Allosteric mechanisms in normal and pathological nicotinic acetylcholine receptors

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Recent chemical and advanced structural studies on site-directed and naturally occurring pathological mutants of individual members of the multigene family of nicotinic acetylcholine receptors have yielded structure–function relationships supporting indirect 'allosteric' interactions between the acetylcholine-binding sites and the ion channel in signal transduction.

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Current Opinion in Neurobiology 2001, 11:369–377

0959-4388/01/$ – see front matter

Abbreviations
ADNFLE autosomal dominant nocturnal frontal lobe epilepsy
NAChR nicotinic acetylcholine receptor

Introduction

Membrane receptors for transmitters, peptides and pharmacological agents are central to signal transduction. They selectively recognize chemical effectors (neuronal or hormonal) and transduce, in an 'indirect' allosteric manner [1], binding recognition into biological action though the activation of ligand-gated ion channels (LGICs) and/or G-protein-coupled receptors (GPCRs). Since their initial isolation and characterization in the early 1970s as integral membrane proteins, about 2000 different receptor species have been cloned and sequenced. In the *C. elegans* genome, for example, there are up to 90 LGIC genes, and about 5% of all *C. elegans* genes encode a GPCR, including about 1000 orphan receptors that may be chemoreceptors [2]. Many of these receptor genes are conserved through evolution [3]. About 5% of the known human genes are assigned to receptors, which are the targets of major drugs, and are responsible for several important human pathologies.

In this review, we discuss new insights gained in the much studied model provided by the nicotinic acetylcholine receptor (nAChR), and in addition make reference to recent observations made in other systems concerning general receptor mechanisms.

With respect to neurotransmitter signaling, the classical example of the neuromuscular junction — where ACh is liberated in less than 0.2 ms as a high local concentration pulse (up to 10^{-3} M) over a postsynaptic dense layer of nAChR molecules — may not be as general as initially thought. Acetylcholine, as well as other neurotransmitters and peptides, may be released in a volume or paracrine mode [4] at the level of varicosities, thus modulating, in a 'tonic' manner, a widely dispersed population of high-affinity receptors. For example, in the spinal cord, endogenous ACh tonically modulates serotonin release, in part through nAChRs present on inhibitory γ-amino butyric acid (GABA)ergic neurons [5].

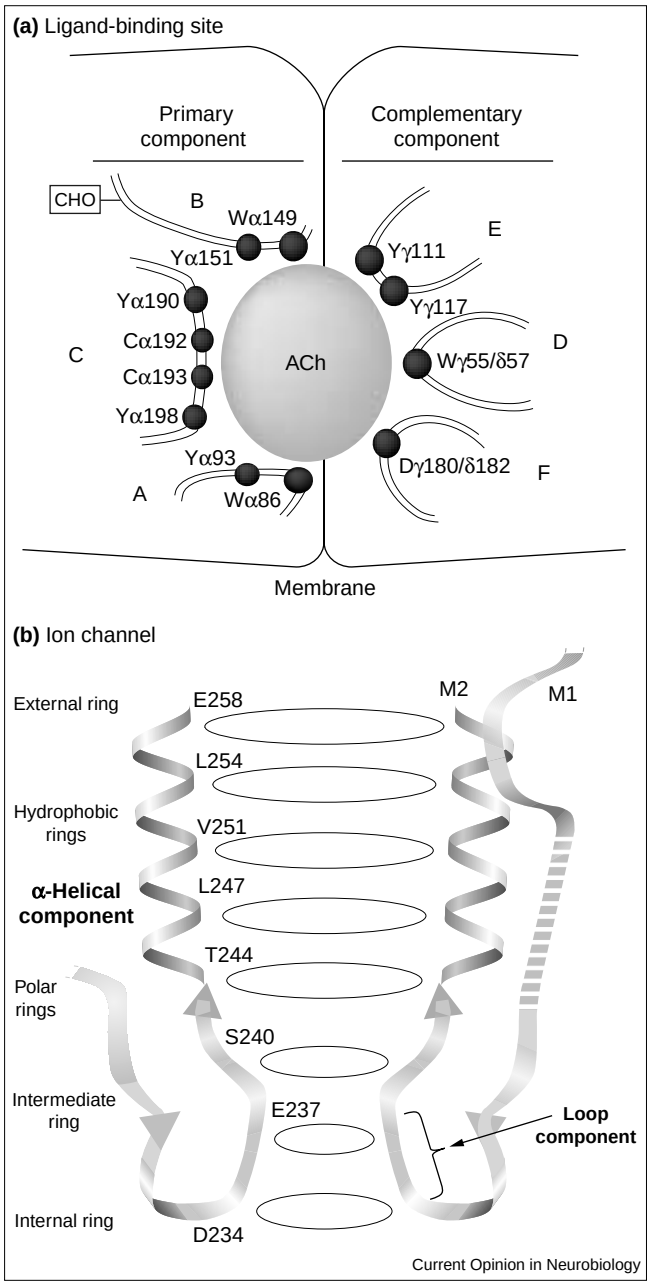
Subunit composition and distribution of neuronal nAChR

