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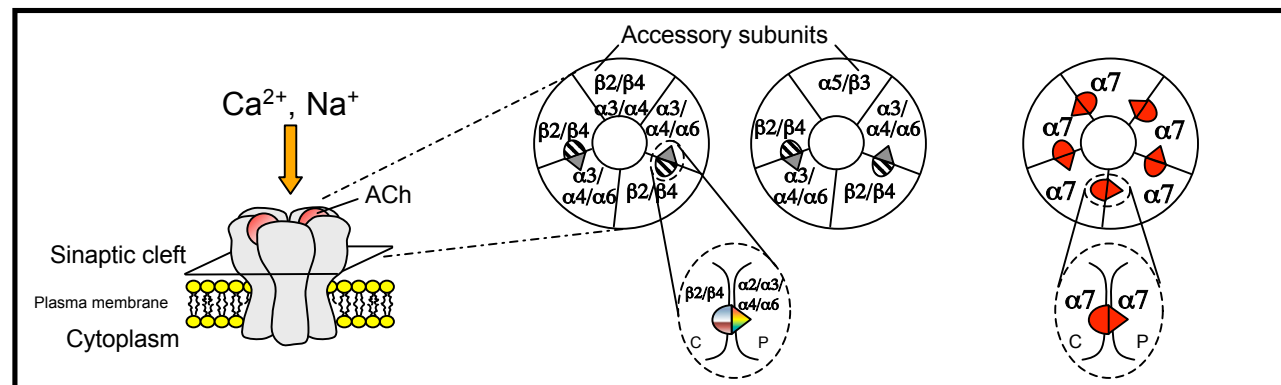
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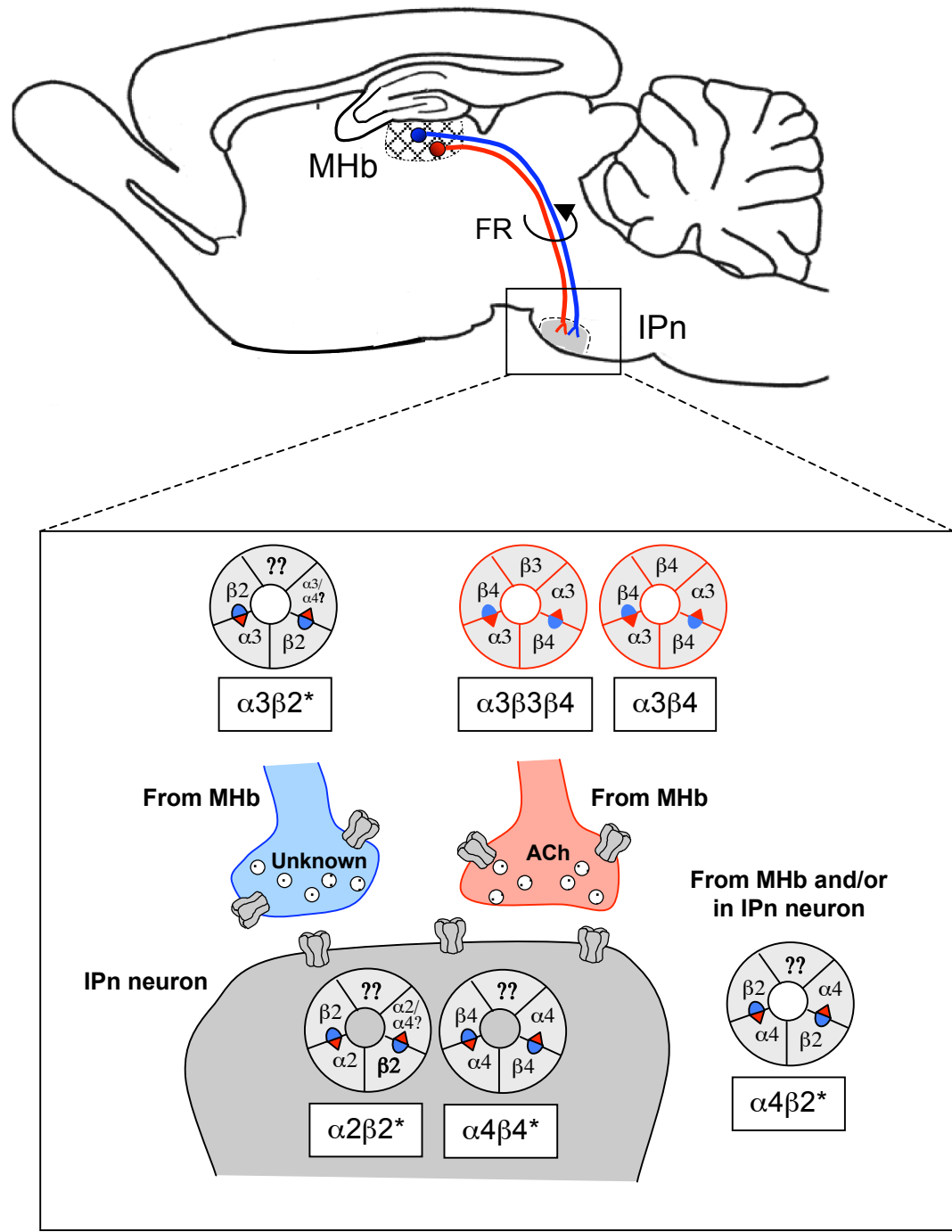
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SPECIAL ISSUE

