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Heterogeneity and complexity of native brain nicotinic receptors

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ABSTRACT

Neuronal cholinergic nicotinic receptors (nAChRs) are a heterogeneous class of cationic channels that are widely distributed in the nervous system that have specific functional and pharmacological properties. They consist of homologous subunits encoded by a large multigene family, and their opening is physiologically controlled by the acetylcholine neurotransmitter or exogenous ligands such as nicotine. Their biophysical and pharmacological properties depend on their subunit composition, which is therefore central to understanding receptor function in the nervous system and discovering new subtype-selective drugs.

We will review rodent brain subtypes by discussing their subunit composition, pharmacology and localisation and, when possible, comparing them with the same subtypes present in the brain of other mammalian species or chick.

In particular, we will focus on the nAChRs present in the visual pathway (retina, superior colliculus and nucleus geniculatus lateralis), in which neurons express most, if not all, nAChR subunits. In addition to the major $\alpha4\beta2$ and $\alpha7$ nAChR subtypes, the visual pathway selectively expresses subtypes with a complex subunit composition. By means of ligand binding and immunoprecipitation and immunopurification experiments on tissues obtained from control and lesioned rats, and wild-type and nAChR subunit knockout mice, we have qualitatively and quantitatively identified, and pharmacologically characterised, the multiple complex native subtypes containing up to four different subunits.

1. Introduction

By mimicking the endogenous neurotransmitter acetylcholine (ACh), nicotine acts on a variety of neuronal nicotinic acetylcholine receptor (nAChR) subtypes widely distributed in the central and peripheral nervous systems, and has different effects on a number of brain functions (ranging from cognitive enhancement to reinforcement) depending on the subtype with which it interacts, and the localisation of the target nAChR subtypes in the neural circuits [1–5].

nAChRs belong to the large superfamily of homologous receptors that also includes muscle-type AChRs, and the $GABA_A$, $GABA_C$, glycine and serotonin $5HT_3$ receptors[6,7].

The first studies designed to characterise nAChRs were based on binding assays using nicotinic radioligands in different brain areas and these studies demonstrated that at

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least two classes of putative nAChRs exist in the nervous system: one consisting of receptor molecules that bind ^{3}H-nicotinic agonists with nM affinity but not αBungarotoxin (αBgtx), and the other of receptor molecules that bind nicotine and nicotinic agonists with microM affinity, and αBgtx with nM affinity (reviewed in [8]). The pharmacological heterogeneity revealed by these ligand studies was later confirmed and extended by means of the molecular cloning of a family of genes encoding twelve subunits differentially distributed in the CNS [6].

nAChR subtypes consist of five subunits assembled in the plasma membrane to form a channel and their variety is mainly due to the diversity of the genes encoding the subunits.

Twelve nAChR subunit genes with a common ancestor have so far been cloned and classified into two subfamilies of nine alpha (α2–α10) and three beta subunits (β2–β4). Two main classes of nAChR subtypes have so far been identified: the homomeric or heteromeric αBgtx-sensitive receptors, which are made up of the α7, α8, α7–α8, α9 and/or α10 subunits, and the heteromeric αBgtx-insensitive receptors consisting of the α(α2–α6) and β(β2–β4) subunits [3]. The αBgtx-insensitive nAChRs, consist of combinations of α_2, α_3, α_4 or α_6 subunits with either β_2 or β_4 (e.g., $\alpha_4\beta_2$ and $\alpha_3\beta_4$) or with addition of α_5 and β_3 subunits (e.g., $\alpha_4\alpha_2\beta_3$). The α and β subunits both contribute towards the pharmacological properties of the receptor binding site(s). The binding site has principal and complementary components, and lies at the interface between two identical subunits in homomeric α7 receptors, or between an α (α2, α3, α4 or α6) and a β (β2 or β4) subunit in the case of heteromeric αBgtx-insensitive receptors. The α5 and β3 subunits do not carry the primary or the complementary components of the ACh binding site, and it is generally assumed that they do not directly participate in its formation. It is thought that homomeric receptors have five identical ACh-binding sites per receptor molecule (one on each subunit interface), and the hetero-meric receptors two binding sites per receptor (reviewed in [7]) (Fig. 1B).