Nicotinic acetylcholine receptors exist as 'homopentamers' made up of α7, α8 or α9 subunits, or 'heteropentamers' comprising various combinations of α2–α6 with β2–β4 subunits, α9 with α10 subunits, or precisely arranged $(\alpha1)_2$–β1–γ/ε–δ subunits in muscle. (For the latest findings, see articles in the special issues of *Eur J Pharmacol* **393**:1-320 [2000], and *Neuropharmacology* **39**:2515-2855 [2000], as well as the *Soc Neurosci Abstr* [2000].)

New subunit combinations have been discovered recently by Groot-Kormelink *et al.* [6] in experiments using a *ménage-à-trois* reporter mutation in β3 to detect interactions with the α3β4 couple. Similarly, modified single-channel properties of α3 and β2 or β4 have been observed by Nelson and Lindstrom [7•] in the presence of α5, and α6 has been found to interact with various combinations of α (including α5) and β subunits [8,9]. Activity changes also have revealed interactions of α7 with β3 [10•]. Specific subunit combinations were revealed by single-cell PCR in conjunction with electrophysiological recordings in the locus ceruleus, possibly α6β3β2(α4) [11], and in the substantia nigra and ventral tegmental area, where non-α7-containing receptors with a putative $\alpha4\alpha6\alpha5(\beta2)_2$ composition were found to be sensitive to 1 nM methyllycaconitine [12].

The distribution of nAChR proteins in the brain may not resemble the highly clustered localization at the motor endplate, where a number of specific proteins (e.g. AGRIN, 43K rapsyn, MuSK, MASC) have been identified and shown to interact with postsynaptic nAChR using mouse gene knockouts (for review see [13]). However, none of these proteins has been implicated directly in modulating the signaling response, although mice with a knockout of the nAChR ε subunit have revealed that the receptor may have an indirect role in maintaining the highly ordered structure of the postsynaptic membrane [14]. Direct evidence for subsynaptic clustering of neuronal nAChR has been obtained in chick ciliary ganglion [15,16,17•], and for the α4 subunit in the substantia nigra [18,19]. It is plausible that as yet unidentified cytoplasmic proteins resembling gephyrin [20,21] or PSD-95 [22•] for glycine or glutamate receptors, respectively, may be found to interact with neuronal nAChRs.

Figure 1



Representation of the ligand-binding site and the ion channel. (a) Ligand-binding site, as viewed from the exterior of the receptor along the plane of the membrane. Residues identified by affinity labeling and site-directed mutagenesis are indicated in the primary site present on α-type subunits and the complementary site present on γ or δ subunits of muscle receptor, or on α subunits of homopentameric neuronal receptors or β subunits of heteromeric neuronal receptors (for addition information, see [1,32]); the schematic arrangement of the loops is consistent with the recent structural studies (K Brejc *et al.*, personal communication). (b) Ion channel. Residues from M2 and the cytoplasmic loop component implicated in the selectivity filter are presented; adapted from [32].

