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Abstract: Neuronal nicotinic acetylcholine receptors (nAChRs) are a heterogeneous family of pentameric ligand-gated cation channels that are expressed throughout the brain and involved in a wide range of physiological and pathophysiological processes.The nAChR subtypes share a common basic structure, but their biophysical and pharmacological properties depend on their subunit composition, which is therefore central to understanding their function in the nervous system and discovering new subtype selective drugs.The development of new technologies and the generation of mice carrying deletions or the expression of gain-of-function nAChR subunits, or GFP-tagged receptor genes has allowed the in vivo identification of complex subtypes and to study the role of individual subtypes in specific cells and complex neurobiological systems but much less is known about which native nAChR subtypes are involved in specific physiological functions and pathophysiological conditions in human brain.We briefly review some recent findings concerning the structure and function of native nAChRs, focusing on the subtypes identified in the rodent habenulo-interpeduncular pathway, a pathway involved in nicotine reinforcement and withdrawal. We also discuss recent findings concerning the expression of native subtypes in primate brain.

Cover letter

Editorial Office of
Neuropharmacology

Dear sirs,

Ref. No.: NEUROPHARM-D-14-00758
Title: **Diversity of native nicotinic receptor subtypes in mammalian brain**

In reply to your letter of October 2 concerning the above cited manuscript, we carefully considered your and the reviewer's comments and changed the text accordingly (changes in the text are highlighted in red).

We do hope that you will now find the new version of the paper suitable for publication in Neuropharmacology and look forward to hearing from you in due course.

Yours faithfully,

Dr. Cecilia Gotti

Point by point reply to the referee

Some information concerning the structure and function of neuronal nIcotinic receptors are known from long time and to cite all the original data is almost impossible. For this reason we cited the most updated and important reviews. We have, however, added reviews from our groups to widen the perspective on these issues.

We have corrected all the mistakes that were present in the references and in the text as indicated by the reviewer.

***Response to Reviewers**

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Highlights

nAChRs are a heterogeneous family of pentameric ion channels that are ubiquitously expressed in the nervous system

The subunit composition of each nAChR subtype determines its distinct pharmacological, function and kinetics properties

nAChRs contribute to cognitive function, and changes in their expression or dysfunctions are involved in various neuropsychiatric diseases.

The cellular localization of nAChR subtypes in some neuronal circuits is remarkably conserved from rodents to primates suggesting that these subtypes play specific and important functional roles.

*Title page

Diversity of native nicotinic receptor subtypes in mammalian brain

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Abstract

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The nAChR subtypes share a common basic structure, but their biophysical and pharmacological properties depend on their subunit composition, which is therefore central to understanding their function in the nervous system and discovering new subtype selective drugs.

The development of new technologies and the generation of mice carrying deletions or the expression of gain-of-function nAChR subunits, or GFP-tagged receptor genes has allowed the *in vivo* identification of complex subtypes and to study the role of individual subtypes in specific cells and complex neurobiological systems but much less is known about which native nAChR subtypes are involved in specific physiological functions and pathophysiological conditions in human brain.

We briefly review some recent findings concerning the structure and function of native nAChRs, focusing on the subtypes identified in the rodent habenulo-interpeduncular pathway, a pathway involved in nicotine reinforcement and withdrawal. We also discuss recent findings concerning the expression of native subtypes in primate brain.

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1. Introduction

In 1905, Langley (Langley, 1905) reported that nicotine produces short-term stimulation followed by long-term blockade of skeletal muscle, and concluded that it interacted with a *receptive substance* expressed in skeletal muscle. Langley postulated his theory thirty years before it was demonstrated that acetylcholine (ACh) is present at neuromuscular junctions (Dale et al., 1936), but it was not until the early 1980s that the structure and functions of nicotine-binding receptors (AChR) were discovered.

Nicotine is a highly lipophilic compound present at high level in tobacco leaves which can be smoked, chewed, or sniffed. Once in the bloodstream, nicotine rapidly crosses the blood–brain barrier and accumulates in the brain (Hukkanen et al., 2005), where it binds with high affinity the neuronal nicotinic acetylcholine receptors (nAChRs). The nAChRs in the brain represent a heterogeneous family of ubiquitously expressed pentameric ion channels, whose responses to endogenous neurotransmitter ACh and exogenous nicotine are involved in a number of physiological processes and pharmacological effects (Albuquerque et al., 2009; Dani and Bertrand, 2007; Hurst et al., 2013).

nAChR activation excites target cells and mediates fast synaptic transmission in autonomous ganglionic neurons and in some brain areas. Anatomical and functional evidence suggests that most of these receptors have a presynaptic and/or preterminal localisation, and modulates the release of almost all neurotransmitters, but some also have a somatodendritic post-synaptic localization (Albuquerque et al., 2009; Gu and Yakel, 2011). In brain, the nAChRs regulate the release of both excitatory and inhibitory neurotransmitters, as a consequence, the activation of nAChRs can have opposite modulatory effects on the same circuit depending on whether they are expressed on excitatory or inhibitory neurons (Jensen et al., 2005; Albuquerque et al., 2009).

nAChRs and nicotinic mechanisms contribute to cognitive function and their decline or dysfunction has been observed in several neuropsychiatric diseases (Lewis and Picciotto, 2013; Higley and

Picciotto, 2014). In addition, genetic studies have linked nAChRs to epilepsy and schizophrenia, and studies of mutant (knock-out, Ko, or knock-in, Kin) mice have shown that they are involved in pain mechanisms, anxiety depression and nicotine addiction (Picciotto et al. 2001; Champtiaux and Changeux, 2002; Gotti and Clementi, 2004; Changeux, 2010b; Drenan and Lester, 2012; Hurst et al., 2013).