When defining the receptor subtypes, we use an asterisk to indicate the possible presence of unidentified nAChR subunits, otherwise all subunits are given and the subtype exactly defined.

nAChRs are permeable to the monovalent Na^+ and K^+ ions, and also to Ca^{2+}. The cation permeability of the subtypes is influenced by their subunit composition (reviewed in [9]).

nAChR activation excites target cells and mediates fast synaptic transmission (e.g., in autonomous ganglionic neurons and restricted brain areas), but anatomical and functional evidence suggests that nAChRs are preferentially located at the preterminal and presynaptic sites regulating neurotransmitter secretion in several brain regions. In particular, presynaptic nAChRs have been found to be involved in the release of ACh, noradrenaline (NA), dopamine (DA), glutamate and GABA (reviewed in [10]) (Fig. 1A). Modulation of the release of all these neurotransmitters can explain why nicotine has such diverse and often profound effects even though the expression of nAChRs in the nervous system is not very high in comparison with other neurotransmitter receptors (reviewed in [11,12]).

The exact stoichiometry of native heteromeric nAChRs is still unclear. Biochemical and electrophysiological studies showed that the chick α4β2 and human α4β2 and α3β4 subtypes have a stoichiometry of 2α and 3β when expressed in oocytes or cells injected with cRNAs or cDNAs in a ratio of 1/1 (α/β) (reviewed in [3]). Moreover, recent studies in oocytes have shown that changing the α4/β2 cDNA ratio leads to a variety of subtypes: a ratio of 1:10 favours the expression of $(\alpha4)_2(\beta2)_3$ subtype that is highly sensitive to ACh, a ratio of 10:1 favours the expression of the $(\alpha4)_3(\beta2)_2$ subtype, which is less sensitive to ACh [13,14]. The $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ subtypes also have different Ca^{2+} permeability with the latter having much higher Ca^{2+} permeability than the former [15]. Extreme conditions such as exposure to low temperature, chronic exposure to nicotine, or boosting the number of β2 subunits by means of additional transfection also favour the expression of the highly ACh sensitive $(\alpha4)_2(\beta2)_3$ subtype [13,16]. However,

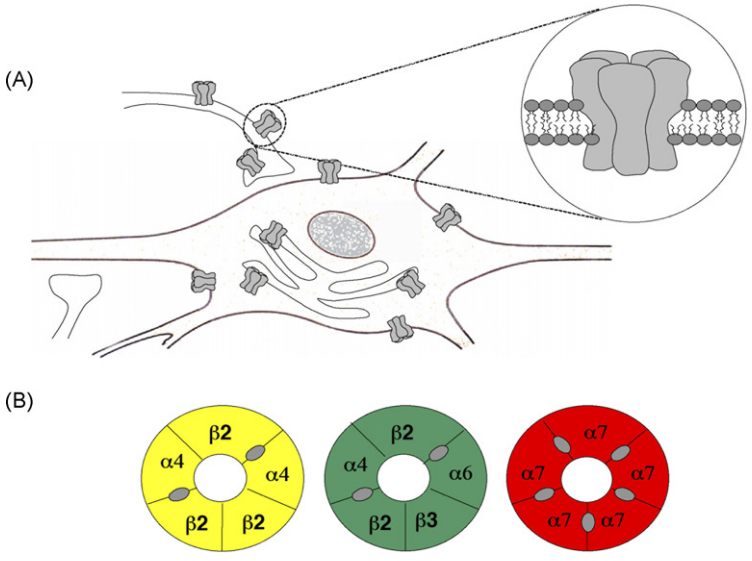

Fig. 1 – Localisation and subunit composition of three nAChR subtypes. (A) Illustration of the possible cell localisation of nAChR subtypes: presynaptic, preterminal and postsynaptic. (B) Subunit arrangement and localisation of the ACh binding sites in homomeric α7 subtype, and heteromeric α4β2 subtype or α4α6β3β2 subtype.

although there is some evidence of the presence of different stoichiometries of the brain α4β2 subtype they have not yet been clearly demonstrated biochemically.

We will now critically discuss the methods used in identifying expression pattern of native receptor subtypes and their caveats and then summarise data on the identification and characterisation of native nAChR subtypes in the various areas of the CNS. Moreover as an example of receptor complexity we will report the results obtained in the characterisation of nAChRs in the visual pathway an area where all known neuronal subunits are expressed and an important role of nAChRs both during development and postnatally has been demonstrated [2,4,6,9,17–20].

2. Caveats and tools used to characterize native subtypes

Studies aimed at characterising the structure and composition of native nAChR subtypes have been complicated by the large number of nAChR subtypes, the expression of multiple subtypes within a given tissue or cell type, and the lack of subtype specific pharmacological tools.