In the brain, many nAChR have been localized at presynaptic positions [23] where they facilitate neurotransmitter release by allowing the calcium ion entry directly through the nicotinic channels, or indirectly through voltage-sensitive calcium channels [24]. Presynaptic nAChRs might also mediate local neuromodulation by ACh at the level of 'synaptic triads' [25], which are postulated to serve as elementary components for temporal learning. For example, in the hippocampus, the nAChR can modulate neural circuits by exciting inhibitory interneurons [26] or by reducing the responsiveness of *N*-methyl D-aspartate (NMDA) glutamate receptors [27].

Structure–function relations updated

Our current understanding of the nAChR molecule is based mainly on chemical and genetic approaches combined with predictive methods and the limited structural information available. The success in determining the three-dimensional structure of the binding region of a glutamate receptor [28,29•] — with the details largely anticipated by models based on homologies with bacterial periplasmic binding proteins [30] — underlies the predictive capacities of secondary structure computations from first principles, for which the most advanced methods have been applied recently to the nAChR by Le Novère *et al.* [31••]. The final consensus secondary structure includes 9 α helices and 17 β strands: the large extracellular domain is predicted to comprise β strands with only two α helices at the α-amino group end, with resemblance to the immunoglobulin domain [32]; the transmembrane segments are predicted to be in a mixed α/β topology, with the α helices predominantly linked, on the cytoplasmic side, through a stretch of variable length and sequence to two well-conserved amphipathic helices whose function deserves investigation.

The location of the 'agonist binding sites' at the interface between subunits where each subunit provides three loops of amino-acid residues, A, B and C for the principal, and D, E and F for the complementary component, respectively (see Figure 1a), is supported by recent data (see [32]). The cation–π interactions that stabilize agonist binding have been investigated by Zhong *et al.* [33], who used tryptophan derivatives (as well as a tethered quaternary ammonium group) incorporated into the receptor by an *in vivo* nonsense-suppression method [33]. The orientation requirements for activation by covalent agonists have been provided by Sullivan and Cohen [34•], who used ligands tethered via cysteine substitution in conjunction with sulfhydryl-reactive reagents. Striking differences in the environment of the two ACh sites carried by the muscle nAChR have been revealed by a novel method of time-resolved fluorescence spectroscopy [35•]. Improved definition of the residues in the amino-terminal domain involved in desensitization [36] has emphasized the contribution of a short loop in the vicinity of the main immunogenic region in conferring fast desensitization from the β2 to the β4 subunit [37].

The crystal structure at 2.7 Å resolution of the acetylcholine-binding domain from a novel glia-derived soluble analogue of the neuronal nAChR synaptic domain that

occurs naturally in the snail *Lymnea stagnalis* (AB Smit *et al.*, personal communication) reveals a homopentameric organization, with the ACh (and α toxin) site made up of the predicted aromatic amino acids and double cysteine of loops A, B, C and D, E, F at the subunit interface, and with each monomer folding, as anticipated [32], into an immunoglobulin-like topology with long and twisted β-strands (K Brejc *et al.*, personal communication).

New insights into the functional organization of the channel by site-directed mutagenesis distinguish the transmembrane M2 α-helical component, which lines a hydrophilic pore (independently of the charge on the amino-acid chains), from an SF loop (which may be analogous to a phosphate or 'P' loop), which serves as a 'selectivity filter' at (or near) the cytoplasmic extremity of the channel (see Figure 1b) [38••], but also possibly as a gate [39]. This role is also confirmed by mutations that convert the glycine receptor from anionic to cationic selectivity [40]. These results take on added importance in relation to the K^+ channel, the structure of which has been determined by X-ray crystallography [41], which shares possible geometric features with the nAChR ion channel, although in an inverted disposition [32].

As in the case of the K^+ channel, the direct participation of backbone atoms in establishing the properties of the channel should be considered. In this respect, of particular relevance is England *et al.*'s [42••] study of backbone mutations in transmembrane regions M1 and M2. These authors used a nonsense suppression technique using α-hydroxy (rather than α-amino) acids to replace the usual peptide backbone bond by an ester.

