The Diversity of Subunit Composition in nAChRs: Evolutionary Origins, Physiologic and Pharmacologic Consequences

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ABSTRACT: Nicotinic acetylcholine receptors are made up of homologous subunits, which are encoded by a large multigene family. The wide number of receptor oligomers generated display variable pharmacological properties. One of the main questions underlying research in molecular pharmacology resides in the actual role of this diversity. It is generally assumed that the observed differences between the pharmacology of homologous receptors, for instance, the EC_{50} for the endogenous agonist, or the kinetics of desensitization, bear some kind of physiologic relevance *in vivo*. Here we develop the quite challenging point of view that, at least *within a given subfamily* of nicotinic receptor subunits, the pharmacologic variability observed *in vitro* would not be directly relevant to the function of receptor proteins *in vivo*. *In vivo* responses are not expected to be sensitive to mild differences in affinities, and several examples of functional replacement of one subunit by another have been unravelled by knockout animals. The diversity of subunits might have been conserved through evolution primarily to account for the topologic diversity of subunit distribution patterns, at the cellular and subcellular levels. A quantitative variation of pharmacological properties would be tolerated within a physiologic envelope, as a consequence of a near-neutral genetic drift. Such a "gratuitous" pharmacologic diversity is nevertheless of practical interest for the design of drugs, which would specifically tackle particular receptor oligomers with a defined subunit composition among the multiple nicotinic receptors present in the organism.

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INTRODUCTION

The nicotinic acetylcholine receptors (nAChRs) are well characterized transmembrane allosteric proteins involved in the physiological responses to acetylcholine (Changeux and Edelstein, 1998). They are composed of five identical (homopentamers) or different (heteropentamers) polypeptide chains arranged symmetrically around an axis perpendicular to the membrane. The agonist binding sites are located at the interface between two subunits (Corringer et al., 2000; Sine, this issue). nAChRs may spontaneously exist under several discrete interconvertible conformational states: basal or resting (closed), active (open) or desensitized (closed) (Edelstein et al., 1996). Nicotinic ligands, agonists or competitive antagonists, but also allosteric effectors binding to sites distinct from the ACh binding site, may differentially affect the equilibrium established between the various conformations.

In addition to their primordial role in neuromuscular and motor autonomous transmission, nAChRs are involved in several central functions, including control of voluntary motion, memory and attention, sleep and wakefulness, reward and pain, anxiety, and sen-

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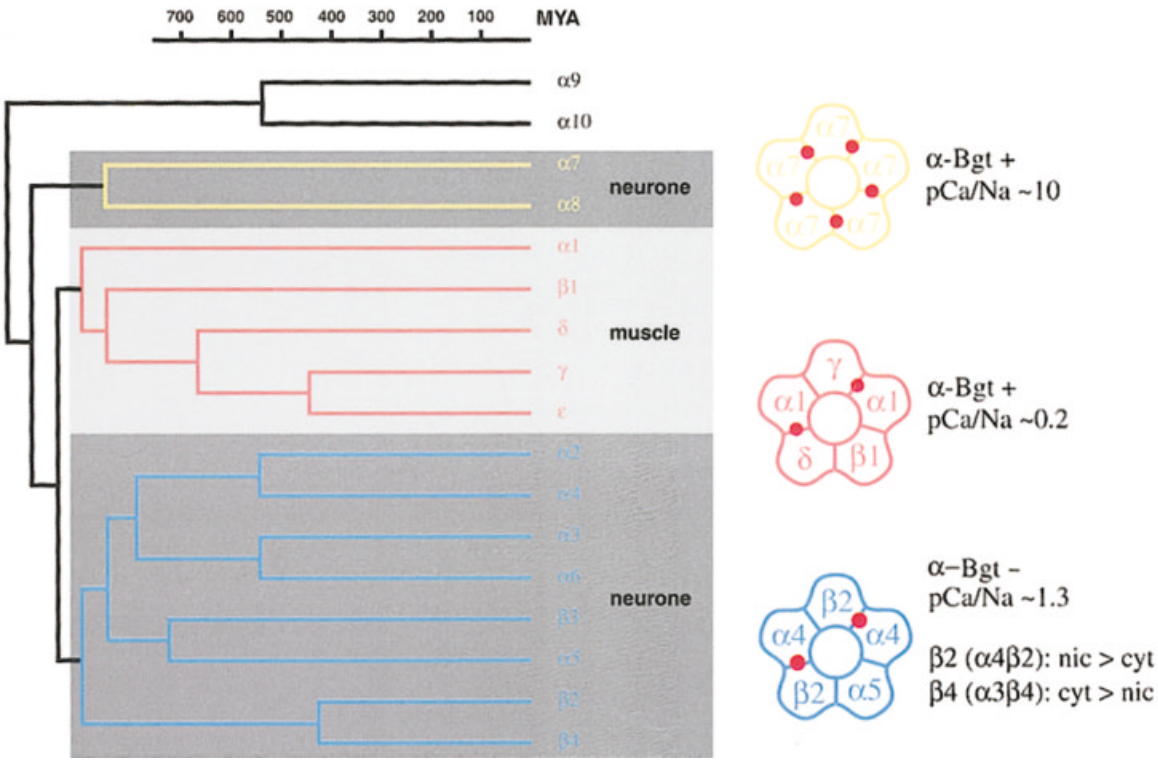

Figure 1 Relationship between the phylogeny of amniote subunits and the properties of receptors. The dates of the most recent gene duplications are calculated from neighbor-joining reconstructions (Saitou and Nei, 1987), according to the most recent estimated dates of species divergence (Kumar and Hedges, 1998). α-Bgt, α-Bungarotoxin. pCa/Na, relative calcium/sodium permeability.

sory gating (Picciotto et al., 1995; Cordero-Erausquin et al., 2000). Nicotinic agonists thus exhibit multiple pharmacologic actions. Several human neuropathologies have recently been shown to be caused by genetic alterations of nAChR genes, including congenital *myasthenia*, autosomal dominant frontal lobe nocturnal epilepsy, and possibly a schizophrenic syndrome (Léna and Changeux, 1997; Lindstrom, 1997; Bertrand, this issue). These receptors are also involved at various degrees in several neuropathologies such as *Parkinson* and *Alzheimer*'s diseases and *Gilles de la Tourette*'s syndrome (Picciotto, this issue). However, the most widespread human pathology associated with neuronal nAChRs is the addiction to nicotine (Picciotto et al., 1998; Dani et al., 2001; McGehee, this issue).