The large majority of nAChRs ligands are not selective as the majority of classical nicotinic agonists (e.g., epibatidine, nicotine and cytisine) are not subtype specific *in vivo*. They do have different rank orders of potency on the different α/β combinations of heterologously expressed receptors, but this is not sufficient to discriminate the various subtypes *in vivo*. Recently a new agonist A8530 that binds with high affinity β2* but not β4* subtypes[21] has been developed. The competitive antagonist dihydro-β-erythroidine has sub-micromolar affinity for α4β2 and α3β2 and it is 10–50 times less potent on α3β4 and α7 receptors expressed in oocytes. Mecamylamine is a non subtype-specific noncompetitive antagonist that penetrates CNS very well and, for this reason, is frequently used in behavioural studies. The αBgtx antagonist is specific for α7*, α8*, α9*, and α10* receptors, but cannot distinguish between them. Some snail toxins such as MII or PIA αconotoxins are selective for the α6/α3β2* receptors (reviewed in [21]).

However the use of genetically engineered knockout (Ko) or knockin (Kin) mice in which one or more nAChR subunit genes of interest are silenced or mutated makes it possible to analyse the structure, pharmacology and functional role of native nAChRs in complex neurobiological systems, and our group has devised a powerful complementary approach by preparing a series of antibodies (Abs) that specifically recognise all the cloned subunits of native nAChR subtypes [3,10].

The specificity of our Abs has been checked in various ways. Quantitative immunoprecipitation or immunopurification experiments using nAChRs from different areas of the CNS of wild type (WT) and Ko mice allowed us to select Abs specific for the subunit of interest, and establish the immunoprecipitation capacity of each of them [22–24]. Their specificity was also tested by Western blotting which showed that some of the Abs were less specific for this technique than in the immunoprecipitation experiments. We found that specificity is not only sequence related (the same peptide can be used to raise Abs with different degrees of specificity in different rabbits) but could also change over time in the same rabbit.

The specificity of the anti-peptide Abs have proven relatively poor in immunocytochemical experiments in which very few specific Abs, have been confirmed. This situation also has been pointed out by other researchers who used Abs in Western blotting and immunocytochemistry [25,26].

The fact that many Abs are highly specific in immunoprecipitation, but not Western blotting and/or immunohistochemistry, experiments is possibly due to the relatively low subunit expression in the brain (too low for accurate detection by Western blotting or immunohistochemistry) or to the fact that in immunoprecipitation experiments the antigens are initially selected by nAChR subunit binding to radiolabelled ligands. If an Ab recognises both nAChR and non-nAChR epitopes, this cannot affect the immunoprecipitation of ^{3}H-epibatidine radiolabelled receptors (the only dangerous cross-reactivity for this technique is that between different subunits), whereas it may greatly affect the Western blot and/or immunocytochemistry experiments.

Specificity problems were also encountered in studies on subunit mRNA regional and cellular localisation. Several *in situ* hybridisation (ISH) studies on nAChR subunit mRNAs were performed with hydrolysed riboprobes or digoxigenin-labeled riboprobes. Although these approaches may have higher sensitivity and cellular resolution than ISH with oligoprobes, probe specificity is difficult to determine and these techniques are more prone to false positivities (see e.g. [27]). Therefore, in the following section we will divide ISH studies on the basis of the probe used.

RT-PCR and single-cell PCR techniques are highly sensitive and specific, but give only all-or-none results. They may therefore overestimate the relevance of certain subunits (e.g., compare the data obtained using oligoprobes, riboprobes and single-cell PCR for α2, α3 and α6 in midbrain DA neurons, with those obtained in α3 and α6 Ko mice and immunoprecipitation studies [24,27–31].

3. Native nicotinic acetylcholine receptor subtypes

The early pharmacological and electrophysiological studies of β2 Ko mice led to the identification of four subtypes of nAChRs in the mouse brain [32]. The availability of new Ko mice, new pharmacological tools and new purification techniques has helped define the subunit composition of a much larger number of native nAChRs. The results obtained so far provide a very complex, although possibly still incomplete, picture of brain nAChR subtypes and open up new insights into nicotinic cholinergic functions in normal and pathological brain.

We will here describe the major rodent brain subtypes by discussing their subunit composition, pharmacology and localisation and, when possible, comparing them with the same subtypes present in the brain of other mammalian species or chick.

3.1. *α2* receptors*

α2 mRNA has a limited expression pattern in rodent brain, with a moderate signal in the retina and interpeduncular nucleus (IPN) as detected with oligoprobes [27,33].