Unwin and co-workers [43••] have obtained 4.6-Å resolution electron microscopy images of *Torpedo* nAChR, which now attach to the receptor cytoplasmic domain (on the basis of five-fold symmetry organization) densities formerly interpreted as belonging to 43K rapsyn. This suggests — still hypothetically — that there is ion flow through slits adjacent to the two α subunits, with the M3–M4 loop contributing charged residues that might influence selectivity (α subunit residues E377, E384, E391 and E398).

Production of chimeric γ–ε subunits has revealed that elements of the γ subunits in the M3–M4 region can influence the open times of channels in excised patches [44]. Several myasthenic syndrome mutants with altered functional properties have also been reported in the M3–M4 region (see below), as well as a deletion that alters subunit interactions [45•], suggesting that this portion of the molecule also participates in intersubunit contacts. The 4.6-Å resolution data for subunit organization continue to be interpreted by England *et al.* [43••] as the β subunit located between the two α subunits, although the bulk of the available evidence places the γ subunit between the two α subunits [45•,46].

In any case, the structure–function studies demonstrate unambiguously that the ACh-binding site and the ion channel belong to distinct and far distant domains, and thus that their interaction is indirect or allosteric.

Modes and models of signal transduction

Single nAChR molecules respond to ACh by undergoing a conformational transition to an open-channel state, associated with slower 'modulatory' transitions to (or from) desensitized states. These properties are readily described by an allosteric model (see Figure 2a) that is based on concerted transitions between pre-existing conformational states (implying a mechanism involving 'rigid body motion') [1]. Nevertheless, because of their simplicity, standard sequential models of two ACh bindings followed by a gating transition (implying a mechanism involving 'plastic motion') are still used to fit experimental data, as in several recent examples [44,47,48]. In certain cases a variant involving mono-liganded gating is also invoked [48]. In our opinion, four sets of experimental data strongly argue in favor of transitions between a small number of conformations that exist prior to ligand binding.

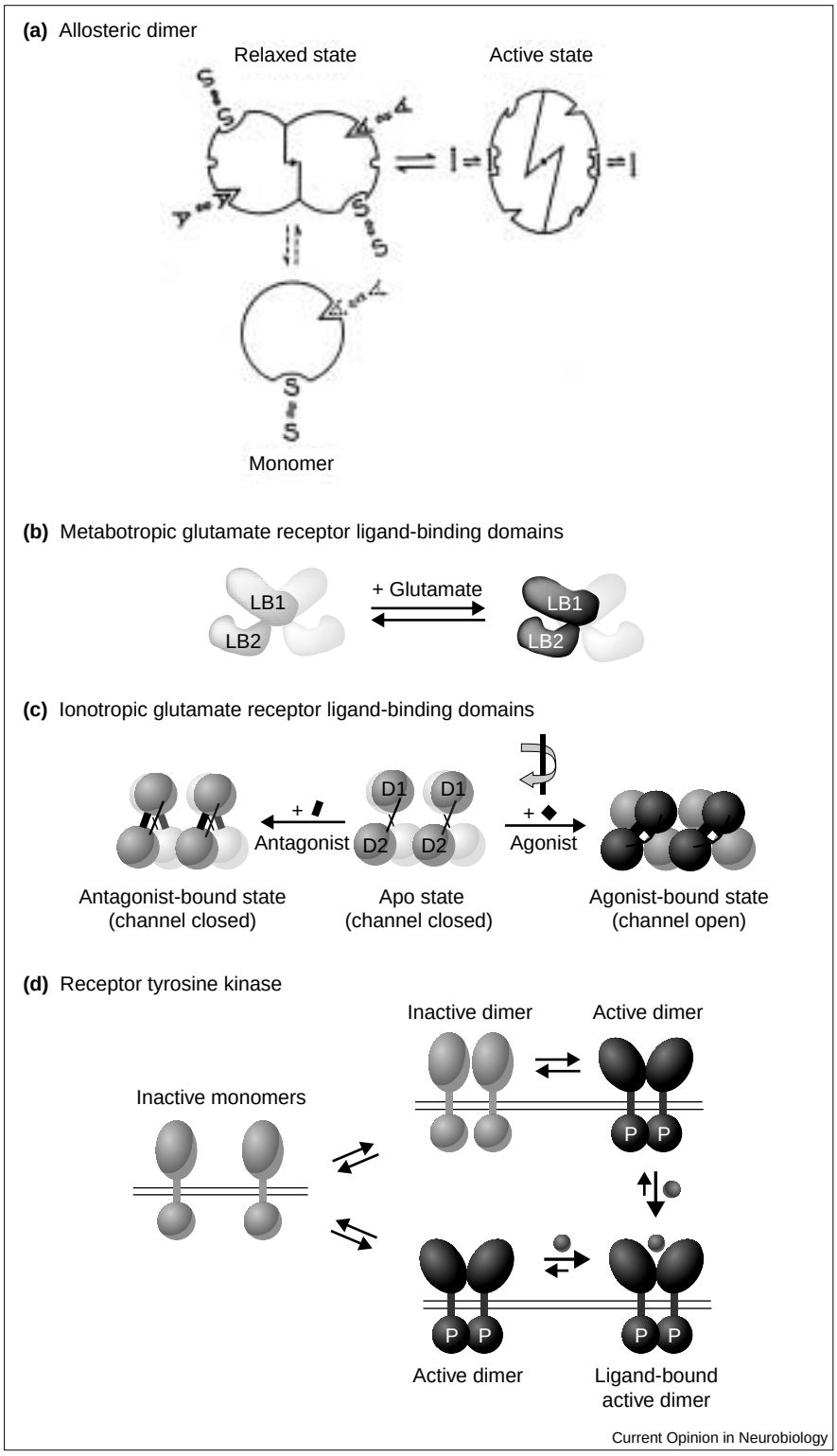
First, observations on the distance separating the ion channel and the ACh-binding site in the range of 20–40 Å [43••,49]; these data confirm that a direct contact is not possible and that coupling of binding and channel opening must occur by an indirect allosteric-type mechanism.

