting its cholinergic (red) and non-cholinergic components (blue).
Lower panel: Subunit composition and putative stoichiometry of the main nAChR subtypes expressed in the presynaptic cholinergic (α3β4 and α3β3β4) and non-cholinergic (α3β2*) IPn terminals. The α2β2* and α4β4* receptors are localised on intrinsic IPn neurons, whereas the α4β2* subtype may have a presynaptic or postsynaptic localisation in the IPn. The proposed localisations are based on the results and references reported in [64].

αBgtx receptors have been affinity purified from the brain of various species, and are pentamers with a single α7 subunit in rat and chick, and homomeric α8 or α7–α8 receptors in chick [14]. Studies using heterologous systems have shown that the α7 subunit can also form functional channels with the α5, β2 or β3 subunits [41,54,55]. Its particular association with the β2 subunit leads to the expression of an α7β2 receptor with distinct pharmacological and functional properties [41,54,55]. It has recently been shown that the α7 and β2 subunits are co-expressed in rat basal forebrain cholinergic neurons and form a novel heteromeric α7β2 subtype [96]. This subtype has different biophysical and pharmacological properties from those of the homomeric α7 receptors expressed by VTA neurons and is highly sensitive to functional inhibition by oligomeric forms of amyloid Aβ1–42. Characterisation of the α7* receptors present in the forebrain cholinergic neurons of β2 Ko mice has further confirmed that the expressed α7β2 subtype has different biophysical and pharmacological properties from those of wild-type basal forebrain neurons, thus indicating that the α7β2 subunits probably assemble to form a new subtype in the former [96].

7. Regulation of native subtypes by chronic nicotine exposure

Chronic nicotine exposure gives rise to neural adaptations that change whole cell physiology and behaviour, mainly due to its interaction with nAChRs.

The effects of nicotine may be due to nAChR activation or desensitisation because, also in the latter case, nicotine can alter neuronal function by interrupting the transmission of endogenous ACh [30,97]. As nAChR subtypes are not equally responsive to nicotine activation and desensitisation, this can influence their functional and behavioural responses (reviewed in [30,97]). In particular, it has been shown that the α7 subtype is more susceptible to inactivation than the β2* or β4* subtypes, but it is not yet clear whether the same subtype may be differently sensitive to activation depending on its pre- or post-synaptic localisation and/or the cell on which it is expressed.

Studies of the brains of tobacco smokers and animals chronically exposed to nicotine have shown that long-term exposure often triggers an increase in the number of nAChRs (so-called up-regulation) [98], and the fact that there is no increase in α2, α3, α4, α5 or β2 mRNA levels in mouse brain chronically exposed to nicotine suggests that post-transcriptional mechanisms are responsible for this [99]. Moreover, nAChR up-regulation is independent of the cells on which the receptors are expressed: i.e. it is "cell autonomous" [100].

In vitro studies of cells transfected with nAChR subtypes have shown the nicotine-induced up-regulation of homomeric α7, and various heteromeric α3β2, α4β2, α6β2 and α3β4 receptors [101–103], and *in vivo* studies of animals chronically treated with nicotine and the brains of human smokers have shown that the most up-regulated receptor is the α4β2 subtype [104]. This up-regulation has been mainly measured by binding studies using membrane permeable ligands that bind both intracellular and surface receptors, and have been performed at nicotinic ligand concentrations in the Kd range that mainly bind the desensitised inactive state of the receptors (see [30] and references therein). Binding studies using membrane impermeant ligands have shown that approximately 85% of the receptors in transfected cells and neurons are intracellular [105,106], and that chronic nicotine treatment increases both intracellular and surface receptors [15].

Various mechanisms have been hypothesised in order to explain α4β2 subtype up-regulation, including increased receptor assembly, decreased surface turnover, increased surface receptor

traffic, decreased receptor degradation, and an induced conformational switch into high affinity receptors that become activated more easily (reviewed in [15,30,106]). Recent data have shown that the presence of the α5 subunit makes the α4β2 subtype in rat hippocampus, striatum, cerebral cortex and thalamus resistant to up-regulation *in vivo* [107].

The effect of chronic nicotine exposure on other nAChR subtypes is less well established. The α7 receptors, which have lower nicotine affinity, are up-regulated to a lesser extent than the α4β2 subtype and in only a few regions [108]. The α3β4* subtype, which is prominent in the pineal gland, Hb, IPn and autonomic ganglia, seems to be resistant to up-regulation [104].

A number of studies have tried to correlate the increase in receptors following chronic nicotine exposure with a possible increase in nAChR function. After nicotine exposure, oocyte-expressed α4β2 receptors reduce ACh-induced currents, whereas the same receptors expressed in mammalian cell lines show increase in these currents and are more sensitive to ACh than controls (reviewed [97]).

Chronic exposure to nicotine of the α7 and α3 subtypes in oocytes, leads to the almost total functional inactivation of the α7 receptors, but only partial inactivation of the α3 subtype, [109]. On the contrary, in cultured cortical neurons, there is an increase in the number of α7 receptors and the whole cell response, with no evidence of long-lasting desensitisation even after long-term nicotine exposure [110].

Nicotine can activate and regulate several subtypes involved in the presynaptic release of various neurotransmitters, and chronic nicotine treatment can differently affect these different subtypes [110]: for example it has been reported that the subtypes involved in the release of dopamine may be functionally increased [111], decreased [112] or unaffected [113].

In conclusion, the data concerning *in vivo* nAChR function after chronic exposure to nicotine are contradictory insofar as they depend on the subtype, the cell systems expressing the subtype, and the functional assays used to measure the activity of the nAChRs.