In addition, studies using ISH with riboprobes have shown intense signal in cells of the olfactory bulb, dorsal and ventral tegmental nuclei of the pons, median raphe, bulbar reticular formation and ventral horn of the spinal cord, and moderate to weak signal in scattered cells of many brain regions (e.g., some amygdaloid nuclei, medial septum and basal forebrain nuclei, inferior and superior colliculus, some hippocampal subregions, cerebral cortex [28,34–37].

In view of the importance of this region for nicotine addiction and other neuropsychiatric diseases, we discuss in more detail α2 mRNA distribution in midbrain dopaminergic neurons. ISH with oligoprobes did not reveal any signal in this region [27], while digoxigenin-labeled riboprobes showed few positive cells in the ventral tegmental area (VTA) [35] and hydrolysed riboprobes showed positive signal in almost all dopaminergic neurons [28]. Evidence from other technical approaches support the most conservative ISH approaches, since α2 mRNA was not detected in any dopaminergic neuron using single cell PCR technique [31] and α2 protein was not detected in dopaminergic striatal terminals using immunoprecipitation technique [30]. Moreover, nicotinic binding in the mesostrial system and nicotine-elicited dopamine release from striatal synaptosomes were not detected in double α4/α6 knockout mice [24]. In primates, the distribution of α2 mRNA is much more widespread and is comparable to that of α4 mRNA [38]. Its distribution in chick is restricted to the lateral spiriform nucleus, but this is not homologous to rodent IPN.

Immunopurification experiments have shown the presence of α2α6β2* α2α4β2*and α2β2*subtypes in retina [33], and an α2β2* subtype in the IPN (Gotti and Zoli unpublished results). The α2* subtype is present very early in rat retina, and its expression is highly regulated during postnatal development. It is selectively transported from retinal ganglionic cells to a retinal target region, the nucleus geniculatus lateralis (LGn), but not to another target region, the superior colliculus (SC) [22] (Fig. 2).

In agreement with the mRNA data, cortical α2* subtype expression is species-specific among mammals, being undetectable in rodent but present in 21% of the high affinity epibatidine receptors in monkey, where it forms an α2α4β2* subtype [39]; it is also present in 10% of the receptors in Broadman area 21 of human cortex [40] An α2α5β2 subtype is highly expressed in chick optic lobe, where it is closely regulated developmentally [41] but there is still no evidence of its existence in mammals. The pharmacological profile of the purified α2α5β2 subtype is very similar to that of the α4β2 subtype purified from the same tissue.

3.2. α3* receptors

α3 mRNA is expressed at high to very high levels in the pineal gland, medial habenula (MHb), dorsal nucleus of the vagus nerve, retinal ganglionic neurons and at low to intermediate levels in the anterior thalamus, nucleus of the solitary tract, area postrema, dopaminergic ventral midbrain and noradrenergic locus coeruleus [29,32,33,37,42]. ISH with riboprobes reveals a much wider distribution, including several cortical areas and hippocampal or parahippocampal regions, several thalamic and hypothalamic nuclei, and the brainstem motor nuclei [34].

Pharmacological studies of heterologously expressed α3β2 and α3β4 subtypes have shown that both bind the agonist epibatidine with high affinity, but the α3β4 subtype has a lower affinity for the agonists cytisine and A85380, and the antagonist αconotoxin MII (αCntxMII) [43].

*α3β2**

This subtype has been identified in the visual pathway (in the retina and in its target tissues, SC and LGn), and represents an important fraction (up to 11%) of the epibatidine binding sites in SC. It seems to be preferentially transported from the retina to these target regions, as eye-enucleation leads to its disappearance from the SC [22] (Fig. 2). It also contains the α5 and/or β3 subunits. SC α3β2* receptors bind αCntxMII with high nanomolar affinity (50 nM), which is about 50 times lower than the affinity of the native α6β2* (1 nM) subtype [22].

Sequential immunoprecipitation experiments have recently identified an α3β2* subtype in rat cerebellum and retina [44,45], and in monkey [39], but not in rodent, striatum [30]. In this latter region, it is reduced by around 50% after dopaminergic denervation with the toxin MPTP [39].