EVOLUTIVE ORIGINS OF SUBUNIT DIVERSITY IN VERTEBRATE nAChRs

Sequence analyses have revealed that the numerous nAChR subunits are homologous proteins that belong, together with the $GABA_{A,C}$, Glycine, $5\text{-}HT_3$, and some invertebrate glutamate receptors, to the "cys-loop" superfamily of Ligand-Gated Ion Channels (Galzi and Changeux, 1994; Le Novère and Changeux, 1995, 1999). Based on sequence similarities and resemblance of gene structures, nAChR subunits have been divided in four subfamilies in vertebrates (Le Novère and Changeux, 1995; Ortells and Lunt, 1995, updated trees can be found on the WorldWide Web, http://www.pasteur.fr/recherche/banques/LGIC/catphylogen.html). These subfamilies of subunits, shown in Figure 1 for the amniotes, are congruent with the groups of receptors, defined on the basis of biochemical, functional or pharmacological data. α9 and α10 form homopentamers by themselves and heteropentamers together. Similarly, α7 and α8 make up homopentamers on themselves and heteropentamers together. α2–α6 and β2–β4 are included in a range of complex heteropentamers, mostly present in neurons. Finally α1, β1, γ, δ, and ε form heteropentamers in muscle cells.

Within the two largest subfamilies, one can further define three tribes of subunits, according to their topological position relative to the agonist binding site within the oligomer. A given subunit may carry the

"main component" of the binding site (α1 in muscle, α2, α3, α4, and α6 in neurons), its "complementary component" (γ, δ, ϵ in muscle, β2 and β4 in neurons), or may not contribute at all to the binding site (β1 in muscle, α5 and β3 in neurons).

Several whole genome duplications occurred at the beginning of vertebrate evolution, more than 500 million years ago (Lundin, 1993), and are probably at the origin of the initial diversity within the subfamilies. Although few data are available for more distant lineages of vertebrates, such as cartilaginous fishes (only *Torpedo* muscle subunits) or bony fishes (a subset of neuronal subunits plus α1), estimations of the possible dates of gene duplications can nonetheless be computed. As a consequence, one may infer the gene content of those species. One can show that the most recent duplications leading to the main subunits are probably older than 400 million years. The present set of genes was therefore stabilized by the appearance of tetrapods. Very few duplications occurred afterward, and were limited to a specific phylum (α1 in lissamphibians and β3 in teleosteans). Similarly, very few genes were lost during the evolution of tetrapods. For instance, ϵ seems to be absent in birds, and α8 disappeared in mammals.

We can conclude that the diversity of nAChR subunits is conserved through evolution, and hypothesize that the presence of each individual subunit has probably been positively selected. This situation contrasts with that of the globins (Hardison, 2001) or of the olfactory receptors (Young and Trask, 2002). In those two multiple gene families, multiple paralogous genes systematically exist, but are often not orthologous between the various phyla, revealing much higher rates of gene creation and inactivation in the course of evolution.

The variable transcription of the genes coding for nAChR subunits across the nervous system generates a diversity of distribution and thus of colocalisation of subunits. The combinatorial assembly of these subunits produces a wide structural diversity of receptor oligomers, targeted to different subcellular compartments, which exhibit variable electrical properties (conductance, ion selectivity, rectification), pharmacologic characteristics (affinities for agonists, competitive antagonists and allosteric effectors, potency orders) and kinetics of activation and desensitization.

PHYSIOLOGIC MODES OF ACTION OF ACETYLCHOLINE

It is classical to divide chemical neurotransmission in two different modes, the wiring and the volume transmissions (Zoli et al., 1999). In the former case, the transmitter is released in a synaptic cleft, in close proximity of the postsynaptic receptors. The concentration of neurotransmitter reaches millimolar ranges, sufficient to activate in the millisecond range receptors with a low affinity active state and a fast desensitization rate. In the latter case, the transmitter is released in the intercellular spaces, and receptors with low desensitization rates and high affinity active states are then thought to be involved.

The properties of desensitization and recovery from desensitization of the receptors are indeed crucial to shape the physiologic response (Jones and Westbrook, 1996). nAChRs exhibit variable desensitizing behaviors (Lester and Quick, this issue). From the fast desensitization of α7 to the quasi-absence of desensitization of α2β2, a wide range of desensitization kinetics are observed (Chavez-Noriega et al., 1997). Such desensitization properties are directly related to the subunit composition. For instance, within the neuronal hetero-oligomeric subfamily, an αiβ2 oligomer desensitizes much faster than an αiβ4 oligomer (Papke and Heinemann, 1991; Bohler et al., 2001). Similarly α3βj oligomers desensitize faster than α2βj and α4βj receptors. It is striking that there does not seem to be a clearcut correlation between the desensitization properties of nAChRs and their EC_{50} for acetylcholine (Table 1), which could allow the assignment of the various receptor oligomers to the major modes of neurotransmission described above. Indeed, neuronal nAChRs display variable combinations of the two properties. In mammals, α7 possesses a fast desensitization rate and an active state with a low affinity for ACh, α3β4 a slow desensitization rate and a low affinity active state, α3β2 a fast desensitization rate and a medium affinity active state, and α4β2 a medium affinity but a slow desensitization rate. In addition, several chick nAChRs display a relatively high affinity active state.