Neuronal Nicotinic Receptor-based Therapeutics

to be published in *Biochemical Pharmacology*

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**STRUCTURAL AND FUNCTIONAL DIVERSITY OF NATIVE BRAIN
NEURONAL NICOTINIC RECEPTORS.****Cecilia Gotti^a, Francesco Clementi^a, Alice Fornari^b, Annalisa Gaimarri^a, Stefania Guiducci^b, Irene Manfredi^a, Milena Moretti^a, Patrizia Pedrazzi^b, Luca Pucci^a and Michele Zoli^b**^aCNR, Institute of Neuroscience, Cellular and Molecular Pharmacology, Department of Medical Pharmacology, University of Milan, Milan, Italy. ^bDepartment of Biomedical Sciences, Section of Physiology, University of Modena and Reggio Emilia, Modena, Italy**Corresponding author:**

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Abstract

1
2 Neuronal nicotinic acetylcholine receptors (nAChRs) are a family of ligand-gated ion channels
3 present in the central and peripheral nervous systems, that are permeable to mono-and divalent
4 cations. They share a common basic structure but their pharmacological and functional
5 properties arise from the wide range of different subunit combinations making up distinctive
6 subtypes.
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10 nAChRs are involved in many physiological functions in the central and peripheral nervous
11 systems, and are the targets of the widely used drug of abuse nicotine. In addition to tobacco
12 dependence, changes in their number and/or function are associated with neuropsychiatric
13 disorders, ranging from epilepsy to dementia.
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17 Although some of the neural circuits involved in the acute and chronic effects of nicotine have
18 been identified, much less is known about which native nAChR subtypes are involved in specific
19 physiological functions and pathophysiological conditions.
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23 We briefly review some recent findings concerning the structure and function of native nAChRs,
24 focusing on the subtypes identified in the meso-striatal and habenulo-interpeduncular pathways,
25 two systems involved in nicotine reinforcement and withdrawal. We also discuss recent findings
26 concerning the effect of chronic nicotine on the expression of native subtypes.
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33 **Key words: neuronal nicotinic receptors, subtypes, subunit composition, stoichiometry,**
34 **meso-striatal pathway, habenulo-interpeduncular pathway**
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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 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 $[\text{Ca}^{2+}]$ [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 localization [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 (knockout mice, Ko) or mutations in critical receptor domains (knockin 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 ($\alpha 2$ - $\alpha 10$) and three β ($\beta 2$ - $\beta 4$) subunits [15]. Unlike the β subunits, all nine α subunits have adjacent cysteines, analogous to cysteines 192-193 of the α subunit of muscle-