*α3β4**

The pineal gland expresses a high level of epibatidine receptors, virtually all of which contain the α3 and β4 subunits [46]. The affinities of nicotinic drugs for these native binding sites are very similar to those for heterologously expressed receptors [46]. α3β4 and α3β2β4 subtypes have also been identified in the cerebellum [44], retina [45], IPN and MHb (Gotti and Zoli, in preparation). After immunodepleting β2* containing receptors from the MHb and IPN, we have also identified an α3β3β4 subtype that is selectively present in this habenulo-interpeduncular pathway (Gotti and Zoli, in preparation).

In the hippocampus, the α3β4* subtype is involved in NA release because the αconotoxin AuIb (which is specific for the α3β4 subtype in heterologus systems) blocks 20–35% of nicotine-stimulated NA release in hippocampal synaptosomes [47]. It may also play a major role as an autoreceptor in the neurons of the habenulo-interpeduncular pathway, as there is no difference in nicotine-induced ACh release from the IPN between WT and β2 Ko mice [48].

It has been reported that a recently discovered conotoxin (BuIA) blocks both α3β2* and α3β4* subtypes [8] but its unblocking kinetics are much lower for the α3β4* than the α3β2* receptors [49].

3.3. α4* receptors

α4 subunit mRNA is present in the majority of brain areas, although less broadly distributed than the β2 subunit. The two subunits colocalise in most brain areas (with the highest levels in most thalamic nuclei, the isocortex, the dorsomedial hypothalamic nucleus and the ventral midbrain) and in the spinal cord of rodents [1,50], as well as of primates [35].