Because of the difficulty to find authentic fast acting nicotinic synapses outside the peripheral nervous system, it has often been considered that ACh was acting in the brain as a "neuromodulator" and that nAChRs were involved in "volume" transmission. Recent studies nevertheless revealed authentic fast acting nicotinic synapses. The most investigated interneuronal nicotinic synapse is engaged in the motor autonomic transmission at the level of the peripheral ganglia. It is a complex synapse containing aggregated α3β4α5 receptors (20% of which also contain β2) surrounded by scattered α7 receptors (see below, about the subcellular targeting). Synapses involving α7 receptors have been demonstrated in the hippocampus (Alkondon et al., 1998; Frazier et al., 1998)

Table 1 EC_{50} (μM) for Acetylcholine of Human, Rat, and Chick nAChRs Reconstituted in *Xenopus* oocytes

Receptor	$\alpha2\beta2$	$\alpha2\beta4$	$\alpha3\beta2$	$\alpha3\beta4$	$\alpha4\beta2$	$\alpha4\beta4$
Homo	68.7[h]	82.6[h]	26[e] 28[g] 443[h]	160[e] 163[g] 203[h]	68.1[h] 100[j]	19.7[h]
Rattus	87[c]	44[c]	24[c] 11[d] 70[f]	77[c] 219[d] 210[f]	65.6[i]	
Gallus			5.6[a] 5.2[b] 4.1[e]	158[a] 53[e]	0.77[a] 0.3[b] 0.48[e]	4.8[a]

[a] Couturier et al. (1990).
[b] Gross et al. (1991).
[c] Cachelin and Rust (1994).
[d] Cohen et al. (1995).
[e] Gerzanich et al. (1995).
[f] Harvey and Luetje (1996).
[g] Wang et al. (1996).
[h] Chavez-Noriega et al. (1997).
[i] Zwart and Vijverberg (1998). Some data are also coming from transfected human cell HEK293.
[j] Chavez-Noriega et al. (2000). This list is by no means an exhaustive report of all the publications on the subject. $\alpha6$ containing receptors, as well as receptors containing $\alpha5$ or $\beta3$ are not reported in the table because of the very few data available.

and the supraoptic nucleus (Hatton and Yang, 2002). In the dorsolateral septum (Wong and Gallagher, 1991) and the visual cortex (Roerig et al., 1997), synapses have been uncovered that are insensitive to α-bungarotoxin, a specific blocker of $\alpha7$ in the brain. The dopaminergic neurons of the substantia nigra exhibit nicotinic synapses with $\alpha4$ containing receptors (Sorenson et al., 1998). Other synapses have been located, for instance, on the Renshaw cell of the spinal chord (Dourado and Sargent, 2002), which may contain $\alpha4\beta2$, and in the ambiguus nucleus of the brainstem (Zhang et al., 1993). Thus, authentic fast nicotinic transmission may take place in several neuronal systems that mobilizes a broad range of nAChR oligomers with different subunit composition, physiologic and pharmacologic properties.

VARIABILITY IN THE SENSITIVITY TO ACETYCHOLINE

A major reccurent question about the combinatorial diversity of ligand-gated ion channel subunits is the physiologic significance of the associated pharmacologic variability. Such a significance is most often unquestioned, with the hidden "adaptationist" assumption that a diversity of functions emerged from the diversity of structures, and had to be precisely selected to be maintained throughout evolution.

A first issue to consider is the actual conservation of the pharmacologic diversity in the course of evolution. An exhaustive compendium of the pharmacologic characteristics for all known nAChRs subunit combination is out of the current article focus. However, in Table 1, we compiled the available EC_{50} for acetylcholine of several neuronal nAChRs heterooligomers, from three different amniote species. Many studies were performed in different experimental conditions. In particular, the ionic strengths were rarely comparable, this factor being known to affect the EC_{50} values (Cachelin and Rust, 1994). The first obvious conclusion is the difficulty to get consistent results across different studies, and therefore to compare them. However, it is quite clear that the average values are not conserved, in particular between chick and mammalian receptors. In addition, the interspecies differences lie not only in the absolute EC_{50} values (compare the various $\alpha3\beta2$ or $\alpha4\beta2$), but also in the rank orders. For instance, chick $\alpha4\beta4$ is less sensitive to ACh than chick $\alpha4\beta2$, whereas human $\alpha4\beta4$ is more sensitive than human $\alpha4\beta2$. The variability of the sensitivity for acetylcholine thus does not seem "frozen" through evolution, and hence is not expected to result from a long term selective pressure. If there is undoubtedly conservation of the subunit diversity, this is not the case of the pharmacologic diversity, which would then result from a contingent, near-neutral, evolutionary process.

The same observations hold for exogenous agonists such as nicotine, curare, and competitive antag-

onists, although obviously without evolutionary correlates. Each particular oligomeric assembly of subunits possesses a unique spectrum of pharmacologic properties. This particular signature is of considerable practical interest to specifically target a given nAChR oligomer, and thus dissect a particular subset of cholinergic circuits (see, for instance, Albuquerque, this issue).

PHYSIOLOGIC RELEVANCE OF PHARMACOLOGIC VARIABILITY

The physiologic conditions of neurotransmission *in vivo*, for instance a synaptic regime, notably differ from the conditions of the oocyte recording. For instance, the EC_{50}, as well as all the other pharmacologic parameters, are determined with transfected cells on a large population of receptors with homogeneous solutions of ligands. The measurements are most often interpreted in terms of the mass-action law, and all the derived formalism. These experimental conditions strikingly differ from those under which receptors are functional *in vivo*. The action of the endogenous ligands on receptors takes place in confined volumes. Tens of thousands molecules of ligands react with a few thousands of receptors, which in addition are heterogeneously distributed. It is therefore difficult, if possible, to transpose the differences in EC_{50} observed *in vitro* to *in vivo* conditions.

On the other hand, in the case of classical synaptic transmission, it can be reasonably assumed that the amplitude of the electrical response depends on the presynaptic quantum size (the absolute number of acetylcholine molecules), the number of receptors present in the postsynaptic density (Faber et al., 1992), and of the fraction of receptors in the activatable conformation (Heidmann and Changeux, 1982), rather than on the affinity of the active state for the transmitter. Because the numbers of neurotransmitter and receptor molecules are small, large stochastic fluctuations of the response may arise. Those fluctuation can depends on geometric parameters independent of the receptor intrinsic properties, such as the percent of the synaptic cleft occupied by receptors (Kruk et al., 1997).

Finally, synapses are highly plastic complex systems with homeostatic properties (Marrone and Petit, 2002; Zucker and Regehr, 2002), such that a small change in the quantity of current triggered by an EPSP could be counterbalanced by adaptative cellular mechanisms. Consequently, mild changes of intrinsic receptor response may not have major consequences on the efficiency of synaptic transmission, and a significant variability of pharmacologic properties might be tolerated at the system level.