Nicotine binding activates nAChRs, but bound nAChRs can also be desensitised and inactivated by nicotine sometimes regardless of nAChR activation (reviewed in Picciotto et al., 2003). Moreover chronic nicotine exposure leads to neural adaptations that may be due to nAChR activation and/or desensitisation and, in the latter case, nicotine can alter neuronal function by interrupting the transmission of endogenous neurotransmitter ACh (Changeux, 2010a; Colombo et al., 2013).

The advent of new technologies such as optogenetics, the generation of mice carrying deletions or the expression of gain-of-function nAChR subunits and the use of lentiviral vectors to re-express nAChR subunits in selected brain regions of Ko mice or specific receptor subtype genes, tagged with GFP (Drenan and Lester, 2012; Fowler and Kenny, 2012) has greatly increased our understanding of the functional role of specific receptor subtypes in nervous system physiology and pathology.

As a number of comprehensive reviews have described the structure and function of nAChRs (Picciotto et al. 2001; Gotti and Clementi, 2004; Albuquerque et al., 2009; Jensen et al., 2005; Dani and Bertrand, 2007 Changeux, 2010a; Hurst et al., 2013).

the aim of this article is to provide a short overview of some recent aspects of the structure and function of nAChR subtypes, particularly those present in the habenulo-interpeduncular (Hb-IPN) pathway and to compare the expression of nAChR subtypes in rodents and primate brains.

2. Structure of nicotinic receptor subtypes

Studies using numerous complementary approaches have made it possible to define the structure function and pharmacology of nAChRs. Ligand binding assays have been used to identify and characterize brain nAChRs for more than 40 years (Patrick and Stallcup, 1977) and radioactive ligand have shown that ^{125}I-αBungarotoxin (αBgtx) and ^{3}H-nicotine bind to receptors that have different anatomical and pharmacological distributions (Clarke et al., 1985).

The pharmacological heterogeneity of nAChRs revealed by these ligand studies was later confirmed and extended by means of the molecular cloning of a family of genes encoding twelve subunits and by studies of their expression in heterologous systems (Dani and Bertrand, 2007; Albuquerque et al., 2009 ; Changeux, 2010a).

nAChRs belong to the large superfamily of cys-loop homologous receptors, that also includes muscle-type AChRs, and $GABA_{A/C}$, glycine and serotonin (5-HT_3) ionotropic receptors (Taly et al., 2009; Miller and Smart, 2010). Like all of the other members of the cys-loop ligand-gated ion channel superfamily, all nAChR subunits have an approximately 200 amino acid long relatively hydrophilic extracellular amino terminal portion that carries the ACh binding site, followed by three hydrophobic transmembrane domains (M1-M3), a large intracellular domain which varies significantly from one subunit to the other, and then a fourth hydrophobic transmembrane domain (M4) (Figure 1A) (Albuquerque et al., 2009). M1 and M3 shield M2 from the surrounding lipid bilayer and M4 is the most exposed to lipids. Both the amino- and carboxy- terminals are extracellular.

The subunits are arranged around a central pore and site-directed mutagenesis experiments have shown that the transmembrane M2 domain lines the ion channel and identifies the residues important for the ion selectivity, permeability and channel gating of the receptors (reviewed in Albuquerque et al., 2009; Changeux, 2010a).

In vertebrates the genes that have been cloned so far (CHRNA2-CHRNA10, and CHRNB2-CHRNB4) code for subunits that are divided into two subfamilies of nine α (α2-α10) and three β (β2-β4) subunits expressed in the nervous system, cochlea and a number of non-neuronal tissues (Hurst et

al, 2013). All the nine α subunits have adjacent cysteines (analogous to cysteines 192-193 of the α1 subunit of the muscle-type AChR) whereas the β subunits do not have those cysteines.

The variety of subtypes in the mammalian nervous system, is mainly due to the diversity of the possible combinations of the eleven neuronal subunits (all but α8) expressed in this animal class. nAChR subtypes can be divided into two main classes: 1) αBgtx-sensitive subtypes which can be homomeric or heteromeric, and are made up of the α7, α9 and α10 subunits; and 2) αBtgx-insensitive receptors, which are heteromeric combinations of α2-6 and β2-4 subunits that bind nicotine with high affinity but not αBgtx (Figure 1C) (Gotti et al., 2009; Changeux et al., 2010a).

All five subunits in the pentameric complex contribute to channel kinetics, ion conductance and selectivity (Albuquerque et al., 2009) and the subunit composition of each nAChR subtype determines the pharmacological characteristics of the ligand binding sites and the cation preference of the channel. The functional properties of each subtype are unique, but overlap sufficiently to make them very difficult to distinguish using pharmacological agents especially when the subtypes have subunits in common or contain different subunits with a high degree of homology (e.g., α3 and α6, or α2 and α4).

nAChRs are not only permeable to monovalent Na^+ and K^+ ions, but also to Ca^{2+} ions. Heterologous expression of homomeric α7 nAChRs has revelead a fractional Ca^{2+} current comparable to that estimated for N-methyl-D-aspartate (NMDA) receptors and much higher than that of heteromeric nAChRs (Fucile et al., 2004). The ability of nAChRs to alter intracellular calcium by activating different downstream intracellular pathways plays a pivotal role in neuronal signaling and plasticity (reviewed in Dajas-Bailador and Wonnacott, 2004).

2.1 ACh binding site

A significant contribution to the identification of the ligand binding site in nAChRs has been made by analyzing the crystal structure of the ACh binding protein (AChBP) from the freshwater snail (Celie et al., 2005; Rucktooa et al., 2009). This homopentameric soluble protein, which is 210 residues

long, binds ACh and is secreted by snail glial cells into cholinergic synapses and has an affinity spectrum resembling that of homomeric α7 or α9 receptors.