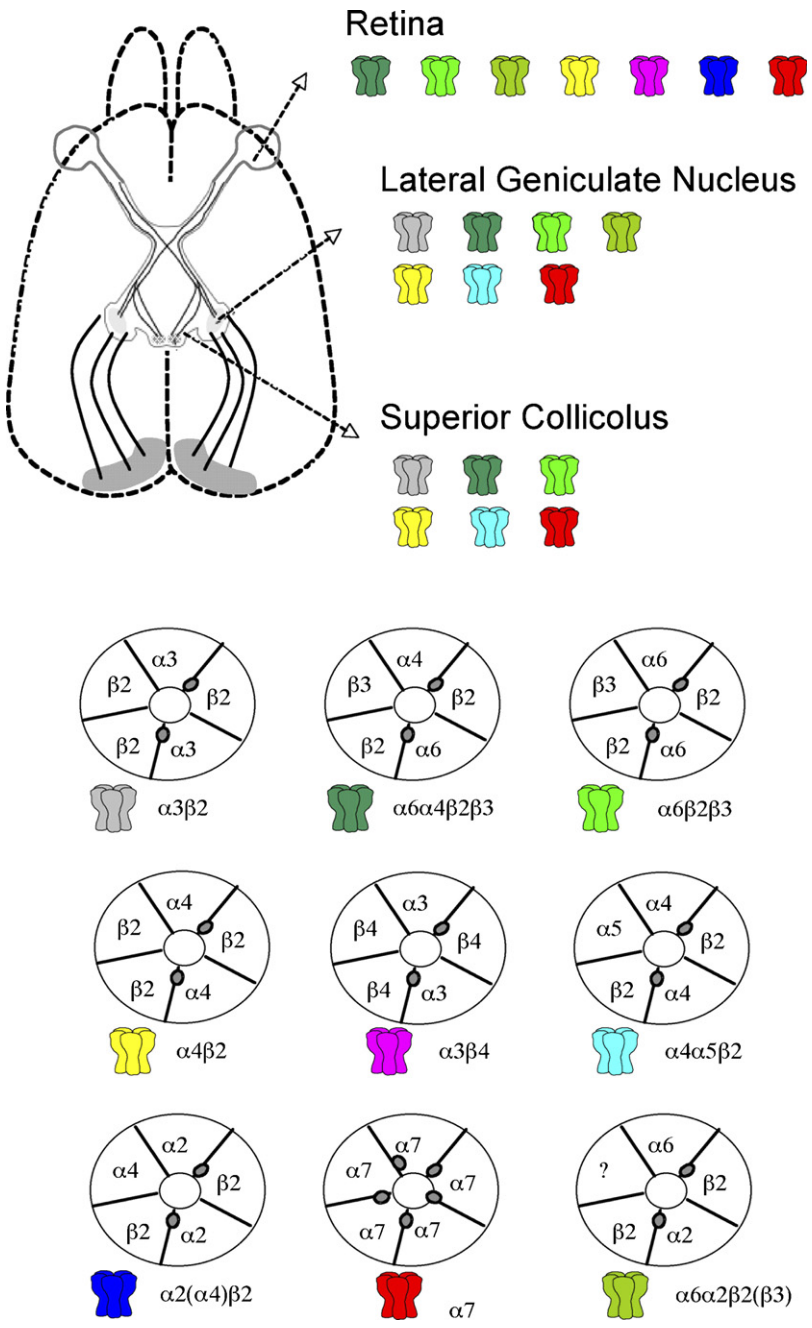

Fig. 2 – nAChR subtypes in the retina and its projections to the superior colliculus (SC) and nucleus geniculatus lateralis (LGn). (Upper panel) A simplified illustration of the retinal pathway. (Lower panel) Subunit composition and putative stoichiometry of the nAChR subtypes expressed in retina, retinal terminals and SC and LGn cell bodies according to the results reported in [22,33].

*α4β2**

The α4β2* subtype binds with high affinity to the most common nicotinic agonists, and knocking down the α4 or β2 subunits abolishes high affinity nicotinic agonist binding in most brain regions [1]. It is the subtype that is most strongly up-regulated by nicotine exposure, so that chronic nicotine treatment up-regulates markedly the number of α4β2* subtypes in rodent brain and mimicks the effect observed in the post-mortem brains of human smokers (reviewed in [17]).

This native subtype was the first to be biochemically and pharmacologically characterised in total rat brain (reviewed in [8]) and subsequently in specific subregions such as the cerebral cortex, striatum, SC, LGn and cerebellum [22,30,44]. In the striatum and cortex, a subpopulation of this subtype contains the α5 subunit. In striatum, the α4β2 subtype is present in both dopaminergic and non dopaminergic cells, whereas the α4α5β2 subtype is specifically expressed by dopaminergic terminals [30]. The α4β2 subtype is involved in mediating GABA release from thalamic slices and the GABA

cells innervating dopaminergic cells in the VTA, and DA release from striatal synaptosomes and slices (reviewed in [51]).

The α4β2* subtype is selectively reduced in the cortex of patients with Alzheimer's disease as measured by both binding and immunoprecipitation studies [40,52]

Both the α4β2 and α4α5β2 subtypes are present in chick brain, where their number is highly regulated during embryonic development [53].

*α4β4**

Although the α4 and β4 subunits form a functional channel in heterologous expression systems, there is still no evidence of the presence of this subtype in mammalian brain. It was first identified in chick retina, where it accounts for a relatively high proportion of the heteromeric αBgtx-insensitive receptors present on postnatal day 1 and, after immunopurification, its pharmacological profile was shown to be almost identical to that of the heterologously expressed subtype [54]. Based on binding studies in β2 Ko mice, it was suggested the presence of α4β4 subtype in the IPN [31], and recent immunoprecipitation results suggest that it may be present in rat cerebellum and retina [44,45].

3.4. *α6* receptors*

α6 subunit mRNA is highly expressed in catecholaminergic nuclei (substantia nigra (SN), VTA and locus coeruleus), and a few other regions including retina and to (a lesser extent) the thalamic reticular nucleus. These are all regions in which it colocalises with the mRNA for the β3 subunit [24,27,37,55].

Genetic deletion of the α6 subunit does not change the level of mRNA for the α3, α4, α5, α7, β2 and β4 subunits, but leads to the almost complete disappearance of high affinity αCntxMII binding and the partial loss of high affinity epibatidine binding in the visual pathway [56].

α6α4β2β3 and α6β2β3

In agreement with ISH studies, immunopurification experiments have identified two major α6* subtypes in rodent striatum [24,30], rat retina [33] and SC [22], α6α4β2β3 and α6β2β3, with the former representing 40–60% of all α6* receptors. These two subtypes have indistinguishable binding affinities for various classical nicotinic agonists and antagonists, but different binding affinities and sensitivity for αCntxMII and methyllycaconitine (MLA) [24,30]. Competition binding experiments on striatal α6* receptors from WT or α4 Ko mice show a biphasic αCntxMII displacement of epibatidine binding with a high and low affinity site in WT mice (α4α6β2β3 and α6β3β2 subtypes), but only a single high-affinity site in α4 Ko mice (subtype α6β2β3) [24]. This indicates that one of the two epibatidine binding sites in a fraction of WT striatal α6* receptors (subtype α4α6β2β3) is at the α4β2 interface with a low affinity for αCtxMII and MLA, whereas the other (with high affinity for the αCtxMII and MLA) is at the α6β2 interface which is the only interface in the α6* receptors of α4 Ko mice.