This is not to say that no constraint limits the pharmacologic properties of the receptors. The introduction in mice $\alpha7$ subunit of mutations in the residues lining the ionic pore, generating opened "desensitized states" (Revah et al., 1991), decreases EC_{50} for ACh about three rank orders, and results in increased sensitivity to nicotine-induced seizures (Gil et al., 2002). Also, a variety of "gain of function" mutations in the $\alpha4$ and $\beta2$ subunits causes autosomal dominant frontal lobe epilepsies in humans (Bertrand, this issue). nAChRs can thus accommodate EC_{50} for ACh only within an envelope of values compatible with the function of cholinergic systems.

SOME FUNCTIONAL EQUIVALENCES REVEALED BY KO ANIMALS

When several subunits of the same tribe of neuronal heteromeric receptors (Fig. 1) are expressed in the same cell, the inactivation of genes coding for particular nAChR subunits yields in general mild phenotypes.

A striking example concerns motor autonomic transmission. In addition to the $\alpha7$ subunit, which forms receptors on its own, the motor cells of the autonomic ganglia express $\alpha3$, $\alpha5$, $\beta4$, and $\beta2$ (De Biasi, this issue). The three former subunits assemble into receptor oligomers that are the main contributors to motor autonomic transmission. Twenty percent of these receptors also contain $\beta2$. Inactivation by gene targeting of $\alpha3$ triggered major autonomic defects, resulting in 50% of lethality at birth, the remaining animals dying during the first 3 months of life (Xu et al., 1999a). Yet, the inactivation of either $\beta2$ or $\beta4$ did not cause lethal autonomic problems (Xu et al., 1999b). On the opposite, the double mutants $\beta2^{-/-}$/$\beta4^{-/-}$ displayed a phenotype comparable to the $\alpha3^{-/-}$ mice. It is important to note that the EPSP were strongly decreased in $\beta4^{-/-}$ mice, the peak current being only 2% of the wild-type, but nevertheless without any major consequence for global autonomic transmission. Thus, although $\alpha3$ cannot be replaced by any other subunit of the same tribe (because none are expressed in the motor autonomic neuron), $\beta4$ can be functionally replaced by $\beta2$. This apparent subunit redundancy in the wild-type animal is a consequence of the transcriptional regulation of both subunits (see section on promoters below).

Another example of possible functional replacement has been noted with the $\alpha4$ and $\alpha6$ subunits in the dopaminergic neurons of the ventral mesenceph-

alon. In our laboratories, the inactivation of the $\alpha 6$ gene resulted in an upregulation of the $\alpha 4$ protein, which replaced the missing $\alpha 6$ protein (to be published). This effect does not seem linked to a transcriptional regulation and could just be due to a relieve of the titration of $\beta 2$ by $\alpha 6$.

A final example is given by the ϵ null mice (Witzemann et al., 1996). In the wild-type animals, γ is expressed all over the embryonic muscle fiber. This subunit disappears after birth to be replaced by ϵ, expressed only by the subsynaptic nuclei (Duclert and Changeux, 1995). In the $\epsilon^{-/-}$ mice, the "embryonic" subunit remains expressed longer than in wild-type mice. Its presence compensates the lack of ϵ and rescues the animals up after weaning, even if the electrophysiological properties of $(\alpha 1)_2\beta 1\gamma\delta$ receptors differ markedly from those of $(\alpha 1)_2\beta 1\epsilon\delta$ oligomers.

Therefore, different subunits of the same tribe may mutually replace each other within hetero-oligomeric receptors, yet with the obvious condition that they are expressed at the same cellular and subcellular locations. The maintenance of the subunit diversity within a subfamily should therefore not be looked for solely at the level of their pharmacologic properties. The patterns of receptor subunits distribution become then of primary importance in the understanding of nAChR function.

PROMOTERS AND REGULATION OF EXPRESSION

In situ distribution of nAChR subunit mRNAs in the adult nervous system revealed a broad diversity of expression patterns. Some subunits are widely expressed at medium or low levels, like $\alpha 4$ and $\beta 2$ (for the rodent, see Wada et al., 1989) while others like $\alpha 2$, $\alpha 3$, $\alpha 6$, or $\beta 4$, are expressed at high levels yet in a limited number of structures (for the rodent, see Wada et al., 1989; Dineley-Miller and Patrick, 1992; Le Novère et al., 1996). In addition, the study of developmental patterns revealed transient mRNAs subunit expressions (such as $\alpha 3$ mRNA in the cortex, Zoli et al., 1995). Note that, if the diversity of expression patterns is conserved across species, the distribution of a particular subunit may vary. For instance, the pattern of expression of $\alpha 2$ gene varies considerably from one species to the other. In the rat brain, $\alpha 2$ is expressed exclusively in the olfactory bulb and the interpeduncular nucleus (Wada et al., 1989). Similarly, in the chick brain, it is observed only in the lateral spiriform nucleus (Daubas et al., 1990), but not in the interpeduncular nucleus. In sharp contrast, in the brain of the rhesus monkey, $\alpha 2$ is strongly expressed all over the brain (Han et al., 1997).

Such a wealth of transcriptional behaviors have to be encoded in the promoters that control the expression of the subunit genes (see Duclert and Changeux (1995) and Schaeffer et al. (2001) for model system of the motor endplate and Bessis et al. (1995) for the neuronal nAChRs). However, a given stretch of DNA can only encrypt a limited amount of transcriptional information. The information carried within a given promoter is indeed rather limited. For instance, the promoter of $\beta 2$ directs the expression of the gene to every neurons and represses it elsewhere (Bessis et al., 1997). To achieve the complex patterns actually observed, one need several promoters. This can be achieved for instance by gene duplication. The presence of several paralogs multiplies the number of promoters, and accordingly, the possibilities of regulation.

This phenomenon has been unravelled for several multigene families of transcription factors. In *Drosophila, paired*, *gooseberry*, and *gosseberry neuro* are paralogous genes involved in different developmental steps. It has been shown that the coding sequence of these genes were functionaly interchangeable, despite their large sequence divergence (Li and Noll, 1994). Any of these coding sequence might be replaced by any other, as long as the *cis*-regulatory region where kept constant. One may even replace the coding sequence of the *Drosophila* genes by the coding sequence of PAX3, a mouse ortholog of *paired* (Xue and Noll, 1996). Recently, it has also been shown in mice that the coding sequence of a Hox gene can replace its *trans*-paralog on another complex without consequences (Greer et al., 2000), even if the two genes have completely different sequences and functions in development.