1 type nAChRs [16]. The subunits have been cloned from neuron-like cells or cDNA libraries
2 obtained from vertebrate brain, but it has recently been shown that some of them are expressed
3 by non-neuronal cell types throughout the body and have various functions. However, the
4 nAChRs expressed in non-neuronal tissues are beyond the scope of this review and interested
5 readers are referred elsewhere [17-21].
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8
9 Neuronal nAChRs belong to a large superfamily of homologous receptors, the so called
10 Cys-loop ion channel receptors, which also include muscle-type nAChRs and GABA_A, GABA_C,
11 glycine and serotonin 5HT₃ receptors (reviewed in [16]). Recent crystallisation and structural
12 determinations of ACh binding proteins (homopentameric soluble proteins whose affinity
13 spectrum resembles that of homomeric $\alpha 7$ receptors) from *Lymnaea stagnalis* and *Bulinus*
14 *truncates* snails [22, 23], and the saltwater mollusc *Aplysia californica* [24], have helped define
15 the molecular details of the receptor binding sites. Moreover, the crystallisation of prokaryotic
16 ligand-gated cation channels from the bacterium *Erwinia chrysanthemii* (ELIC) [25, 26] and the
17 proton-opened pentameric ligand-gated ion channel homologue from the bacterium *Gloeobacter*
18 *violaceus* (GLIC) have further helped to define the molecular details of the receptor structure
19 [27].
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22 Like all of the other members of this superfamily of ligand-gated ion channels, nAChR
23 subunits have a relatively hydrophilic extracellular amino terminal portion that carries the ACh
24 binding site and faces the synaptic cleft, followed by three hydrophobic transmembrane domains
25 (M1-M3), a large intracellular loop, and then a fourth hydrophobic transmembrane domain (M4)
26 (reviewed in [28]). The M1–M4 transmembrane domains are arranged in concentric layers
27 around the central aqueous pore: the M2 domain lines the pore membrane, M1 and M3 shield
28 M2 from the surrounding lipid bilayer, and M4 is the most exposed to lipids.
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31 Upon agonist binding to pentameric nAChRs, the domains of each of the five subunits
32 are rearranged in such a way as to open the central pore and allow ion flux through the channel
33 for a few milliseconds, after which the receptor closes to a non-conducting state [29]. Chronic
34 exposure to a low nicotine concentration (such as that present in the blood of smokers) leads to
35 consistent receptor desensitisation which stabilises the receptor in a closed state that is
36 unresponsive to agonists (reviewed in [30]).
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42 Binding studies using radioactive ligands have allowed the identification of two principal classes
43 of nAChRs in the central nervous system: one class binds nicotine and other nicotinic agonists
44 with nM affinity but not α Bungarotoxin (α Bgtx); the other binds nicotine and nicotinic agonists
45 with μ M affinity, and α Bgtx with nM affinity [2]. The pharmacological heterogeneity of the
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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 α Bgtx-sensitive receptors can be homomeric or heteromeric, and are made up of the $\alpha 7$, $\alpha 8$, $\alpha 9$, $\alpha 7$ - $\alpha 8$, or $\alpha 9$ - $\alpha 10$ subunits whereas the α Bgtx-insensitive receptors are heteromeric and consist of combinations of α ($\alpha 2$ - $\alpha 6$) and β ($\beta 2$ - $\beta 4$) 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 $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$, $\alpha 8$ or $\alpha 9$ subunits (which have two adjacent cysteines), and contributes three loops from discontinuous sections of the primary sequence; the complementary face is carried by the $\beta 2$, $\beta 4$, $\alpha 7$, $\alpha 8$, $\alpha 9$, or $\alpha 10$ subunits and also contributes three discontinuous loops. The $\alpha 10$ subunit has been classified as an α -type subunit, but is cannot act as a primary subunit at the agonist binding site, and only works when it is associated with the $\alpha 9$ subunit [32, 33]. Similarly, $\alpha 5$ 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 $\alpha 7$, $\alpha 8$ or $\alpha 9$ 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 site (accessory) subunits in pentameric receptors

In heteromeric α Bgtx-insensitive receptors, the accessory subunits are those that do not directly participate in forming the binding site. Heterologous expression studies have shown that the $\alpha 5$ and $\beta 3$ 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 $\alpha 3$ or $\alpha 4$ and $\beta 2$ or $\beta 4$ 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 $\alpha 4\beta 2^{*1}$ subtypes in which the presence of different accessory subunits ($\alpha 5$, $\beta 3$, $\alpha 4$, $\beta 2$) changes their pharmacological and biophysical properties, their sensitivity to allosteric modulators and their sensitivity to up-regulation [37-40].

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.

1 The $(\alpha 4\beta 2)_2\alpha 5$ subtype has the highest Ca^{2+} permeability, whereas the $(\alpha 4\beta 2)_2\beta 2$ subtype has the
2 greatest affinity for ACh and nicotine activation, and is also the most sensitive to nicotine
3 desensitisation [37]. Moreover, the presence of the $\alpha 5$ subunit in the $\alpha 4\beta 2^*$ subtype confers
4 sensitivity to the allosteric modulator galantamine [37]. The inclusion of the $\alpha 5$ subunit also
5 affects the pharmacological and functional properties of other subtypes: for example in the
6 $\alpha 3\beta 2^*$ and $\alpha 3\beta 4^*$ subtypes, it increases desensitisation and Ca^{2+} permeability, and alters
7 agonist-stimulated responses [39].