Second, pleiotropic phenotypes for which a single-point mutation in the channel domain increases the apparent affinity for agonists at the distant ACh-binding site, such as the myasthenic mutant εT264P; the data are quantitatively accounted for by an allosteric mechanism assuming a decrease of the isomerization constant from 10^9 to 10^3 between basal and active states [50].

Third, spontaneous openings of single nAChR channels recorded by voltage clamp in the absence of ligand [51,52]; the data can only be described by an allosteric model that includes opening of unliganded receptors [50]; spontaneous openings in muscle and neuronal nAChR with mutations in other subunits have also been reported recently [53•].

Fourth, conversion of competitive agonists to competitive antagonists, as first observed in the nAChR α7 mutant L247T [54] (or vice-versa, as in the case of glycine receptors with a startle disease mutation [55]). Both phenotypes are readily understood in the allosteric framework, but not in the sequential one [1]. (Similar properties have recently been reported by Taverna *et al.* [56••] for the Lurcher mutation that enhances potency of glutamate and converts an antagonist to an agonist.) In addition, for the γ subunit W55L mutant (of *Torpedo*), *d*-tubocurarine (*d*TC) binding at the α–γ site acts not as a competitive antagonist but as a coactivator or partial agonist [57]. The data can be accounted for by an allosteric model that assumes that the γ subunit W55L mutation alters ACh binding at the α–γ but not at the α–δ site, while *d*TC binding to both sites is unaltered

Figure 2



Allosteric models for enzymes and membrane receptors. **(a)** The initial formulation of the allosteric model, in terms of a dimeric enzyme (used with permission from [103]). **(b)** Tyrosine receptor kinase, showing aggregation of momomers to form inactive and active dimers; the latter are preferentially stabilized by ligand (adapted from [62]). **(c)** The two domains (D1 and D2) contributing to the GluR2 ligand-binding core; the domains (lobes) undergo rotation and closure of the site that is stabilized by smaller agonist and opposed by larger antagonist (adapted from [29]). **(d)** Extracellular ligand-binding region of the mGluR1 receptor; two domains (lobes) are present, as in (c), identified as LB1 and LB2 with closer contact of the domains stabilized by glutamate (adapted from [61]).