Similarly, the maintenance of subunit diversity in nAChRs may, in part, result from the presence of several promoters, allowing the wealth of observed expression patterns. In agreement with that idea is the presence of 27 subunits of nAChR in *Cænorhabditis elegans* (11 more than in human) (Sattelle et al., 2002). In this species each neuronal cell is strictly identified and the organism does not experience any cellular epigenesis during development. By comparison, only 10 genes are found in *Drosophila melanogaster*, another ecdysozoa which, in contrast, experiences cellular epigenesis throughout development.

The existence of several paralogs and their independent promoters, in turn, may generate a redundancy not required by function. nAChR subunit $\beta 2$ has been so far found in every neurons, this wide-

spread expression being due to its particular promoter (Bessis et al., 1997). At the same time $\beta 4$ is coexpressed with $\alpha 3$ because the two genes are located in a cluster and share common regulatory elements (McDonough and Deneris, 1997). Consequently, every neuron that expresses $\alpha 3$ will also express $\beta 2$ and $\beta 4$, even if the presence of the two latter subunits is not required (see the section on KO animals). Note that other clusters of subunits exists, like the one carrying $\alpha 6$ and $\beta 3$, which suggests that such a case could not be unfrequent.

However, in addition to their transcription in a given cell type, receptor subunits need to be addressed to specific subcellular locations in order to fulfill their physiologic role.

INTRACELLULAR TARGETING AND RECEPTOR CLUSTERING

Interneuronal synapses are rather complex structures, which markedly differ from the classical neuromuscular junction. The postsynaptic domain may contain several different kinds of receptors (Dumoulin et al., 2000; Tsen et al., 2000). These receptors, among which nAChRs, are heterogeneously distributed, and this distribution may influence their contribution to the signal transmission (Wilson Horch and Sargent, 1995; Shoop et al., 1999; Berg and Jacob, this issue).

Moreover, neurotransmitter receptors in general, and the nAChRs in particular, are embedded in complex supramacromolecular assemblies (Sheng, 1998), which could be crucial for synaptic function (Grant and O'Dell, 2001). In particular, receptors have been suggested to be connected together through cytoplasmic linkers, such as the 43K-rapsyn (Sobel et al., 1977), and form molecular networks (Cartaud and Changeux, 1993; Kneussel and Betz, 2000). Such networks of receptors are present in bacteria (Shimizu et al., 2000), where they are involved in chemotactic responses. In the latter system, the coupling of receptors through intra-cellular linkers has strong functional consequences. Interestingly, the bacterial networks exhibit a topology similar to the network proposed by Kneussel and Betz (2000) for the clusters of GABA and glycine receptors. Furthermore, the receptors are not frozen in this complex environment and their distribution patterns can be modified by activity (Renger et al., 2000). Neurotransmitter receptors can move across the plasma membrane, and aggregate (Meier et al., 2001; Sergé et al., 2002), and even can be transferred back and forth to an intracellular pool (Barry and Ziff, 2002).

The targeting of $GABA_A$ (Connolly et al., 1996) or glutamate receptors depends on the identity of the subunits forming the receptors. In addition, the composition in subunits also controls the local movements of glutamate receptors (Shi et al., 2001).

Few examples of subunit-specific targeting are well documented for nAChRs. Immunocytochemichal experiments show that $\beta 3$ subunit is preferentialy addressed to the neurites, in particular to the axons (Léna et al., 1999). In $\beta 3^{-/-}$ mice, the binding sites for α-conotoxin MII, specific of the $\alpha[3|6]\beta 2$ interface, disappear from the striatum (Booker et al., 2000). Therefore, $\beta 3$ seems responsible of the targeting of $\alpha 6\beta 2\beta 3$ receptors to the dopaminergic terminals, while the $\alpha 6\beta 2$ receptors unassociated with $\beta 3$ would stay in the somatodendritic compartment (Champtiaux et al., 2002).

At the subcellular level, subunit-dependent specific targeting has also been observed. As described previously, the motor autonomic synapse is made up of clusters of $\alpha 3\beta 4\alpha 5(\beta 2)$ receptors located in postsynaptic densities, surrounded by scattered $\alpha 7$ receptors (Shoop et al., 1999, 2002). The constructions of chimeric $\alpha 7$ subunits, carrying the cytoplasmic portion of $\alpha 3$, triggered a retargeting of the receptors formed by the chimera to the postsynaptic densities (Williams et al., 1998). Chimeric $\alpha 7$ subunits carrying the cytoplasmic portion of $\alpha 5$ are not redirected to the postsynaptic densities, while this is the case for $\alpha 5$ when assembled with $\alpha 3$.

nAChR function at the system level critically depend on the differential targeting of receptor oligomers at the cellular and subcellular levels. The incorporation of subunits able to specifically target nAChR oligomers to different domains of the plasma membrane could be one of the constraints helping to maintain different subfamilies such as $\alpha 7$–$\alpha 8$, in addition to the hetero-oligomeric receptors. In the latter subfamily, the axonal versus somato-dendritic targetting could also account for the maintenance of $\alpha 5$ and $\beta 3$, in addition to the classical α and β.

CONCLUSIONS AND PREDICTIONS

The functions of nAChRs rely on their intrinsic properties, but also on their cellular and subcellular distribution. We propose here that, *within a given subfamily* of nAChR subunits, the maintenance of several possible interchangeable subunits within receptor oligomers is not directly related to the difference of their intrinsic electrophysiologic and pharmacologic properties. Variation of these features might be driven by near-neutral evolution, and would be tolerated within a physiologic envelope acceptable for the survival of

the species. The diversity of subunits would rather be maintained by the necessity to correctly transcribe the gene encoding subunits in a wide variety of neuronal structures and to address particular receptors to specific subcellular compartments. In other words, it would suffice for a given neuronal cell to possess a cationic channel controlled by acetylcholine, at the right place, at the right time. The synapse and the whole network might then adapt to encompass the differences of receptor intrinsic properties.