Studies of β3* Ko mice have confirmed that β3 subunit does not participate to the high affinity αCntxMII binding site because, although β3 subunit deletion greatly reduces the expression of α6* receptors, the residual α6β2* receptors maintain the same affinity and sensitivity to the toxin [23,55,57].

The α6* receptors in the mesostriatal pathway are presynaptic and localised on dopaminergic cells because striatal DA denervation with the selective toxin 6-hydroxydopamine, leads to the disappearance of the α6α4β2β3 and α6β2β3 subtypes, as well as αCntxMII binding [24,30].

α6* receptors mediate αCntxMII-sensitive DA release from striatal terminals, but do not seem to be involved in the DA release induced by systemic nicotine because *in vivo* microdialysis studies of freely moving mice have shown no difference in basal DA levels or in DA level changes elicited by systemic nicotine treatment in the ventral striatum of WT and α6 Ko mice [55,58].

α4α6β2β3 and α6β2β3 subtypes are also present in the retinorecipient areas, SC and LGn, where they are expressed by terminals of retinal ganglion cells since eye-enucleation leads to their complete disappearance [22] (see below).

Striatal α6* receptors seem to be conserved across species as α4α6β2β3 and α6β2β3 subtypes with similar αCntxMII binding affinity and functional properties are expressed on dopaminergic terminals in monkey, rodent and human caudate [39,40,59–61]. Moreover ligand binding and immunoprecipitation studies have shown that there is a selective reduction of αCntxMII and A85380 binding sites [62] together with a loss of α6β3* receptors [40] in the striatum of Parkinsonian patients.

The available evidence shows that α6* nAChRs in both the visual [33] and mesostriatal [24] pathways are enriched on nerve terminals rather than in the cell body/dendrite compartment thus suggesting that the α6 subunit or another subunit regularly associated with α6 (e.g., β3) influences receptor targeting. This may also be the case for the α5 subunit in some α4* nAChRs [24]. Support for a targeting role of β3 subunit comes from studies of β3 Ko mice showing that α6* nAChR expression is significantly reduced in both the DA cell body (ventral midbrain) and terminal (striatum) regions, but more markedly in the latter (76% versus 42% in the ventral midbrain) [23].

3.5. *α7 receptors*

The localisation of nAChRs containing the α7 subunit is less controversial, because the almost irreversible and specific binding of αBgtx has allowed their clear and precise localisation at both cellular and subcellular level (reviewed in [1]). They are highly expressed in brain, particularly in the cortex, hippocampus and subcortical limbic regions, and at low levels in the thalamic regions and basal ganglia. The distribution of α7 mRNA and αBgtx binding is wider in primate brain [35,38] suggesting a more important role of these receptors in primates.

αBgtx receptors have been affinity purified from the brain of various species, thus confirming that they are pentamers with a single α7 subunit in rat and possibly the presence of α8 subunit in chick [8].

The α7 subtype undergoes rapid activation and desensitisation. It has a presynaptic localisation [63] (where it is

involved in the direct, glutamate [10,51], or indirect, NA, release of neurotransmitters [64] or a postsynaptic or somatic localisation, where its high calcium permeability can have long-term effects on metabolic pathways and gene expression.

Recent studies have shown that the α7 subunit can also form functional channels with the subunits of the αBgtx-insensitive subfamily in heterologous systems, but no biochemical evidence for such a receptor composition *in vivo* is yet available (reviewed in [3]). An α7 gene that incorporates a unique 87-base pair cassette exon has recently been identified: when expressed in Xenopus oocytes, it forms channels with a slower kinetics and reversible αBgtx binding [65]. Immunolocalisation studies suggest that this subtype constitutes a distinct subset of α7 receptors.

4. Nicotinic receptors expressed in the visual pathway

Neuronal nAChRs are highly expressed in the visual system, where they play important roles in the retina itself, and in retinal target tissues (reviewed in [66]).

In 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 [67–69]. β2 Ko mice have retinal waves with altered spatiotemporal properties and retinofugal projections to the dorsal LGn and SC that do not segregate into eye-specific areas [67]. Furthermore, recent anatomical and functional studies of LGn in β2 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 [70]. Ko mice also have reduced visual acuity and functional expansion of the binocular subfield of the primary visual cortex [67].

We and others have recently shown that αBgtx-sensitive and insensitive nAChRs are highly expressed in vertebrate retina (reviewed in [66]). The temporal pattern of expression and subunit composition of the principal subtypes is species-specific, and shows increased heterogeneity and complexity during development and in adulthood [33,71,72].