Both homomeric and heteromeric nAChRs have a pentameric structure with the subunits organised around a central channel: the homo-oligomeric receptors have five identical (orthosteric) ACh-binding sites per receptor molecule (Figure 1B) (Palma et al., 1996) located at the interface between two adjacent subunits, whereas hetero-oligomeric receptors have two or three α subunits and three or two β subunits, and therefore two orthosteric binding sites per receptor molecule located at the interface between the α and β subunits (Taly et al., 2009) (see Figure 1B**).** Each orthosteric ACh binding site has a principal (or ‘‘plus’’) and a complementary (or ‘‘minus’’) component. In heteromeric nAChRs, the principal component is carried by the α2, α3, α4 or α6 subunits with the complementary site carried by the β2 or β4 subunits, whereas each subunit in the homomeric receptors contributes to both the principal and complementary components, which are present on opposite sides of the same subunit (reviewed in Corringer et al., 2000; Taly et al., 2009) (see Figure 1B).
In order to elicit stable nAChR activation ACh needs to bind two binding sites in heteromeric receptors, and three binding sites at non-consecutive subunit interfaces in homomeric receptors (Rayes et al., 2009).

In heteromeric αBgtx-insensitive receptors, the subunits that do not directly participate in forming the binding site are called accessory subunits. Presently, only 6 subunits (α5, β3, α3, α4, β2, β4) have been shown to be able to occupy the accessory position in functional receptors. The role of accessory subunits has been investigated in the α4β2*[1] subtypes in which the presence of different accessory subunits changes their pharmacological and biophysical properties, their sensitivity to allosteric modulators and their sensitivity to up-regulation (Moroni et al., 2006; Tapia et al., 2007;

[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.

Kuryatov et al., 2008; Moroni et al., 2008).

Recent studies have revealed further complexity in the definition of binding sites and the possible subunits involved. The use of concatenated receptors whose DNA was linked covalently led to the expression of receptors whose subunits were in a specified order, and whose pharmacological properties were similar to those of the non-linked receptors (Nelson et al., 2003; Zhou et al., 2003). Studies of the concatameric α4β2α4β2α4 subtype have shown that in addition to the two orthosteric binding sites at the α4β2 interface they also have an additional binding site at the α4α4 interface (Moroni et al., 2008; Mazzaferro et al., 2011) as shown in Figure 1B right panel. Nicotinic ligands may also have different efficacy towards the two stoichiometries: for example the selective α4β2 sazetidine binds with high affinity to both stoichiometries being a full agonist of $(\alpha4)_2(\beta2)_3$ nAChRs, but a partial agonist, with an efficacy of only 6%, of $(\alpha4)_3(\beta2)_2$ nAChRs (Zwart et al., 2008).

Also the α3β4 subtype can exist in two stochiometries but in this case the agonist sensitivity of the two α3β4 stoichiometries is similar. However, only the subtype with two α3 subunits is susceptible to enhancement by low zinc concentrations, and the two stoichiometries have markedly different single-channel conductance and kinetics (Krashia et al., 2010)

Jin and colleagues (2014) using the concatameric approach, expressed either the dimeric constructs of α4 and β2 subunits with a free α5 subunit or concatameric pentameric, receptors incorporating a single copy of α5, in different positions, and found that the α5 subunit can occupy the position of a non-binding subunit, or replace a β2 subunit participating in a orthosteric binding site. However, functional receptors apparently cannot be formed with α5 subunit in both canonical binding sites.

Studies in heterologous systems have shown that the α7 subunits in addition to homomomeric

receptors can also form functional channels with the subunits present in non-αBgtx binding receptors such as the α5 (Girod et al., 1999), β2 (Khiroug et al., 2002), β3 (Palma et al., 1999) and β4 subunits (Criado et al., 2012). Fluorescently tagged nAChR α7 and β2 subunits have recently been used to characterize the formation of α7β2 nAChR, and to investigate potential pharmacological differences between α7 and α7β2 receptors (Murray et al., 2012). Co-expression of β2 and α7 subunits caused a significant decrease in agonist-evoked whole cell current amplitudes, but this decrease occurred without affecting the concentration-response characteristics of a range of common agonists and antagonists.

Using linked-subunit approach, three concatameric receptors ($(\alpha7)_5$, $(\alpha7)_4(\beta2)_1$, $(\alpha7)_3(\beta2)_2$) with defined subunit ratios and assembly orders were prepared (Moretti et al., 2014). Each of the three concatameric constructs showed ACh-evoked function. Notably, maximally-stimulating concentration and response to nicotinic agonists and antagonists were statistically-indistinguishable. This supports the hypothesis that α7β2 nAChR agonist activation predominantly or entirely reflects binding to α7α7 subunit interface sites (Murray et al., 2012).

2.2 Allosteric binding sites

In addition to ligands acting at the orthosteric neurotransmitter binding site, nAChRs are modulated by a variety of molecules acting at several distinct allosteric sites (Pandya and Yakel, 2011; Young et al, 2008; Hurst et al., 2013).

These molecules, called allosteric modulators, have a variety of structures from Ca^{2+} ions (Galzi et al., 1996), and small molecules (Pandya and Yakel, 2011; Young et al, 2008; Gill et al., 2013), to proteins (Chimienti et al., 2003; Levitin et al.,2008; Miwa et al., 2011). Allosteric modulators may provide selective potentiation or inhibition of physiological activity and typically they have low intrinsic activity (though, in a few cases, they can act as full agonists) and can reduce the concentration of agonist required to achieve channel opening and enhance the channel opening. Therefore, when they

are administered alone, they usually do not activate nAChRs but increase the activation elicited by endogenous nicotinic agonists, like choline or ACh.