This hypothesis could be tested with an appropriate set of mutant mice. A knock-in of a chimeric $\alpha4/\alpha3$ cytoplasmic-loop subunit into the locus of $\alpha3$ could, for instance, reverse the effects of the inactivation of $\alpha3$. Similarly a knock-in of $\beta4$ in the locus of $\beta2$ might reverse the effects of the inactivation of $\beta2$. Finally, a knock-in of γ in the locus of ϵ might rescue ϵ null mice, as already suggested in (Witzemann et al., 1996).

If true, a trade-off of a neutral diversification of molecular properties would have taken place in the course of evolution, to the benefit of defined spatiotemporal distributions of receptor subunits, at the cellular, subcellular, and even network levels.

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REFERENCES

Alkondon M, Pereira E, Albuquerque E. 1998. α-Bungarotoxin and methylycaconitinesensitive nicotinic receptors mediate fast synaptic transmission in interneurons of rat hippocampal slices. Brain Res 810:257–263.

Barry M, Ziff E. 2002. Receptor trafficking and the plasticity of excitatory synapses. Curr Opin Neurobiol 12:279–286.

Bessis A, Champtiaux N, Chatelin L, Changeux JP. 1997. The neuron-restrictive silencer element: a dual enhancer/silencer crucial for patterned expression of a nicotinic receptor gene in the brain. Proc Natl Acad Sci USA 94:5906–5911.

Bessis A, Salmon AM, Zoli M, Le Novère N, Picciotto M, Changeux JP. 1995. Promoter elements confering neuron specific expression of the beta-2-subunit of the neuronal nicotinic acetylcholine receptor studied in vitro and in transgenic mice. Neuroscience 69:807–819.

Bohler S, Gay S, Bertrand S, Corringer PJ, Edelstein SJ, Changeux JP, Bertrand D. 2001. Desensitization of neuronal nicotinic acetylcholine receptors conferred by n-terminal segments of the beta 2 subunit. Biochemistry 40:2066–2074.

Booker T, Allen R, Marks M, Grady S, Whiteaker P, Smith K, Collins A, Heinemann S. 2000. Analysis of the beta3 nicotinic acetylcholine receptor subunit in mouse brain using beta3 null mice. In: Neuronal nicotinic receptors: from structure to therapeutics, p. 22.

Cachelin A, Rust G. 1994. Unusual pharmacology of (+)-tubocurarine with rat neuronal nicotinic acetylcholine receptors containing $\beta4$ subunits. Mol Pharmacol 46: 1168–1174.

Cartaud J, Changeux J. 1993. Post-transcriptional compartimentalization of acetylcholine receptor biosynthesis in the subneural domain of muscle and electocyte junctions. Eur J Neurosci 5:191–202.

Changeux JP, Edelstein S. 1998. Allosteric receptors after 30 years. Neuron 21:959–980.

Chavez-Noriega L, Gillespie A, Stauderman K, Crona J, Claeps B, Elliott K, Reid R, Rao T, Velicelebi G, Harpold M, Johnson E, Corey-Naeve J. 2000. Characterization of the recombinant human neuronal nicotinic acetylcholine receptors $\alpha3\beta2$ and $\alpha4\beta2$ stably expressed in hek293 cells. Neuropharmacology 39:2543–2560.

Chavez-Noriega LE, Crona JH, Washburn MS, Urrutia A, Elliott KJ, Johnson EC. 1997. Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors h$\alpha2\beta2$, h$\alpha2\beta4$, h$\alpha3\beta2$, h$\alpha3\beta4$, h$\alpha4\beta2$, h$\alpha4\beta4$ and $\alpha7$ expressed in *Xenopus* oocytes. J Pharmacol Exp Ther 280:346–356.

Cohen B, Figl N, Quick M, Labarca C, Davidson N, Lester H. 1995. Regions of $\beta2$ and $\beta4$ responsible for differences between the steady state dose–response relationships of the $\alpha3\beta2$ and $\alpha3\beta4$ neuronal nicotinic receptors. J Gen Physiol 105:745–764.

Connolly C, Wooltorton J, Smart T, Moss S. 1996. Subcellular localization of γ-aminobutyric acid type a receptors is determined by receptor β subunits. Proc Natl Acad Sci USA 93:9899–9904.

Cordero-Erausquin M, Marubio L, Klink R, J-P C. 2000. Nicotinic receptor function: new perspectives from knockout mice. Trends Pharmacol Sci 21:211–217.

Corringer PJ, Le Novère N, Changeux JP. 2000. Nicotinic receptors at the amino acid level. Annu Rev Pharm Toxicol 40:431–458.

Couturier S, Erkman L, Valera S, Rungger D, Bertrand S, Boulter J, Balli vet M, Bertrand D. 1990. Alpha5, alpha3 and non-alpha3, three clustered avian genes encoding neuronal acetylcholine receptor related subunits. J Biol Chem 265:17560–17567.

Dani J, Ji D, Zhou FM. 2001. Synaptic plasticity and nicotine addiction. Neuron 31:349–352.

Daubas P, Devillers-Thiery A, Geoffroy B, Martinez S, Bessis A, Changeux JP. 1990. Differential expression of the neuronal acetylcholine receptor $\alpha2$ subunit gene during chick brain development. Neuron 5:49–60.

Dineley-Miller K, Patrick J. 1992. Gene transcripts for the nicotinic acetylcholine receptor subunit, beta4, are distributed in multiple areas of the rat central nervous system. Mol Brain Res 16:339–344.

Dourado M, Sargent P. 2002. Properties of nicotinic receptors underlying renshaw cell excitation by alpha-motor

neurons in neonatal rat spinal cord. J Neurophysiol 87: 3117–3125.

Duclert A, Changeux JP. 1995. Acetylcholine receptor gene expression at the developping neuromuscular junction. Physiol Rev 75:339–368.

Dumoulin A, Levi S, Riveau B, Gasnier B, Triller A. 2000. Formation of mixed glycine and GABAergic synapses in cultured spinal cord neurons. Eur J Neurosci 12:3883–3892.

Edelstein S, Schaad O, Henry E, Bertrand D, Changeux JP. 1996. A kinetic mechanism for nicotinic acetylcholine receptors based on multiple allosteric transitions. Biol Cybern 75:361–379.

