The nicotinic acetylcholine receptor: the founding father of the pentameric ligand-gated ion channel superfamily

by

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Abstract: a critical event in the history of biological chemistry was the chemical identification of the first neurotransmitter receptor, the nicotinic acetylcholine receptor. Disciplines as diverse as electrophysiology, pharmacology and biochemistry, joined together in a unified and rational manner with the common goal of successfully identifying the molecular device that converts a chemical signal into an electrical one in the nervous system. The nicotinic receptor has become the founding father of a broad family of pentameric membrane receptors, paving the way for their identification, including that of the GABA_A receptors.

It has been 42 years since the isolation of the nicotinic acetylcholine receptor from fish electric organ, the first ligand–gated ion channel, and first ion channel, ever identified; 25 years since the first GABA-A and glycine receptor subunits were cloned and sequenced and concomitantly their homology with the nicotinic acetylcholine receptors recognized; and 5 years since the discovery that closely homologous ligand–gated ion channels are present in prokaryotes (1). In this review, I briefly retrace the main steps in the discovery of the nicotinic acetylcholine receptor, the titular head of this receptor superfamily.

The concept of receptor and the chemical identification of the acetylcholine receptor

The English physiologist John Newport Langley, working with neuromuscular preparations proposed in 1905 that muscle tissue possesses «a substance that combines with nicotine and curare...receives the stimulus and transmits it». He called the muscle entity the «receptive substance». In the subsequent 50 years, the concept of pharmacological receptors, inspired three main lines of research. Firstly, the pharmacological approach aimed at characterizing the
specificity of the receptor site by using novel chemical ligands (eg the distinction between nicotinic and muscarinic acetylcholine receptors (AChR) by Sir Henry Dale); secondly, the electro-physiological approach exemplified by Bernard Katz and John Eccles aimed at understanding the ionic responses to endogenous neurotransmitter signals; and thirdly, the chemical tradition aimed at the chemical identification of the receptor molecule(s).

In the late 60’s, lipids, polysaccharides, proteins, even nucleic acids, were considered as potential receptors. The early independent efforts of Carlos Chagas, Eduardo de Robertis, and David Nachmansohn to identify the receptor for acetylcholine in the electric organ of the fish *Electrophorus electricus* were abandoned because their tissue extracts lacked specificity (2). However, in the course of these studies, Nachmansohn recognized the extraordinarily rich content of nicotinic synapses in the electric organ (2). He, with Ernest Schoffeniels, devised a method for preparing individual cells, or electroplaques, from the electric organ. This offered the opportunity to investigate, simultaneously, the electrophysiological, pharmacological and altogether with the biochemical characteristics of the response to ACh within the same biological system (2). At this time there were also speculations that the enzyme acetylcholinesterase (AChE) and the physiological receptor site for ACh could reside on the same protein complex.

The introduction of new biochemical methods radically changed the field of receptor identification. One such method is affinity labeling which relies on the use of compounds that are structural homologs of the neurotransmitter and also possess a highly reactive group. This combination allows for specific binding to the receptor site, and once bound the probe covalently links to the protein. For instance, the molecule, p-trimethylammonium benzenediazonium fluoroborate (TDF), carries a trimethylammonium group (as does ACh) as well as a reactive diazonium group (3). As anticipated TDF interacted covalently with *E. electricus* electroplaque as an irreversible competitive antagonist, and curare protected against this covalent attachment (4). The method was subsequently improved upon with the synthesis of 4-(N-maleido)-phenyltrimethylammonium iodide (MPTA).
whereby the diazonium is substituted for by a maleimide group (5). The latter selectively reacts with –SH groups exposed by treating the electroplaque membrane with dithiothreitol. However, at this stage, both the method of tissue preparation and the specificity of the compounds used were insufficient to allow for isolation of the receptor in its active form from the electric organ.