8
9 It has been shown that the $\beta 3$ subunit co-assembles with several nAChR subunit
10 combinations but, in all cases other than $\alpha 3\beta 3\beta 4$, it appears to have a dominant negative effect
11 that leads to the absence of the functional expression of the assembled $\beta 3^*$ receptor complex [41,
12 42]. However our ex-vivo studies [31] indicate the great propensity of $\beta 3$ to assemble with $\alpha 6$
13 subunit, and $\alpha 6^*$ receptor expression in $\beta 3$ knock-out mice is decreased in the cell bodies and
14 nerve terminals of dopaminergic neurons. This decrease suggests that the $\beta 3$ subunit is important
15 for the formation of the majority of $\alpha 6\beta 2^*$ or $\alpha 4\alpha 6\beta 2^*$ receptors, and that its loss causes defects
16 in nAChR assembly, degradation and/or trafficking. The exclusive role of the $\beta 3$ subunit as an
17 accessory subunit has been confirmed using fluorescently labelled $\alpha 6$ and $\beta 3$ subunits and the
18 FRET technique, which has shown that only a single $\beta 3$ subunit is incorporated in pentameric
19 $\alpha 6\beta 2^*$ receptors[43].

20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 **4. Subunit stoichiometry**

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

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42 Two different methodological approaches have shown that heterologously expressed
43 $\alpha 4\beta 2$ subtypes have an $(\alpha 4)_2(\beta 2)_3$ stoichiometry that is highly sensitive to activation by ACh [44,
44 45]. Subsequently it has been found that the $\alpha 4\beta 2$ subtype has biphasic ACh concentration-effect
45 curves in heterologous expression systems [38, 46, 47]. Changes in the ratio of $\alpha 4$ to $\beta 2$ subunits
46 alter agonist sensitivity. The cells receiving excess $\alpha 4$ subunits are less sensitive to ACh ($\text{EC}_{50} \approx$
47 $100 \mu\text{M}$). whereas those receiving excess $\beta 2$ subunits are more sensitive ($\text{EC}_{50} \approx 1 \mu\text{M}$). The
48 injection of mRNAs encoding linked $\alpha 4\beta 2$ subunits with mRNA encoding $\beta 2$ or $\alpha 4$ generates
49 ACh concentration-effect curves indicating respectively higher or lower ACh sensitivity
50 [48]. These results strongly support the hypothesis that variations in α/β stoichiometry generate
51 molecular subtypes with distinctive physiological and pharmacological characteristics.
52 Furthermore, these different stoichiometries also have different Ca^{2+} permeability [39].

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It has been shown that a number of brain regions contain a substantial number of low affinity $\beta 2^*$ epibatidine binding sites that may represent native $(\alpha 4)_3(\beta 2)_2$ receptors [49]. In addition, recent functional and biochemical studies have shown that cortical and thalamic nAChRs in heterozygous $\alpha 4^{+/-}$ and $\beta 2^{+/-}$ mice have different relative expressions of $\alpha 4$ and $\beta 2$ subunits, and that this correlates with differences in the functional properties of native nAChRs [50]. Overall, these findings support the conclusion that $\alpha 4\beta 2$ nAChRs with different stoichiometries are expressed in native tissue.

Finally, different receptor stoichiometries may play a relevant role in pathophysiological states. Studies of transfected cells have shown that chronic exposure to nicotine upregulates the expression of $(\alpha 4)_2(\beta 2)_3$ 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 $\alpha 7$ subtype is mainly a homomeric receptor in neurons that co-express other subunits, but can form heteromeric receptors made up of the $\alpha 7$ subunit with the $\beta 3$, $\alpha 5$ and $\beta 3$ 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 $\alpha 7$, $\alpha 4$ and $\beta 2$ subunits produce the appropriate mRNA, but have very different levels of $\alpha 7$ and $\alpha 4\beta 2$ 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 $\alpha 7$ receptors and heteromeric receptors containing only one type of α and one type of β subunit [2, 4]. The $\alpha 4\beta 2^*$ receptors account for 90% of the high affinity neuronal nAChRs in mammalian brain whereas the $\alpha 3\beta 4^*$ 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 $\alpha 3\beta 4^*$ 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 $\alpha 4\beta 2^*$ nAChRs also contain the $\alpha 5$ subunit, which is widespread in the brain [58]. Deletion of the $\alpha 5$ 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 $\alpha 5$ incorporation is to increase nAChR function without affecting nAChR expression, which may explain why $\alpha 5$ Ko mice are less sensitive to the acute effects of nicotine administration [59, 60].

Besides the widespread $\alpha 4\beta 2^*$ and more restricted $\alpha 3\beta 4^*$ subtypes, other native subtypes have been recently identified in specific brain regions.