2.3 nAChR subunit variants

The α7 subunit is encoded by the 10 exon CHRNA7 gene located on chromosome 15q14 that gives rise to a transcript that is translated into a protein of approximately 57 kDa. Genetic analysis has revealed that in humans exons 5–10 of CHRNA7 are duplicated and fused with three duplicated exons (A, B and C) from a serine/threonine kinase, ULK4 mapping to 3p22.1 and upstream of them to an additional exon (D) of unknown provenance. This partly duplicated gene, subsequently named CHRFAM7A, codes for a protein called α7dup whose molecular weight is 46.2 kDa. The CHRFAM7A protein lacks the signal peptide and the ligand-binding domain for ACh (reviewed in Hurst et al., 2013). Recently published data show that the oocyte expression of CHRFAM7A acts as a dominant negative regulator of α7 nAChR activity by means of a mechanism involving a reduction in the number of functional α7 nAChRs incorporated into the oocyte surface (de Lucas-Cerrillo et al., 2011). Moreover, it has been shown that CHRFAM7A is present in peripheral blood-derived monocytes and macrophages, and in THP-1 monocytic and neuroblastoma cell lines (Benfante et al., 2011). Recently, by using the Förster resonance energy transfer (FRET) technique it has also been confirmed that the α7dup subunits are assembled and transported to the cell membrane together with full-length α7 subunits and these α7-α7dup receptors show altered receptor function (Wang et al., 2014).

A series of linkage analyses, and candidate gene and genome-wide association studies (GWAS) have recently shown that variants in the human α3-α5-β4 nAChR subunit gene cluster on chromosome 15q24-25.1 are involved in the risk of nicotine dependence, smoking and lung cancer. A non-synonymous single nucleotide polymorphism (SNP) rs16969968 in CHRNA5 (the gene that encodes for the α5 subunit), leads to a substitution in position 398 in the TM3-TM4 loop of the α5 subunit

(aspartic acid to asparagine, D398N). Receptors incorporating α5 subunit with this SNP have decreased nAChR function and this is associated with an increased risk of nicotine dependence and smoking-related diseases (reviewed in Beirut et al., 2008; Improgo et al., 2010).

3. Native nAChR subtypes

The approaches currently used to localise and identify nAChR subtypes include techniques for localising subunit mRNA (*in situ* hybridization (ISH) and single-cell PCR) or protein (immunoprecipitation and immunocytochemistry), receptor autoradiography at regional or cellular level, techniques for assessing subtype composition and pharmacology (binding in tissue homogenates, immunoprecipitation, immunopurification and Western blotting) and functional assays (neurotransmitter release from slices or synaptosomes and electrophysiological techniques).

Information concerning the subunit associations of most of the native nAChR subtypes identified in neurons comes from immunoprecipitation and immunopurification studies using subunit-specific antibodies (Abs), whose specificity has been tested in the brain areas of wild type (Wt) and nAChR subunit Ko mice.

This was possible because the native subtypes extracted from the membrane were radiolabelled with nicotinic ligands, and the selection of the antigens was made by receptor binding. The immunoprecipitation of native radiolabelled receptors also ensured that only pentameric receptors were immunoprecipitated because analysis of the sucrose gradients of the different native subtypes solubilised using a non-denaturating detergent (such as Triton x-100) shows that they preserve their pentameric assembly, unlike transfected subtypes in which high affinity binding sites can also be detected with a sedimentation coefficient that is not exclusively compatible with pentameric subunit assembly (Kuryatov et al., 2000).

The immunochemical localisation at optic and electron microscopy levels of neurotransmitter receptors and subunit proteins are important criteria when localizing native subtypes and defining their subunit composition. However, these techniques cannot be easily used in the case of nAChR subunits

because most of the available anti-subunit antibodies are non-specific when used in histochemical preparations, which means that immunocytochemistry labelling leads to similar staining patterns in tissues obtained from the Wt and Ko mice for the subunit putatively targeted by the antibody(Moser et al., 2007). On the other hand, the major drawback of immunoprecipitation and immunopurification is that the spatial resolution obtained is only at regional level.

In summary, although very few nicotinic ligands are subtype-specific, the combined use of ligand binding assays, immunoprecipitation and autoradiographic studies of brain tissues from Wt and subunit Ko mice has provided critical data for identifying and defining the subunit composition, localisation and pharmacology of native subtypes.

3.1. Autoradiographic studies

The earliest autoradiographic ligand binding studies demonstrated that the binding of ^{125}I-αBgtx was distinct from that obtained using the radioactive agonist ligands ^{3}H-nicotine, ^{3}H-ACh and ^{3}H-cytisine (Clarke et al., 1985; Marks and Collins, 1982) and a number of studies showed that ^{3}H-cytisine and ^{3}H-ACh bind with nM affinity to the same sites bound by ^{3}H-nicotine. Badio and Daly (1994) subsequently identified a new ligand, epibatine, that binds with very high affinity (pM) heteromeric receptors to the same sites as those bound by ^{3}H-nicotine. Since then, further saturation binding studies to rodent membranes have shown that epibatidine also binds to low affinity sites and that some of this binding is competed by the presence of αBgtx (Marks et al., 2006).