A second method, that significantly advanced the field was the marked improvement of procedures for fractionation and purification of membrane fragments rich in AChE from *E. electricus* electric organs. Electron microscopic sections of these membrane fragments revealed they formed closed vesicles (6). Inspired by the technique used with bacterial permeases (7), it became possible to measure radioactive Na\(^+\) (or K\(^+\)) ion fluxes with these microsacs by using a simple filtration method (8). The microsacs responded to nicotine agonists with specificities closely resembling those recorded by electrophysiological methods employing intact electroplaques. The signal transduction by the neurotransmitter could be reproduced in a totally acellular system in the absence of energy supply and in a chemically defined environment. Thus, it became possible to study in vitro the chemistry of the physiological response to ACh and of the signal transduction mechanism involved (8). The receptor molecule was evidently present in the purified membranes in a functional state. It was possible now to follow reversible binding to these purified membranes using the nicotinic agonist decamethonium as the radioactive ligand (by the method of equilibrium dialysis that Gilbert & Muller-Hill (9) used to identify the lac-repressor) (Fig 1). The detergent deoxycholate gently extracted the binding protein without denaturing it and bound decamethonium was displaced by various nicotinic agonists and antagonists including curare and flaxedil in the order of their physiological effects (10). Since then, similar receptor-binding assays have been used extensively to characterize the GABA\(_A\) and glycine receptors (this volume).

Third Chen-Yuan Lee, a Taiwanese pharmacologist, had found that a snake venom toxin, \(\alpha\)-bungarotoxin (\(\alpha\)BGT), specifically blocks in vivo neuromuscular transmission in high vertebrates at the postsynaptic level without interacting with AChE (11). Aware of Claude
Bernard’s lesson to use toxic compounds as chemical lancets, I asked Chen-Yuan Lee, who unexpectedly visited me at the Pasteur Institute, for a sample of the toxin. A few days later, I received it and immediately tried it in the three systems just mentioned. The result was remarkable: α-BGT blocked the electroplaque’s electrical response in vivo and the microsac’s ion-flux response to nicotinic agonists in vitro; α-BGT blocked as well the binding of radioactive decamethonium to the detergent extract (Fig 1). This extract contained a protein, sensitive to pronase digestion, that bound nicotinic agonists and the snake venom toxin in a mutually exclusive manner. This nicotinic receptor (nAChR) molecule was shown to be a high molecular weight hydrophobic protein that could be physically separated from AChE.

Then, an α-toxin from *Naja nigricollis*, closely homologous to α-BGT, was covalently coupled to sepharose beads without losing its binding activity. Mixing the toxin beads with the membrane extract revealed that 75–100% of the nAChR protein bound to the toxin beads, whereas 85–100% of AChE remained in the supernatant. The data (13), confirmed that AChE and the nAChR molecule were distinct protein entities. These studies also introduced Cuatrecasas’s technique of affinity chromatography to the nAChR field. Many groups then became aware of these distinct methods (14-16). We (17,18) and others used alternative affinity columns with immobilized quaternary ammonium agonists or antagonists (Fig 2) extending Miledi et al. (20) use of radioactive I$^{131}$-labeled α-BGT (which, according to them, selectively binds to the receptor in its resting state).

Another rather simple technological development, that, retrospectively, had an important impact on nAChR research was the isolation of a novel generation of excitable microsacs exceptionally rich in nAChR (20–40% of total protein) prepared from homogenates of *T. marmorata* electric organ (21), a finding was readily confirmed by other groups. The nAChR-rich membranes made the structural and functional properties of the membrane-bound nAChR accessible to a variety of biochemical and biophysical methods, such as purification in large quantities (22), fluorescence spectroscopy (23), electron spin resonance (24), and X-
ray diffraction (25).