In situ hybridisation studies have shown that the $\alpha 2\beta 2^*$ 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. $\alpha 6\beta 3^*$ subtypes in mesostriatal and visual pathways

Early *in situ* hybridisation studies showed that mRNAs for the $\alpha 6$ and $\beta 3$ subunits co-localise in the soma of dopaminergic cells of the mesostriatal pathway and retina, whereas the medial habenula (Hb) contains only $\beta 3$ 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

1 cells) and the superior colliculus and lateral geniculate nucleus (two retina target regions) have
2 $\alpha 6\beta 2\beta 3^*$ receptors that consist of the $\alpha 6\beta 2\beta 3$ and $\alpha 4\alpha 6\beta 2\beta 3$ subtypes [13, 67].

3
4 The two ACh binding sites are identical in the $\alpha 6\beta 2\beta 3$ subtype, but different in the
5 $\alpha 4\alpha 6\beta 2\beta 3$ subtype, which has both an $\alpha 6\beta 2$ and an $\alpha 4\beta 2$ interface [67]. Purely $\alpha 6\beta 2\beta 3$ or
6 mixed $\alpha 4\alpha 6\beta 2\beta 3$ receptors have different pharmacological and physiological properties, and
7 their cell biology may also be different. The $\alpha 4\alpha 6\beta 2\beta 3$ subtype has higher affinity for nicotinic
8 agonists than $\alpha 6\beta 2\beta 3$ [68], and chronic nicotine [69] has opposite effects on the expression of
9 these subtypes in the striatum.

10
11 The $\alpha 6\beta 2^*$ receptors are localised presynaptically in both visual and mesostriatal
12 pathways and, together with the $\alpha 4\beta 2^*$ subtype, modulate the release of dopamine from
13 dopaminergic terminals in the striatum. Both receptor populations have indistinguishable binding
14 affinities for various classical nicotinic agonists and antagonists, but different binding affinities
15 and sensitivities for α conotoxin MII, which recognizes only $\alpha 6\beta 2$ interfaces with nM affinity
16 [67].

17
18 The mesostriatal dopamine (DA) pathway is a major brain target for nicotinic agonists and has
19 two principal components : the ventral mesolimbic pathway, which has cell bodies in the ventral
20 tegmental area (VTA), and terminals in the nucleus accumbens (nAc) and tuberculum
21 olfactorium (TO); and the dorsal nigrostriatal pathway, which has cell bodies in the substantia
22 nigra (SN) and terminals in the caudate-putamen (CPu). nAChRs in the dopaminergic neurons of
23 the mesostriatal pathway play an important role in controlling locomotion and the development
24 of some long-lasting adaptations associated with nicotine abuse. Behavioural and functional
25 studies of rats, nicotinic subunit $\beta 2$ Ko mice, and $\beta 2$ Ko mice selectively re-expressing the $\beta 2$
26 and/or $\alpha 6$ subunits in the ventral midbrain [70] [71] have shown that nAChRs in the
27 dopaminergic neurons of the VTA are necessary for the rewarding effects of nicotine. Moreover,
28 locomotion studies of $\beta 2$ Ko mice have shown that an imbalance in DA neurotransmission
29 makes them hyperactive in the open field [72], and that selective re-expression of the $\beta 2$ subunit
30 in the SN rescues this effect. Other studies of mice expressing hypersensitive $\alpha 6^*$ receptors have
31 shown that their activation in DA neurons finely tunes DA release and is sufficient to cause
32 hyperactivity [73].

33
34 Unpublished data from our laboratories show that the subunit composition of $\alpha 6^*$
35 receptors in the different subsystems of mesostriatal DA neurons are partially heterogeneous.
36 The DA terminals of the nigrostriatal pathway exclusively express $\alpha 4\alpha 6\beta 2\beta 3$ receptors, whereas

those of the mesolimbic pathway express a majority of $\alpha 6\beta 2\beta 3$ receptors. In addition, a minor population of $\alpha 4\beta 2\beta 3$ receptors is only expressed in the CPU. The fact that most of the receptors with two $\alpha 6\beta 2$ interfaces are found in the ventral striatum, and that the dorsal striatum expresses only receptors with mixed $\alpha 4\beta 2$ and $\alpha 6\beta 2$ interfaces may partially explain recent evidence indicating the dominance of purely $\alpha 6^*$ sensitive responses in the nAc, and predominantly $\alpha 4$ responses in the CPU [74]. Furthermore, binding studies in striatal tissue have shown that $\alpha 4\alpha 6\beta 2\beta 3$ 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 $\beta 2^*$ 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 $\beta 4$ or $\alpha 5$ or $\alpha 2$ 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 $\beta 2^*$ and $\beta 4^*$ receptors [64]. The $\beta 2^*$ population in the Hb contains the $\alpha 4\beta 2^*$ and $\alpha 3\beta 2^*$ subtypes, some of which also contain the accessory $\alpha 5$ or $\beta 3$ subunits. In the IPn, $\beta 2^*$ nAChRs exist as three populations of approximately equal size: $\alpha 2\beta 2^*$, $\alpha 3\beta 2^*$ and $\alpha 4\beta 2^*$ [64].