Later autoradiographic studies of several ^{3}H-nicotinic ligands and ^{125}I-αBgtx binding using brain from Wt and β2 Ko mice showed the presence of at least four different classes of nAChRs: the first represents the αBgtx receptors whose distribution does not change significantly in β2 Ko mice; the second represents the α4β2 receptors that constitute the vast majority of nAChRs in mouse brain; the third class represents the α3β4 subtype which is highly expressed in the medial habenula (MHb), dorsocaudal medulla oblongata, and pineal gland; and the fourth class (possibly the α4β4 subtype) is

expressed in the medial lateral habenula (LHb) and the dorsal IPN (Zoli et al., 1998).

Over the last 10 years many more ligands specific for the different subtypes have been discovered including A85380 (which is specific for β2* receptors) and αconotoxin MII which is specific for the α3β2∗ and α6β2∗ subtypes. The use of these old (^{125}I-αBgt and ^{125}I-epibatidine) and new (^{125}I-αconotoxinMII, and ^{125}I-A85380) ligands and brain slices obtained from Wt and α2, α4, α5, α6, α7, β2, β3, β4 Ko mice has provided a clear picture of the localization of many subtypes (Baddick and Marks, 2011).

Deletion of the α7 subunit completely and selectively eliminates ^{125}I-αBgtx binding, but deletion of any of the other subunits had no effect. Deletion of the β2 subunit completely eliminates ^{125}I-A85380 binding throughout mouse brain, whereas deletion of the α4 subunit eliminates most ^{125}I-A85380 binding, but residual ^{125}I-A85380 binding sites were found in the dopaminergic pathways, visual tracts, MHb and IPN probably because of the presence of the α6β2* subtype. The binding of ^{125}Iα-conotoxin MII is eliminated in most brain regions by deleting α6 or β2 subunit, and reduced by deleting the α4 or β3 subunit (Baddick and Marks, 2011).

Inhibition of high-affinity ^{125}I-epibatidine binding sites by cytisine is a useful experimental means to separate subsets of cytisine-sensitive receptors mainly represented by α4β2 subtype and cytisine-resistant receptors represented by the α6β2* and α3β4* subtypes. When 100 nM cytisine is included in the incubation of ^{125}I-epibatidine in slices of Wt mice, labelling was almost exclusively observed in the MHb, fasciculus retroflexus, IPN and inferior colliculus; this binding was lost in β4 Ko mice, thus further indicating the presence of an α3β4 subtype in these areas (Baddick and Marks, 2011).

3.2 Biochemical and pharmacological studies of native subtypes

Vertebrate neuronal 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.

3.2.1 αBungarotoxin-sensitive receptors

The availability of the αBgtx specific ligand has allowed the purification and characterization of the αBgtx receptors present in the brain of various species: they are pentamers of only α7 subunits in rat (Drisdel and Green, 2000), but may be homomeric α7 or α8 or heteromeric α7-α8 receptors in chick (Gotti et al., 1994; Keyser et al., 1993). It has recently been shown that α7 and β2 subunits are co-expressed in rat basal forebrain cholinergic neurons, and form a novel heteromeric α7β2 subtype that has different biophysical and pharmacological properties from those of the homomeric α7 receptors expressed by ventral tegmental area neurons. This α7β2 subtype is highly sensitive to functional inhibition by oligomeric forms of amyloid Aβ1-42, and may be relevant in Alzheimer's disease (Liu et al., 2009).

Using the α7-selective ligand αBgtx to affinity purify α7* receptors, we have recently biochemically identified in addition to the α7 homomeric subtype an α7β2 subtype in the basal forebrain of Wt mice and humans. Western blotting with subunit-specific Abs showed that the α7β2* nAChRs were expressed in Wt but not in β2 Ko mice, and that they represent only 3% of all β2* receptors. We found that an α7β2 receptor is also present in human basal forebrain, but not in the cerebellum (Moretti et al., 2014).

3.2.2 αBungarotoxin-insensitive receptors

Various immunological and affinity ligand immunopurification studies have shown that the large majority of nAChRs in the nervous system are heteromeric and contain at least one type of α and one type of β subunit (reviewed in Gotti et al., 2006b). α4β2∗ receptors account for 90% of the high affinity nAChRs in mammalian brain, whereas α3β4∗ receptors are the major subtype in the ganglia,

adrenal medulla, and few CNS regions such as the MHb, the dorsal motor nucleus of the vagus nerve (Wada et al., 1989; Zoli et al., 1998), the motor nucleus of the trigeminal nerve (Wada et al., 1989), and the pineal gland (Hernandez et al., 2004). Some $\alpha4\beta2^*$ receptors in the cortex (10-15%), hippocampus (37%), striatum (24%), thalamus (10%), superior colliculus (11%) and mesencephalon (32%) (Mao et al., 2008; Gotti et al., 2010) and $\alpha3\beta4^*$ receptors in the habenula (5-10%) or IPN (16%) (Grady et al., 2009; Scholze et al., 2012) may also contain the α5 subunit, whose presence is believed to increase the rate of channel desensitisation and calcium permeability (reviewed in Fucile, 2004).

In addition to these major central and peripheral nAChR subtypes, many other native nAChR subtypes with more complex subunit compositions have been identified in the rodent mesostriatal pathway (substantia nigra, ventral tegmental area, nucleus accumbens and caudate-putamen) and visual pathways (retina, superior colliculus and lateral geniculate nucleus) including the α6β2β3* receptors that consist of the α6β2β3 and α4α6β2β3 subtypes (reviewed in Changeux, 2010b). The two ortostheric ACh binding sites are identical in the α6β2β3 subtype, but different in the α4α6β2β3 subtype, which has both an α6β2 and an α4β2 interface (Champtiaux et al., 2003)

Data from our laboratory have shown that the subunit composition of α6* receptors in the different areas of mesostriatal dopamine system are partially heterogeneous. The dopamine 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 caudate-putamen (Gotti et al., 2010). Furthermore, binding studies in striatal tissue of primates have shown that α4α6β2β3 is the subtype preferentially vulnerable to nigrostriatal damage as it is lost in the striatum of animal models of Parkinson's disease and human patients (Quik and Wonnacott, 2011).