Much attention has recently been given to α6* receptors, which bind αconotoxin MII. Rodent studies have shown that intravenous nicotine self-administration leads to an increase in the number of α6* receptors in rats [114], but a decrease in the number of striatal α6* receptors in rodents chronically treated with nicotine by means of minipumps [115,116] or receiving it in drinking water [117]. These apparently discrepant results may be due to the different modalities of nicotine administration leading to differences in nicotine concentration in the brain and/or the kinetics or duration of receptor exposure, which may differently affect α6* nAChRs. The result of a recent experiment has added further complexity. It was found that the decrease in overall αconotoxinMII binding observed after oral nicotine administration is due to two opposite effects on different α6* subtypes: a decrease in the α4α6β2* subtype and an increase in the α6(nonα4)β2* subtype [118]. Accordingly, α4−/− mice chronically treated with oral nicotine show increased αconotoxinMII binding in striatum [118].

As mentioned above, the α4β2* and α6β2* subtypes are the main subtypes modulating dopamine release in rodent striatum. *Ex vivo*, studies have shown that α6β2* receptors mediate 30–50% of ^{3}H-dopamine release in the CPu and nAc [71], and voltammetry studies indicate that they are responsible for most of the dopamine release in the nAc obtained with the burst stimulation of striatal slices [74]. Chronic oral nicotine treatment, which up-regulates the α6(non-α4)β2 subtype but leads to the loss of α4α6β2, abolishes α6*-mediated effects on burst firing, thus suggesting that is the former subtype that primarily modulates this type of α6β2*-mediated dopamine release [118].

Studies of non-human primates have shown that, although both α4β2 and α3/α6β2* subtypes are present at similar levels in monkey striatum, the latter determines 70% of the dopamine release. Long-term nicotine treatment selectively modifies dopamine release in distinct striatal sub-regions: i.e. ventral but not the dorsal putamen [119]. The same treatment up-regulates αconotoxinMII-resistant ^{125}I-epibatidine binding (putative α4β2* receptors) without changing ^{125}I-αconotoxinMII binding (α3/α6β2* receptors) in both the dorsal and the ventral putamen. It would be interesting to assess whether the regional heterogeneity in nicotine-mediated dopamine release stems from alterations in α6* composition, as has been shown in rodents.

Although chronic nicotine exposure decreases the number of α6* receptors in the striatum, it has no effect on those in the superior colliculus, where they are highly expressed, and does not change the number of β3* receptors in either region [116] notwithstanding the fact that α6* receptors contain the β3 subunit in both [67,83]. This suggests that α6β3* receptors are more resistant to nicotine-induced down-regulation than α6(nonβ3)* receptors, or that the β3* receptors assemble with other α subunits.

It is not known why nicotine has such different effects on α6* receptors. *In situ* hybridisation and single-cell PCR studies have shown that there is a mixture of α4, α6, β2 and β3 subunits in midbrain dopaminergic neurons [65,120]. If the number of β2 subunits is limited, it is possible that nAChR subunits compete for assembly in the endoplasmic reticulum of these neurons. By acting as a preferential chaperone on α4β2 receptors, nicotine may favour their formation and thus decrease the pool of β2 subunits available for assembly with the α6 subunit (see also [118] for a similar hypothesis). In the case of the superior colliculus, it is not known whether the α4, α6, β2 and β3 subunits are present in the same retinal ganglionic cells, and so it is possible that there is no competition between α4 and α6 subunits.

The role of nAChR up-regulation in inducing and/or maintaining nicotine dependence is still uncertain. Up-regulation can be achieved in animals by means of administration routes that are different from those of smokers and/or as nicotine doses that are much higher. Yet, it has been shown that smokers have higher cortical levels of nicotinic agonist binding and α4 subunit than non-smokers [121,122]. As the level of up-regulated subtypes persists for days after nicotine administration is stopped, it is possible that nAChR up-regulation plays a role during nicotine withdrawal. Decreased nicotine levels can allow nAChRs to recover from desensitisation and be more responsive to endogenous ACh, which may contribute to withdrawal symptoms or craving. This possibility is also suggested by the results of neuroimaging studies showing that smokers have high β2* receptor levels for at least seven days after stopping smoking [123]. The levels of β2 nAChRs do not correlate with the severity of dependence or withdrawal, but only with the urge to smoke in order to relieve withdrawal symptoms.

The vast majority of the different effects of nicotine are determined by the functional features and location of the nAChR subtypes with which it interacts in specific neuronal circuits, but recent findings have shown that nicotine affects not only the number of nAChRs, but also that of other receptors and proteins important for the development of synaptic plasticity, either by partially blocking the proteosome, or through other mechanisms [98,104,124,125]. It therefore seems that nicotine induces behavioural effects via a complex interplay of different signal transduction pathways that can be different in different nervous circuits.

8. Conclusions

The fine molecular structure of nAChRs has been better clarified over recent years mainly as a result of very different methodological approaches.

It has been shown that there is a substantial number of native subtypes, although native nAChRs are assembled into functional pentamers made up of a relatively restricted number of subunit combinations. Moreover, the *in vivo* characterisation of new and unsuspected subtypes (heteromeric $\alpha7\beta2$) has increased the complexity of studying native subtypes.

Much has been learned concerning the role of accessory subunits in the function and pharmacology of the different subtypes, but we still do not know their precise role in nAChR localisation and traffic and the subunit stoichiometry of the heteromeric subtypes remains a largely unsolved question.

Finally the mechanisms by which chronic nicotine affects the function and number of native subtypes are still elusive and this will be an important future step in our understanding of the role of nAChRs in tobacco dependence and neuropsychiatric diseases.

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