In agreement with binding studies [80-82], our immunoprecipitation studies found that the $\beta 4^*$ nAChR population in both regions is mainly associated with the $\alpha 3$ subunit, and a significant fraction of the $\alpha 3\beta 4^*$ nAChRs contain accessory, mostly $\beta 3$ subunits. The $\beta 3$ subunits are associated with $\alpha 6\beta 2^*$ nAChR in the mesostriatal dopaminergic and visual pathways [67, 83] but the presence of $\beta 3$ subunits not associated with $\alpha 6$ subunits in the Hb-IPn pathway is in line with the findings of *in situ* hybridisation studies showing high levels of $\beta 3$ but not $\alpha 6$ subunit mRNA in the Hb [65, 66, 84]. This is a novel subtype because ganglionic

1 $\alpha 3\beta 4$ *nAChRs contain $\alpha 5$ as an accessory subunit [85]. $\beta 3$ subunit mRNA is not expressed in
2 the IPn [66] and so nAChRs containing $\beta 3$ cannot be synthesised in the IPn.

3
4 Among the subtypes present in the Hb-IPn pathway, only the $\alpha 3\beta 4$ and $\alpha 3\beta 3\beta 4$ subtypes
5 mediate [^3H]-ACh release in mouse IPn. The deletion of the $\beta 3$ subunit gene does not affect the
6 level of expressed subtypes in the Hb, but decreases the number of $\alpha 3$ and $\beta 4$ subunits in the IPn
7 by ~50%, and similarly decreases the [^3H]-ACh release in mouse IPn with no obvious change in
8 EC_{50} . This underlines the fact that, as has been suggested in the case of the $\alpha 6\beta 2\beta 3$ * receptors in
9 the visual and mesostriatal pathways, the $\beta 3$ subunit may play a targeting role in the Hb [13, 37].

10
11 The role of the other subtypes in the Hb-IPn system is difficult to establish, but studies of
12 Rb^+ efflux from IPn synaptosomes indicate that $\beta 2$ * nAChRs are functional [64]. These
13 receptors are not involved in ACh release, and may modulate the release of other
14 neurotransmitters in non-cholinergic inputs to the IPn [86, 87].

15
16 In Hb synaptosomes, Rb^+ efflux indicates that $\beta 2$ * nAChRs may be the only functional
17 presynaptic subtype [64] and may mediate the release of several neurotransmitters as
18 dopaminergic [88], GABA, glutamatergic [89] and noradrenergic terminals [90] have all been
19 identified in this nucleus.

20
21 The IPn contains $\alpha 2\beta 2$ *, $\alpha 3\beta 2$ * and $\alpha 4\beta 2$ * nAChRs. The $\alpha 2\beta 2$ * nAChRs are expressed
22 by intrinsic IPn neurons because $\alpha 2$ mRNA is only expressed by the IPn [91]. The
23 $\alpha 3\beta 2$ *nAChRs may only be located presynaptically as $\alpha 3$ mRNA is not expressed by intrinsic
24 neurons, whereas $\alpha 4\beta 2$ * nAChRs may be expressed on intrinsic IPn neurons and/or afferents to
25 the IPn, and may therefore have both pre- and postsynaptic locations (see lower part figure 1).

26
27 In conclusion the essential role of $\alpha 3\beta 4$ and $\alpha 3\beta 4\beta 3$ in mediating ACh release has
28 been established (see figure 1) but many more studies using different approaches are necessary to
29 define the cellular and subcellular localisation and function of all of the subtypes identified in the
30 Hb-IPn pathway.

31 **6.3. Homomeric and heteromeric $\alpha 7$ receptors**

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33 Homomeric $\alpha 7$ receptors are the most widely expressed α Bgtx-binding receptors in mammalian
34 brain, particularly in the cortex, hippocampus and subcortical limbic regions, and (at low levels)
35 in the thalamic regions and basal ganglia. These $\alpha 7$ receptors may have a presynaptic
36 localisation where they are involved in the direct release of glutamate in the hippocampus and
37 VTA, and of excitatory amino acid in the prefrontal cortex [92], or the indirect release of DA
38 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.