3.2.2.1. β4* receptors in olfactory bulb and hypothalamus

Recent electrophysiological and pharmacological studies have shown that β4* receptors are expressed in the olfactory bulb (OB) and in the hypothalamus.

The OB receives cholinergic input from the horizontal limb of the diagonal band of Broca, and findings of behavioural studies suggest that ACh and nAChRs play an important role in olfactory perceptual learning, odour discrimination and odour detection (Mandairon et al., 2006; Rushforth et al., 2010). Ligand binding autoradiographic studies using ^{125}I-epibatidine have revealed the strong expression of neuronal heteromeric nAChRs in the OB of adult Wt mouse, particularly in the granule cell and glomerular layers. However this binding disappears completely in the granule cell layer and is reduced by 88% in the glomerular layer of β2 Ko mice thus indicating that the glomerular layer also contains β4 heteromeric receptors (Mechawar et al., 2004).

D'Souza and Vijayaraghavan (2012) used electrophysiological techniques to demonstrate that it is the α3β4 subtype that mediates direct excitation of mitral cells (output neurons) in the OB. Their results indicate that α3β4* receptors gate incoming olfactory nerve input so that weak input stimuli are filtered out, and strong stimuli are transmitted via mitral cells.

The hypothalamus is another functionally characterised localisation of a minor population of β4* receptors. Mineur et al. (2011) used a combination of pharmacological, molecular genetics, electrophysiological, and feeding studies, to show that the activation of hypothalamic β4* nAChRs leads to the activation of pro-opiomelanocortin neurons, and these and the subsequent activation of melanocortin 4 receptors mediate the anorexigenic effects of nicotinic agonists leading to decreased food intake in mice (Mineur et al., 2011).

3.2.2.2. nAChR subtype structure and function in the habenulo–interpeduncular pathway

The Hb-IPN system is a central relay station between forebrain and midbrain structures. The Hb is a diencephalic structure that is divided into the LHb and MHb (Hikosaka 2010), The MHb projects almost exclusively to the IPN through the fasciculus retroflexus (Herkenham,and Nauta, 1979; Klemm, 2004), whereas the LHb projects to a number of midbrain regions in addition to the IPN (Klemm, 2004; Lecourtier and Kelly, 2007; Geisler and Trimble, 2008). The MHb contains neurons that produce ACh or substance P (Cuello et al., 1978; Eckenrode et al., 1987), but recent optogenetic studies indicate that MHb cholinergic neurons also contain glutamate and that the two transmitters are co-released by different modes of stimulation (Ren et al., 2011).

The nAChRs present in the Hb-IPN pathway regulate nicotine intake and participate in nicotine-related behaviours such as nicotine withdrawal, and so, deleting or impairing the function of nAChRs in the habenular complex has a large impact on nicotine dependence in rodents. Fowler et al. (2011) showed that receptors containing the α5 subunit play an important role in regulating nicotine intake by demonstrating that α5 Ko mice markedly increase their nicotine intake and self-administer nicotine at doses that normally elicit aversion in Wt mice. This increased intake was blocked by selectively re-expressing the α5 subunit in the MHb, thus indicating that MHb α5* nAChRs are necessary for the appearance of the negative effects of nicotine. They also showed that blocking general and NMDA receptor-mediated transmission in the MHb–IPN pathway increases nicotine self-administration in rats. Taken together, these data indicate that, by activating α5* nAChRs directly and NMDA receptors indirectly, nicotine gives a negative motivational signal that blocks its intake, whereas the absence of the signal in α5 Ko mice induces the self-administration of more nicotine. Moreover, certain allelic variations in the gene encoding the α5 nAChR subunit decrease receptor function and increase susceptibility to tobacco addiction (Fowler et al., 2011).

The aversive properties of nicotine are also regulated by β4* nAChRs (Frahm et al., 2011) whose over-expression in mouse brain leads to a decrease in the oral consumption of nicotine and a strong

aversion to nicotine during the conditional place aversion procedure. A number of SNPs have been found in the β4 subunit gene associated with cigarette consumption in human and the injection of lentiviruses carrying the wild-type β4 subunit or these β4 variants in the MHb of mice leads to aversion or preference for nicotine depending on whether the variants increase or decrease nicotine currents (Slimak et al., 2014).

The Hb-IPN system expresses 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. The β2* population in the Hb contains the α4β2* and α3β2* subtypes, some of which also contain the accessory α5 or β3 subunits; the β2* nAChRs in the IPN, consist of three populations of approximately equal size: α2β2*, α3β2* and α4β2* (Grady et al., 2009; Scholze et al., 2012). In agreement with the binding studies described above, our immunoprecipitation studies showed that the β4* nAChR population in both regions is mainly associated with the α3 subunit, and that a significant fraction of the α3β4* nAChRs contain accessory (mainly β3) subunits. The β3 subunits are associated with α6β2* nAChRs in the mesostriatal dopaminergic and visual pathways (see above) but the presence of β3 subunits not associated with α6 subunits in the Hb-IPN pathway is in line with the findings of ISH studies showing high levels of β3 but not α6 mRNA in the Hb.

The drawback of the immunoprecipitation studies is that their spatial resolution is only regional but the recent generation of Kin mouse strains expressing α3, α4, α6, β2, β3, or β4 nAChR subunits fused to green fluorescent protein (GFP) has made it possible to determine the precise localisation of the receptors -containing these subunits (Fowler and Kenny, 2012).