Using ligand binding and immunoprecipitation techniques with subunit-specific Abs, we analysed the subtypes expressed in the rodent retina e in the two retinorecipient areas, SC and LGn, and found that the retinal expression of αBgtx and high-affinity epibatidine receptors is developmentally regulated and increases until postnatal day 21 (P21). The increase in epibatidine receptors is due to a selective increase in the subtypes containing the α2, α4, α6, β2 and β3 subunits. Immunopurification studies revealed three major populations of epibatidine receptors at P21: α6* receptors (26%), which contain the α6β3β2, α6α4β3β2 and α6α3/α2β3β2 subtypes; α4(nonα6)* receptors (60%), which contain the α2α4β2 and α4β2 subtypes; (nonα4/nonα6)* receptors (14%), which contain the α2β2/β4 and α3β2/β4 subtypes.

Rat SC and LGn, express partially different nAChR subtypes, and analysis of the tissues of eye-enucleated rats allowed us to identifiy the subtypes that are expressed by retinal afferents. Three major nAChR subtypes were detected on retinocollicular afferents: α6β2* (about 45% of the total nAChRs on retinal afferents), α4α6β2* (about 35%) and α3β2* (about 20%). On retinogeniculate afferents, heteromeric nAChRs were more diverse including α4α6β2* (about 40%), α6β2* (about 30%), α4β2* (about 20%), α2α6β2* (about 5%) and α3β2* (about 5%). Some subtypes, such as α4(nonα6)β2* and α2α6β2* subtypes were only detected in the LGn.

The α7 subtype is expressed in both the retina and retinorecipient regions. Its number does not decrease after eye enucleation in target tissues, thus indicating that it does not have a presynaptic localisation in these regions.

Comparison of subtype expression in SC and LGn retinal afferents and retina [33] suggests that some nAChR subtypes are preferentially expressed on retinal axons rather than in the ganglionic cell body/dendrite compartment. The α3β2* and α6β2* subtypes seem to be preferentially transported to retinal nerve terminals, whereas the α2α4β2* and α4(nonα6)β2* receptors are minimally or not transported to the axonal compartment.

Similar compartmental segregation has been previously observed in midbrain DA neurons, that express a larger percentage of α6β2* nAChRs on striatal terminals than on midbrain cell bodies [24]. This suggests that the presence of the α6 subunit or a subunit regularly associated with α6 such as β3 preferentially targets this subtype to the axonal compartment (see above).

5. Concluding remarks

The data that native pentameric subtypes can consist of up to four different subunits indicate that the number of biologically relevant receptor subtypes is larger than previously thought, and this may have important functional and pharmacological implications. The presence of a certain subunit can modify

(i) receptor localisation (e.g., the β3 subunit seems to play a role in the localisation of α6 receptors in the mesostriatal DAergic pathway);
(ii) biophysical and functional properties (e.g., the presence of the α5 subunit in α4β2* or α3β4* subtypes greatly changes their Ca^{2+} permeability, response to nicotinic drugs and receptor desensitisation) [9];
(iii) pharmacological properties (e.g., the α6β2* and α4β2* subtypes respond very differently to αCntxMII [24,57];
(iv) the developmental or adult regulation of expression (e.g., by making available a large variety of promoters, as proposed by [27].

On the other hand, the lack of a subunit may lead to the loss of expression of one subtype and/or the compensatory up-regulation of others (in terms of number and/or function and/or expression of new subtypes not normally present in WT mice).

Knowing their exact subunit composition is a prerequisite for understanding the role of native nAChRs and rational design of new subtype-selective drugs.

We have reported the species differences in receptor subtype brain distribution, in particular between rodents

and primates. The scope of these remarks is to remind that a certain degree of specie specificity in the pattern of nAChR subtype regional expression is present and becomes more and more evident as reliable data appear on the subtypes distribution in primate brains. On the other hand, it seems that the major biophysical and pharmacological properties of nAChR subtypes are conserved among species. Therefore, the cellular and animal models of nAChRs are still extremely valid for the investigation of the functional role of nAChR subtypes and for testing new selective drugs in integrated systems.

However, it is possible that in humans some brain pathways or circuits express nAChR subtypes that are different from those present in rodents (e.g., $\alpha 2^*$ nAChRs) and/or that chronic drug treatments or pathologies affect the subtypes expressed in manners that are peculiar of the human brain. This aspect deserves more extensive investigations and must be taken into account in the development of new nicotinic drugs for the treatment of human diseases.

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