α Bgtx receptors have been affinity purified from the brain of various species, and are pentamers with a single $\alpha 7$ subunit in rat and chick, and homomeric $\alpha 8$ or $\alpha 7$ - $\alpha 8$ receptors in chick [14]. Studies using heterologous systems have shown that the $\alpha 7$ subunit can also form functional channels with the $\alpha 5$, $\beta 2$ or $\beta 3$ subunits [41, 54, 55]. Its particular association with the $\beta 2$ subunit leads to the expression of an $\alpha 7\beta 2$ receptor with distinct pharmacological and functional properties [41, 54, 55]. It has recently been shown that the $\alpha 7$ and $\beta 2$ subunits are co-expressed in rat basal forebrain cholinergic neurons and form a novel heteromeric $\alpha 7\beta 2$ subtype [96]. This subtype has different biophysical and pharmacological properties from those of the homomeric $\alpha 7$ receptors expressed by VTA neurons and is highly sensitive to functional inhibition by oligomeric forms of amyloid A β 1-42. Characterisation of the $\alpha 7^*$ receptors present in the forebrain cholinergic neurons of $\beta 2$ Ko mice has further confirmed that the expressed $\alpha 7\beta 2$ subtype has different biophysical and pharmacological properties from those of wild-type basal forebrain neurons, thus indicating that the $\alpha 7\beta 2$ 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 $\alpha 7$ subtype is more susceptible to inactivation than the $\beta 2^*$ or $\beta 4^*$ 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 $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ or $\beta 2$ 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].

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In vitro studies of cells transfected with nAChR subtypes have shown the nicotine-induced up-regulation of homomeric $\alpha 7$, and various heteromeric $\alpha 3\beta 2$, $\alpha 4\beta 2$, $\alpha 6\beta 2$ and $\alpha 3\beta 4$ receptors [101] [102, 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 $\alpha 4\beta 2$ 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 K_d 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 $\alpha 4\beta 2$ 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 $\alpha 5$ subunit makes the $\alpha 4\beta 2$ 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 $\alpha 7$ receptors, which have lower nicotine affinity, are up-regulated to a lesser extent than the $\alpha 4\beta 2$ subtype and in only a few regions [108]. The $\alpha 3\beta 4^*$ 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 $\alpha 4\beta 2$ 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 $\alpha 7$ and $\alpha 3$ subtypes in oocytes, leads to the almost total functional inactivation of the $\alpha 7$ receptors, but only partial inactivation of the $\alpha 3$ subtype, [109]. On the contrary, in cultured cortical neurons, there is an increase in the number of $\alpha 7$ 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 $\alpha 6^*$ receptors, which bind α conotoxin MII. Rodent studies have shown that intravenous nicotine self-administration leads to an increase in the number of $\alpha 6^*$ receptors in rats [114], but a decrease in the number of striatal $\alpha 6^*$ 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 $\alpha 6^*$ nAChRs. The results of a recent experiment has added further complexity. It was found that the decrease in overall α conotoxinMII binding observed after oral nicotine administration is due to two opposite effects on different $\alpha 6^*$ subtypes: a decrease in the $\alpha 4\alpha 6\beta 2^*$ subtype and an increase in the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ subtype [118]. Accordingly, $\alpha 4^{-/-}$ mice chronically treated with oral nicotine show increased α conotoxinMII binding in striatum [118].

As mentioned above, the $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ subtypes are the main subtypes modulating dopamine release in rodent striatum. *Ex vivo*, studies have shown that $\alpha 6\beta 2^*$ receptors mediate 30-50% of ^3H -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 $\alpha 6(\text{non}\alpha 4)\beta 2$ subtype but leads to the loss of $\alpha 4\alpha 6\beta 2$, abolishes $\alpha 6^*$ mediated effects on burst firing, thus suggesting that is the former subtype that primarily modulates this type of $\alpha 6\beta 2^*$ -mediated dopamine release [118].

Studies of non-human primates have shown that, although both $\alpha 4\beta 2$ and $\alpha 3/\alpha 6\beta 2^*$ 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 α conotoxinMII-resistant ^{125}I -epibatidine binding (putative $\alpha 4\beta 2^*$ receptors) without changing ^{125}I - α conotoxinMII binding ($\alpha 3/\alpha 6\beta 2^*$ receptors) in both the dorsal and the ventral putamen. It would be interesting to assess whether the regional heterogeneity in nicotine-

1 mediated dopamine release stems from alterations in $\alpha 6^*$ composition, as has been shown in
2 rodents.