(SJ Edelstein and JB Cohen, unpublished data). In principle, direct tests of mechanistic alternatives (such as the 'induced fit') could be provided in experiments that independently monitor single-binding and single-channel events [58], but experimental obstacles need to be overcome first.

In contrast, results interpreted as being in conflict with the allosteric scheme have been reported on the basis of indirect biochemical measurements of binding [59] or conformational state [60], but in each case ambiguities concerning the nature of the measurements raise doubts about the conclusions.

Concerning other systems, structural studies by Morikawa and co-workers [61••] of the extracellular ligand-binding region of the seven-transmembrane-helix metabotropic receptor mGluR1 has revealed 'active' and 'resting' conformations (see Figure 2b) that are modulated through the dimeric interface by a packed α-helical structure, with glutamate binding stabilizing both the 'active' dimer and the closed protomer in dynamic equilibrium. In the case of the ionotropic iGluR2 ligand-binding core, dimer formation and symmetrical interdimer interaction with a large conformational change stabilized by agonists as compared with competitive antagonists is, as well, consistent with the basic concept of rigid body motion. The conformational transition would consist of changes in the quaternary organization of protein oligomers with little alteration of the protomer tertiary structure [29•], with individual domains (1 and 2) occupying in this case the roles of protomers (see Figure 2c). A scheme strictly identical to the original two-state model (see Figure 2a,d) has also been adopted by Schlesslinger [62•] for signal transduction by tyrosine kinase receptors.

Functional, cooperative nearest-neighbor interactions between receptor molecules in a membrane two-dimensional lattice were suggested and formalized several decades ago [63], in an attempt to extend allosteric interactions to supra-macromolecular assemblies in biological membranes. Although up to now nAChR function has generally been analyzed in terms of individual receptor molecules (with the possible exception of dimer formation in muscle nAChR [64–66]), evidence is accumulating for many receptor systems that supramacromolecular interactions and clustering contribute to function [67•]. This is the case for bacterial chemotactic receptors, but also for the large receptor complex on T lymphocytes [68], arrays of ryanodine receptor [69], and possibly the intricate lattice of cochlear outer hair cells [70]. Evidence has been presented for molecular interactions between different receptors such as GlyR and α3-containing nAChR clusters under one presynaptic terminal [17•]. In addition, α3β4 nAChR and ATP-gated purine-mediated $P2X_2$ channels influence each other when co-activated [71•], although the precise mechanism of these interactions are not yet elucidated.

nAChR allosteric mutations, deletions and pathological 'states of consciousness'

Natural mutations in the human population are continuing to provide a rich source of complex and surprising phenotypes, particularly in congenital myasthenic syndromes, for which over 50 distinct mutations have been characterized, with most occurring in the ε subunit gene [72]. Roughly half of the well-characterized mutants fall into the category of 'gain-of-function' or 'hyperactive' phenotypes, argued above as supporting the allosteric scheme [50,51]. Moreover, in-frame duplication of six residues in the M3–M4 loops results in abnormal activation kinetics [73] and the point mutation A411P leads to a wide distribution of channel open times [48], reinforcing the arguments that the M3–M4 loop contributes to channel regulation. The α subunit mutation V285I in the M3 domain has also been reported; this amino-acid position shows volume-dependent contributions to channel gating [74].

Concerning neuronal nAChRs, mutations in the α4 subunit produce autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE; reviewed in [75]), although exactly how the functional changes observed in the mutant α4 subunits cause the pathological symptoms, which result in transient alterations in consciousness, remains unclear. A β2 missense mutation V287L in M2 that leads to a 'hyperactive' phenotype manifested by slower desensitization has also been implicated in ADNFLE [76]. Roles for α7 have been suggested in prenatal effects of nicotine on lung cells [77], but also in Alzheimer's disease [78,79], as well as in schizophrenia [80]. In a search for sequence variations that might influence smoking behavior, five novel single nucleotide polymorphisms (SNPs) of β2 were examined, but no correlation with nicotine use was observed [81].