Using a combined electrophysiological/genetic approach and two lines of Kin mice (one expressing

fluorescently tagged α4* nAChRs (α4YFP, Nashmi et al., 2007), and the other hypersensitive α4* nAChRs bearing a leucine to alanine substitution at the M2 9′ position (α4L9′A, Tapper et al., 2004 and Fonck et al., 2005), Fonck et al., (2009) demonstrated that nAChRs containing α4 subunits are selectively localised to cells in the ventrolateral region of the MHb, thus confirming previous ISH results (Le Novère et al., 1996).

Shih et al. (2014) extended this study and used many more GFP-tagged mice to demonstrate high α3 and β4 nAChR subunit levels throughout the ventral MHb, which is consistent with the findings of previous immunoprecipitation and Western blotting (Yeh et al., 2001; Gahring et al., 2004,), radioligand binding (Salas et al., 2004), ISH (Wada et al., 1989; Le Novère et al., 1996, Winzer-Serhan and Leslie, 1997), and transgenic studies (Frahm et al., 2011). α6, β2, β3, and α4 subunits were found only in some areas of the ventral MHb (see also Le Novère et al., 1996) and showed a localisation "gradient" across both the MHb and IPN.

Most neurons expressing α3, β3, and β4 subunits are acetylcholine transferase (ChAT)-immunopositive neurons, which is consistent with their role in regulating ACh release in the IPN (Ren et al., 2011), whereas α6 and α4 subunits are often found in ChAT-negative neurons.

The Hb mediates negative reward and motivation (Matsumoto and Hikosaka, 2007, 2009; Frahm et al., 2011; Lammel et al., 2012), and the cholinoceptive MHb plays an important role in nicotine-related behaviours, especially those that are aversive. MHb cholinergic (but not peptidergic), neurons display spontaneous tonic firing, and acute exposure to nicotine leads to a considerable α3β4-dependent increase in firing frequency, but no change in basal or nicotine-induced firing is observed in the neurons of chronically treated mice. During withdrawal, re-exposure to nicotine doubles the frequency of pacemaking activity in MHb neurons, thus indicating that the heightened nicotine sensitivity of these neurons during withdrawal may contribute to smoking relapse (Gorlich et al., 2013).

Fowler et al. (2011) have shown that habenular nAChRs containing the α5 subunit regulate the consumption of nicotine at normally aversive high doses. Dao et al. (2014) used patch-clamp

electrophysiology in mouse brain slices to show that acute nicotine administration increases the intrinsic excitability of MHb neurons, and that this increase is due to the presence of α5β2*-containing receptors. By activating α5* receptors, acute nicotine exposure facilitates the release of neurokinins on MHb neurons expressing neurokinin receptors type 1 and 3, whose activation increases intrinsic MHb neuron excitability whereas chronic nicotine exposure reduces the responsiveness of α5* nAChRs and the neuromodulatory effect of nicotine on intrinsic excitability probably as a result of nAChR desensitisation, and this may contribute to aversive experiences during nicotine withdrawal.

3.3. nAChR subtypes in primate brain

Research into native primate brain nAChR subtypes cannot benefit from the wealth of technical approaches used to study rodent brain which, together with the difficulty in obtaining primate tissues, explains why little is known about the composition and specific role of native primate brain nAChRs. Figures 2A and 2B summarise the available information concerning the nAChR subtypes in human and monkey brains and allow some regional comparisons of primate and rodent data.

Studies have examined the cerebellar tissue of rats (Turner and Kellar, 2005), mice (Gotti and Marks, unpublished observations) and humans (Moretti et al., 2014). The most thorough analysis involved rat tissue, in which about 35% of the nAChRs are α4β2, about 20% α3β4 and about 10% α3α4β2, α3α4β2, α3β2β4 or α3α4β4, with more than 50% of the nAChRs containing the relatively uncommon β4 subunit (Turner and Kellar, 2005). The same major subtypes are present in mice (although β4* nAChRs maybe less expressed than in rats), whereas no evidence of β4 expression has been found in humans. Although further studies are necessary, these data suggest that nicotinic transmission in cerebellum may be remarkably different in humans and rodents.

The striatal expression of nAChR subtypes seems to be basically the same in rodents (Gotti et al., 2010), monkeys (Quik et al., 2005) and humans (Gotti et al., 2006a), and characterised by the prevalence of the two major subtypes α4β2* and α6β3β2*. This similarity is further underlined by the

preferential decrease in α6β2* nAChRs in rodent and monkey animal models of Parkinson's disease and human Parkinsonian patients (Quik et al., 2011).

The most remarkable difference between rodent and primate nAChR subtypes concerns α2* nAChRs. In mouse and rat, the α2 subunit is highly expressed in the IPN and in scattered cells of few other regions including the hippocampal formation, basal forebrain and amygdala (Ishii et al., 2005) but it is widely expressed in the brain of the *Macaca mulatta*. In particular, in the monkey thalamus, its expression is comparable to that of α4 subunit (Han et al., 2000). It is not yet clear whether this is also the case in human brain, although in the regions that have been quantitatively examined, such as the temporal cortex (Gotti et al., 2006a), α2 expression seems to be higher than in rodents.

One final case that deserves mention is the expression of the rare α7β2 subtype which seems to be expressed in the basal forebrain of mice and humans (Liu et al., 2009; Moretti et al., 2014), but not in many other brain regions. The remarkable conservation of the cellular localisation of this subtype in mammals, suggests that α7β2 nAChRs may play a specific and important functional role in this circuit.

As recently convincingly argued (McCullumsmith et al., 2014), post-mortem tissue is an under-used substrate for genetic and/or preclinical studies. In particular, studies performed on post-mortem tissues from patients with neuropsychiatric illnesses may provide a translational element that is difficult to recapitulate in animal models alone.