Faber D, Young W, Legendre P, Korn H. 1992. Intrinsic quantal variability due to stochastic properties of receptor-transmitter interactions. Science 258:1494–1498.

Frazier C, Buhler A, Weiner J, Dunwiddie T. 1998. Synaptic potentials mediated via α-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal interneurons. J Neurosci 18:8228–8235.

Galzi JL, Changeux JP. 1994. Neurotransmitter-gated ion channels as unconventional allosteric proteins. Curr Opin Struct Biol 4:554–565.

Gerzanich V, Peng X,Wang F,Wells G, Anand R, Fletcher S, Lindstrom J. 1995. Comparative pharmacology of epibatidine: a potent agonist for neuronal nicotinic acetylcholine receptors. Mol Pharmacol 48:774–782.

Gil Z, Sack R, Kedmi M, Harmelin A, Orr-Urtreger A. 2002. Increased sensitivity to nicotine-induced seizures in mice heterozygous for the L250T mutation in the alpha7 nicotinic acetylcholine receptor. Neuroreport 13:191–196.

Grant S, O'Dell T. 2001. Multiprotein complex signaling and the plasticity problem. Curr Opin Neurobiol 11:363–368.

Greer J, Puetz J, Thomas K, Capecchi M. 2000. Maintenance of functional equivalence during paralogous hox gene evolution. Nature 403:661–665.

Gross A, Ballivet M, Rungger D, Bertrand D. 1991. Neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes: role of the α subunit in agonist sensitivity and desensitization. Eur J Physiol 419:545–551.

Han ZY, Le Novère N, Zoli M, Champtiaux N, Hill J, Changeux JP. 1997. Localization of nAChR subunit mRNAs in the brain of *Macaca mulatta*. Eur J Neurosci 12:3664–3674.

Hardison R. 2001. New views of evolution and regulation of vertebrate beta-like globin gene clusters from an orphaned gene in marsupials. Proc Natl Acad Sci USA 98:1327–1329.

Harvey SC, Luetje CW. 1996. Determinants of competitive antagonist sensitivity on neuronal nicotinic receptor β subunits. J Neurosci 16:3798–3806.

Hatton G, Yang Q. 2002. Synaptic potentials mediated by α7 nicotinic acetylcholine receptors in supraoptic nucleus. J Neurosci 22:29–37.

Heidmann T, Changeux JP. 1982. A molecular model for the regulation of synapse efficacy at the postsynaptic level. CR Acad Sci Paris 295:665–670.

Jones M, Westbrook G. 1996. The impact of receptor desensitization on fast synaptic transmission. Trends Neurosci 19:96–101.

Kneussel M, Betz H. 2000. Clustering of inhibitory neurotransmitter receptors at developing postsynaptic sites: the membrane activation model. Trends Neurosci 23:429–435.

Kruk P, Korn H, Faber D. 1997. The effects of geometrical parameters on synaptic transmission: a Monte-Carlo simulation study. Biophys J 73:2874–2890.

Kumar S, Hedges S. 1998. A molecular timescale for vertebrate evolution. Nature 392:917–920.

Léna C, Changeux JP. 1997. Pathological mutations of nicotinic receptors and nicotine-based therapies for brain desorders. Curr Opin Neurobiol 7:674–682.

Léna C, de Kerchove d'Exaerde A, Cordero-Erausquin M, Le Novère N, Arroyo-Jimenez M, J-P C. 1999. Diversity and distribution of nicotinic acetylcholine receptors in the *locus ceruleus* neurons. Proc Natl Acad Sci USA 96: 12127–12131.

Le Novère N, Changeux JP. 1995. Molecular evolution of the nicotinic acetylcholine receptor subunit family: an example of multigene family in excitable cells. J Mol Evol 40:155–172.

Le Novère N, Changeux JP. 1999. The ligand gated ion channel database. Nucleic Acids Res 27:340–342.

Le Novère N, Zoli M, Changeux JP. 1996. Neuronal nicotinic receptor α6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. Eur J Neurosci 8:2428–2439.

Li X, Noll M. 1994. Evolution of distinct developmental functions of three *Drosophila* genes by acquisition of different *cis*-regulatory regions. Nature 367:83–87.

Lindstrom J. 1997. Nicotinic acetylcholine receptors in health and disease. Mol Neurobiol 15:193–222.

Lundin L. 1993. Evolution of the vertebrate genome as reflected in paralogous chromosomal regions in man and the house mouse. Genomics 16:1–19.

Marrone D, Petit T. 2002. The role of synaptic morphology in neural plasticity: structural interactions underlying synaptic power. Brain Res Rev 38:291–308.

McDonough J, Deneris E. 1997. $\beta 3'$: an enhancer displaying neural-restricted activity is located in the 3′-untranslated exon of the rat nicotinic acetylcholine receptor β4 gene. J Neurosci 17:2273–2283.

Meier J, Vannier C, Sergé A, Triller A, Choquet D. 2001. Fast and reversible trapping of surface glycine receptors by gephyrin. Nat Neurosci 4:253–260.

Ortells MO, Lunt GG. 1995. Evolutionary history of the ligand-gated ion-channel superfamily of receptors. Trends Neurosci 18:121–126.

Papke R, Heinemann S. 1991. The role of the β4-subunit in determining the kinetic properties of rat neuronal nicotinic acetylcholine α3-receptors. J Physiol 440:95–112.

Picciotto MR, Zoli M, Léna C, Bessis A, Lallemand Y, Le Novère N, Vincent P, Merlo-Pich E, Brûlet P, Changeux JP. 1995. Abnormal avoidance learning in mice lacking

functional high-affinity nicotine receptor in the brain. Nature 374:65–67.

Picciotto MR, Zoli M, Rimondini R, Léna C, Marubio LM, Merlo-Pich E, Fuxe K, Changeux JP. 1998. Acetylcholine receptors containing the $\beta 2$ subunit are involved in the reinforcing properties of nicotine. Nature 391:173–177.

Renger J, Egles C, Liu G. 2000. A developmental switch in neurotransmitter flux enhances synaptic efficacy by affecting ampa receptor activation. Neuron 29:469–484.