Insights into the functional role of neuronal nAChR subunits have been obtained in mice from a series of knockouts involving the genes for subunits α3, α4, α7, α9, β2 and β4 [82]. The phenotypes observed in these mice include reduced antinociception [83•] and increased exploratory behavior [84] in mice lacking α4; attenuated self-administration of nicotine [85] and increased neurodegeneration during aging [86] in mice lacking β2; and various dysfunctions in mice lacking α3 [87] or both β2 and β4 [88]. Preliminary reports for mutant knockin mice, including α7 L247T [89] and the replacement of leucine by serine at the corresponding position in the α4 subunit [90] offer experimental models of neurodegeneration associated with hyperactivity of nAChR.

Long-term regulation

Considerable attention has been focused recently on the phenomenon of nAChR upregulation, initially in relation to the effects of smoking [91]. Current research has used prolonged exposure to agonists for various human nAChR subunits [92–94]. Some evidence suggests that this phenomenon may be related to desensitization for α4β2 receptors in oocytes [95], although in other studies on α4β2 receptors in M10 cells upregulation occurred at concentrations of agonist 1–2 orders of magnitude higher than their estimated binding constants [96]. However, multiple desensitized states and differences in the kinetic of desensitization and upregulation, as well as ligand-specific differences, may complicate these analyses [97•]. Several specific phosphorylation processes have also been implicated [91].

An important issue is the fraction of agonist binding sites that are intracellular, and the values are surprisingly elevated in certain cases [96], with virtually no α3β2 surface receptors detected by antibody reaction before upregulation in one report [94]. The features of upregulation that appear intimately related to trafficking in

intracellular membranes suggest a 'maturation' model (S Bohler *et al.*, personal communication) that may be related to the specific assembly pathways [98,99].

Conclusions and future prospects

Considerable progress has been made in establishing the stereochemical basis of ligand-binding and ion channel properties for nAChR in general and for the individual variations among members of the multigene family. A fairly coherent picture is emerging on the basis of algorithms for prediction of conformation [31••], medium-resolution structural studies [43••], and astute use of site-directed mutagenesis [32]. In addition, studies on the three-dimensional structure of a soluble molluscan ACh-binding protein provide important new insights (K Brejc *et al.*, personal communication).

For mechanistic studies based on the kinetics of channel opening and closing, a generally accepted framework has yet to be adopted, on the basis of structural data. In principle, the allosteric model of concerted transitions between pre-existing conformational states could serve such a role [1] and in addition would be useful in designing adequate pharmacological treatments for the many novel phenotypes appearing for congenital myasthenic syndromes [72] and ADNFLE [75,76,100].

Animal models of human pathologies are also emerging from the modification of nAChR in transgenic mice [101]. The observation that agonist-dependent upregulation may also play a major role is becoming the focus of considerable research activity in the field of nicotine addiction. At the level of integrating receptors in brain-scale processes [102], paracrine effects [4] have emerged as a plausible mechanism for globally activating receptors, for example, at presynaptic terminals [23]. In all these instances, the signal transduction properties of receptors create a critical step in the bottom-up transfer of information in neuronal networks from the molecular to the cognitive level.

Update

Evidence has been presented for narrowing of the channel involving residues above the M1–M2 loop in the desensitized state [104]. An article describing studies on mice with an α4 channel mutation reported in abstract form [90] has now been published [105]. Progress in analyzing the mode of α7 inhibition by the β-amyloid peptide has been reported by two groups [106,107]. Upregulation has now been shown to involve conductance changes in the case of α4β2 receptors in K-177 cells [108]. Concerning the correlates of acetylcholine with consciousness, the review by Perry *et al.* remains timely reading [109].

Acknowledgements

We thank Pierre Jean Corringer for helpful comments. This work was supported by the Collège de France, the Université de Genève, the Centre National de la Recherche Scientifique, the Association Française contre les Myopathies, the Association pour la Recherche sur le Cancer and the Commission of the European Communities.

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