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Figure Legends

Figure 1:Basic structure of neuronal nicotinic receptors

A: Left) Diagram showing the topography of nAChR subunits.

The extracellular amino terminal portion is followed by three hydrophobic transmembrane domains (M1-M3), a large intracellular loop, and then a fourth hydrophobic transmembrane domain (M4).

Middle) The arrangement of nAChR subunits in an assembled receptor.

Right) Localisation and organisation of the ACh binding sites in a heteromeric receptor.

B. Structure of homopentameric and heteropentameric neuronal subtypes

The pentameric arrangement of nAChR subunits in an α7 homopentameric subtype (left), heteromeric receptor subtype (middle), and the $(\alpha4)_3(\beta2)_2$ subtype (right). The localisations of the subunit interfaces of the orthosteric binding sites are indicated, together with the primary component P(+) carried by the α subunits and the complementary component C(-)carried by an α or non-α subunit. In addition to the two orthosteric sites, the $(\alpha4)_3(\beta2)_2$ subtype has a binding site at the α4α4 interface (star).

Figure 2

A) Regional distribution of nAChR subtypes identified in human brain.

The subtypes in the temporal cortex, cerebellum, striatum and basal forebrain have been identified by means of quantitative immunoprecipitation studies using nAChRs obtained from post-mortem brains, radiolabeled with ^{3}H-Epibatine or ^{125}I-αBungarotoxin and subunit specific antibodies.

B) Regional distribution of monkey nAChR subunit mRNAs.

The localisation of the mRNA of the subunits is based on the results of *in situ* hybridisation or single cell PCR experiments. The striatum and cortex subtypes identified by means of quantitative immunoprecipitation studies are also shown (underlined)

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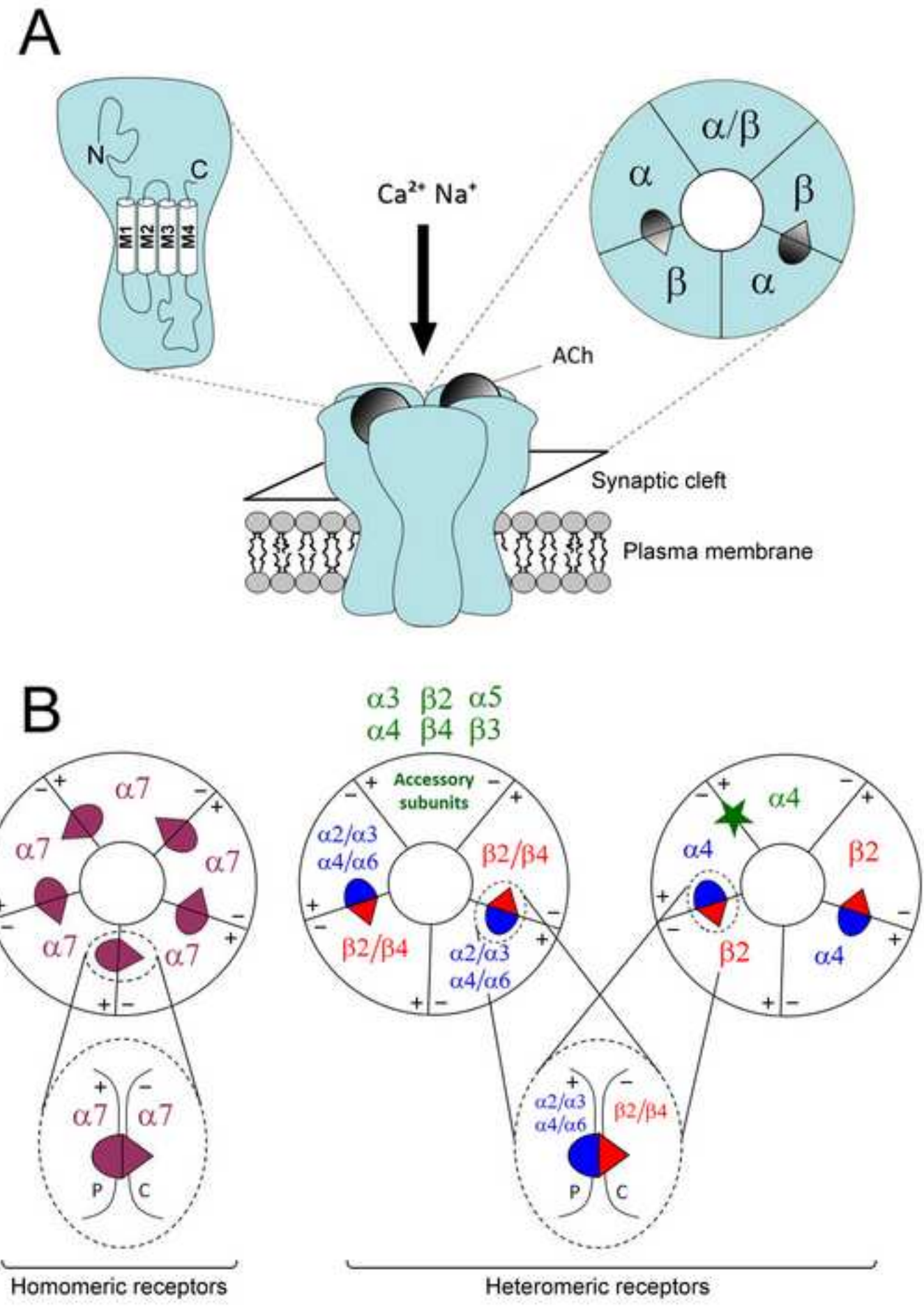

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