Revah F, Bertrand D, Galzi JL, Devillers-Thiery A, Mulle C, Hussy S N Bertrand, Ballivet M, Changeux JP. 1991. Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. Nature 353:846–849.

Roerig B, Nelson D, Katz L. 1997. Fast synaptic signaling by nicotinic acetylcholine and serotonin 5-HT_3 receptors in developing visual cortex. J Neurosci 17:8353–8362.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Sattelle D, Culetto E, Grauso M, Raymond V, Franks C, Towers P. 2002. Functional genomics of ionotropic acetylcholine receptors in *Caenorhabditis elegans* and *Drosophila melanogaster*. Novartis Found Symp 245:240–257.

Schaeffer L, de Kerchove d'Exaerde A, Changeux JP. 2001. Targeting transcription to the neuromuscular synapse. Neuron 31:15–22.

Sergé A, Fourgeaud L, Hémar A, Choquet D. 2002. Receptor activation and homer differentially control the lateral mobility of metabotropic glutamate receptor 5 in the neuronal membrane. J Neurosci 22:3910–3920.

Sheng M. 1998. Molecular organization of the postsynaptic specialization. Proc Natl Acad Sci USA 98:7058–7061.

Shi S, Hayashi Y, Esteban J, Malinov R. 2001. Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. Cell 105: 331–343.

Shimizu T, Le Novère N, Levin M, Beavil A, Sutton B, Bray D. 2000. Molecular model of a lattice of signalling proteins involved in bacterial chemotaxis. Nat Cell Biol 2:792–796.

Shoop R, Esquenazi E, Yamada N, Ellisman M, Berg D. 2002. Ultrastructure of a somatic spine mat for nicotinic signaling in neurons. J Neurosci 22:748–756.

Shoop R, Martone M, Yamada N, Ellisman M, Berg D. 1999. Neuronal acetylcholine receptors with $\alpha 7$ subunits are concentrated on somatic spines for synaptic signaling in embryonic chick ciliary ganglia. J Neurosci 19:692–704.

Sobel A, Weber M, Changeux JP. 1977. Large scale purification of the acetylcholine receptor protein in its membrane-bound and detergent extracted forms from *Torpedo marmorata* electric organ. Eur J Biochem 80:215–224.

Sorenson E, Shiroyama T, Kitai T. 1998. Postsynaptic nicotinic receptors on dopaminergic neurons in the substantia nigra pars compacta of the rat. Neuroscience 87:659–673.

Tsen G,Williams B, Allaire P, Zhou YD, Ikonomov O, Kondova I, Jacob M. 2000. Receptors with opposing functions are in postsynaptic microdomains under one presynaptic terminal. Nat Neurosci 3:126–132.

Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW. 1989. Distribution of alpha2, alpha3, alpha4, and beta2 neuronal nicotinic subunit mrnas in the central nervous system: a hybridization histochemical study in rat. J Comp Neurol 284:314–335.

Wang F, Gerzanich V, Wells GB, Anand R, Peng X. 1996. Assembly of human neuronal nicotinic receptor $\alpha 5$ subunits with $\alpha 3$, $\beta 2$ and $\beta 4$. J Biol Chem 271:17656–17665.

Williams B, Krishna-Temburni M, Schwartz-Levey M, Bertrand S, Bertrand D, Jacob M. 1998. The long internal loop of the $\alpha 3$ subunit targets nAChRs to subdomains within individual synapses on neurons *in vivo*. Nat Neurosci 1:557–562.

Wilson Horch HL, Sargent PB. 1995. Perisynaptic surface distribution of multiple classes of nicotinic acetylcoline receptors on neurons in the chicken ciliary ganglion. J Neurosci 15:7778–7795.

Witzemann V, Schwarz H, Koenen M, Berberich C, Villarroel A, Wernig A, Brenner H, Sakmann B. 1996. Acetylcholine receptor epsilon-subunit deletion causes muscle weakness and atrophy in juvenile and adult mice. Proc Natl Acad Sci USA 93:13286–13291.

Wong L, Gallagher J. 1991. Pharmacology of nicotinic receptor-mediated inhibition in rat dorsolateral septum. J Physiol 436:325–346.

Xu W, Gelber S, Orr-Urtreger A, Amstrong D, Lewis R, Ou CN, Patrick J, Role L, De Biasi M, Beaudet AL. 1999a. Megacystis, mydriasis, and ion channel defect in mice lacking the $\alpha 3$ neuronal nicotinic acetylcholine receptor. Proc Natl Acad Sci USA 96:5746–5751.

Xu W, Orr-Urtreger A, Nigro F, Gelber S, Ballard Sutcliffe C, Amstrong D, Patrick J, Role L, Beaudet A, De Biasi M. 1999b. Multiorgan autonomic dysfunction in mice lacking the $\beta 2$ and the $\beta 4$ subunits of neuronal nicotinic acetylcholine receptors. J Neurosci 19:9298–9305.

Xue L, Noll M. 1996. The functional conservation of proteins in evolutionary alleles and the dominant role of enhancers in evolution. EMBO J 15:3722–3731.

Young J, Trask B. 2002. The sense of smell:genomics of vertebrate odorant receptors. Hum Mol Genet 11:1153–1160.

Zhang M, Wang Y, Vyas D, Neuman R, Bieger D. 1993. Nicotinic cholinocepto-mediated excitatory postsynaptic potentials in rat nucleus ambiguus. Exp Brain Res 96:83–88.

Zoli M, Jansson A, Syková E, Agnati LF, Fuxe K. 1999. Volume transmission in the CNS and its relevance for neuropsychopharmacology. Trends Pharmacol Sci 20: 142–150.

Zoli M, Le Novère N, Hill JA, Changeux JP. 1995. Developmental regulation of nicotinic ACh receptor subunit mrnas in the rat central and peripheral nervous systems. J Neurosci 15:1912–1939.

Zucker R, Regehr W. 2002. Short-term synaptic plasticity. Annu Rev Physiol 64:355–405.

Zwart R, Vijverberg P. 1998. Four pharmacologically distinct subtypes of $\alpha 4\beta 2$ nicotinic acetylcholine receptor expressed in *Xenopus lævis* oocytes. Mol Pharmacol 54: 1124–1131.