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4 Although chronic nicotine exposure decreases the number of $\alpha 6^*$ receptors in the
5 striatum, it has no effect on those in the superior colliculus, where they are highly expressed, and
6 does not change the number of $\beta 3^*$ receptors in either region [116] notwithstanding the fact that
7 $\alpha 6^*$ receptors contain the $\beta 3$ subunit in both [67, 83]. This suggests that $\alpha 6\beta 3^*$ receptors are
8 more resistant to nicotine induced down-regulation than $\alpha 6(\text{non}\beta 3)^*$ receptors, or that the $\beta 3^*$
9 receptors assemble with other α subunits.
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16 It is not known why nicotine has such different effects on $\alpha 6^*$ receptors. *In situ* hybridisation and
17 single-cell PCR studies have shown that there is a mixture of $\alpha 4$, $\alpha 6$, $\beta 2$ and $\beta 3$ subunits in
18 midbrain dopaminergic neurons [65, 120]. If the number of $\beta 2$ subunits is limited, it is possible
19 that nAChR subunits compete for assembly in the endoplasmic reticulum of these neurons. By
20 acting as a preferential chaperone on $\alpha 4\beta 2$ receptors, nicotine may favour their formation and
21 thus decrease the pool of $\beta 2$ subunits available for assembly with the $\alpha 6$ subunit (see also [118]
22 for a similar hypothesis). In the case of the superior colliculus, it is not known whether the $\alpha 4$,
23 $\alpha 6$, $\beta 2$ and $\beta 3$ subunits are present in the same retinal ganglionic cells, and so it is possible that
24 there is no competition between $\alpha 4$ and $\alpha 6$ subunits.
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34 The role of nAChR up-regulation in inducing and/or maintaining nicotine dependence is still
35 uncertain. Up-regulation can be achieved in animals by means of administration routes that are
36 different from those of smokers and/or as nicotine doses that are much higher. Yet, it has been
37 shown that smokers have higher cortical levels of nicotinic agonist binding and $\alpha 4$ subunit than
38 non-smokers [121, 122]. As the level of up-regulated subtypes persists for days after nicotine
39 administration is stopped, it is possible that nAChR up-regulation plays a role during nicotine
40 withdrawal. Decreased nicotine levels can allow nAChRs to recover from desensitisation and be
41 more responsive to endogenous ACh, which may contribute to withdrawal symptoms or craving.
42 This possibility is also suggested by the results of neuroimaging studies showing that smokers
43 have high $\beta 2^*$ receptor levels for at least seven days after stopping smoking [123]. The levels of
44 $\beta 2$ nAChRs do not correlate with the severity of dependence or withdrawal, but only with the
45 urge to smoke in order to relieve withdrawal symptoms.
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56 The vast majority of the different effects of nicotine are determined by the functional features
57 and location of the nAChR subtypes with which it interacts in specific neuronal circuits, but
58 recent findings have shown that nicotine affects not only the number of nAChRs, but also that of
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1 other receptors and proteins important for the development of synaptic plasticity, either by
2 partially blocking the proteasome, or through other mechanisms [98, 104, 124, 125]. It therefore
3 seems that nicotine induces behavioural effects via a complex interplay of different signal
4 transduction pathways that can be different in different nervous circuits.
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8. Conclusions

10 The fine molecular structure of nAChRs has been better clarified over recent years mainly as a
11 result of very different methodological approaches.
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14 It has been shown that there is a substantial number of native subtypes, although native
15 nAChRs are assembled into functional pentamers made up of a relatively restricted number of
16 subunit combinations. Moreover, the *in vivo* characterisation of new and unsuspected subtypes
17 (heteromeric $\alpha 7\beta 2$) has increased the complexity of studying native subtypes.
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20 Much has been learned concerning the role of accessory subunits in the function and
21 pharmacology of the different subtypes, but we still do not know their precise role in nAChR
22 localisation and traffic and the subunit stoichiometry of the heteromeric subtypes remains a
23 largely unsolved question.
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26 Finally the mechanisms by which chronic nicotine affects the function and number of
27 native subtypes are still elusive and this will be an important future step in our understanding of
28 the role of nAChRs in tobacco dependence and neuropsychiatric diseases.
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1 **Figure 1**

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4 **nAChR subtypes in the habenula -interpeduncular pathway**

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7 Upper panel: A simplified illustration of the habenulo-interpeduncular pathway highlighting its
8 cholinergic (red) and non-cholinergic components (blue).

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11 Lower panel: Subunit composition and putative stoichiometry of the main nAChR subtypes
12 expressed in the presynaptic cholinergic ($\alpha 3\beta 4$ and $\alpha 3\beta 3\beta 4$) and non-cholinergic ($\alpha 3\beta 2^*$) IPn
13 terminals. The $\alpha 2\beta 2^*$ and $\alpha 4\beta 4^*$ receptors are localised on intrinsic IPn neurons, whereas the
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