Finally, the nAChR protein purified from *E. electricus* and the purified nAChR-rich membranes from *T. marmorata* were examined by electron microscopy and revealed ring-like particles (8–9 nm in diameter) with a hydrophilic core linked to a compact bundle (26) (Fig 3). Made up of several (5–6) subunits, they formed closely packed two-dimensional assemblies in *T. marmorata* postsynaptic membranes (approximately 8–12 000 μm²) (26,27) (Fig 3). These nAChR images were the first ever of the structure of a neurotransmitter receptor. They were subsequently described in greater details by Nigel Unwin (rev 28) and others. Similar pictures later became available for the GABA<sub>A</sub> and glycine receptors (this volume).

**The pentameric organization of the nicotinic receptor and the complete sequence of the subunits.**

The amount of purified nAChR was sufficient to identify the subunit organisation of the protein. A first study using partial cross-linking of the purified *E. electricus* nAChR, revealed 5 well defined bands suggesting a pentameric organization (29). The pentameric organization was rapidly confirmed by the teams of Karlin and Raftery, who, in addition, discovered that the nAChR molecule is composed of four distinct types of subunits with slight differences of molecular mass, that assemble into an 2α.1β.1γ.1δ heteropentamer (30-33).

Nothing was known about the chemistry of the subunits. However with the recently developed new technology of high-resolution microsequencing, aminoacid sequences could now be determined from small quantities of protein. The sequence of 20-amino-acid comprising the N-terminal domain of the α-subunit of *T. marmorata* receptor was then established in my laboratory (34). A chemical identity card of the receptor was made available, the first ever established for a neurotransmitter receptor. It was confirmed in the Raftery laboratory with the α-subunit of *T. californica* (35) and extended to the N-terminal sequence of the four subunits revealing a number of sequence identities among the subunits (36). Consistent with the Monod-Wyman–Changeux (1965) (MWC) model (37), the nAChR protein was an authentic oligomer, but pseudosymmetrical, with a fivefold axis of rotation perpendicular to the plane of the post synaptic membrane.
Knowledge of the initial sequence data opened the nAChR field to DNA-recombinant technologies. The teams of Shosaku Numa (38-40), Stephen Heinemann (41-42), Eric Barnard (43), as well as by Anne Devillers-Thiéry and Jérôme Giraudat (44-45) in my laboratory, struggled to clone the complementary DNAs of the different subunits from electric organ and muscle and to establish their complete sequence. Experiments by Eric Barnard and Riccardo Miledi had demonstrated that messenger RNA extracted from the electric organ of Torpedo injected into Xenopus oocytes led to the synthesis and incorporation of functional acetylcholine receptors into the membrane of the oocyte (46). Injection of the 4 mRNAs transcribed from the cloned cDNAs yielded functional nAChRs (47) confirming earlier biochemical experiments (48-49) that assembly of the 4 types of subunits suffice to recover a fully operational nAChR.

Examination of the complete cDNA sequences revealed several common structural domains along the sequences of the subunits that led to the first model of transmembrane organization of nAChR subunits (39, 40, 42, 45). It was proposed that the long hydrophilic N-terminal segment, four hydrophobic stretches, and short hydrophilic segment, were organized into an extracellular (synaptic) domain, four transmembrane α-helices, and an intracellular (cytoplasmic) domain. In 1987 closely homologous sequences and the organisation of the subunits – including a cys-loop- were found in GABA<sub>A</sub>, glycine, 5HT3, GluCl and neuronal nAChRs uncluding α<sub>7</sub> and α<sub>4</sub>β<sub>2</sub> nAChRs (50, rev 51) thus creating the superfamily of pentameric receptors that is the subject of the present volume. The recent discovery of cationic orthologs in prokaryotes (52-53) has extended recently the superfamily plunging its evolutionary origins back 3 billion years (1).

**Identification of the acetylcholine-binding sites**

The actual tridimensional topology of the AChR protein and of the various sites it carries still could not be directly inferred from DNA-recombinant technologies. Identification of the amino-acids composing the ACh-binding site and the ion channel relied upon different technologies. The previously mentioned method of affinity labeling proved to be useful at this stage. A first result was obtained by Karlin’s group,
using MPTA (7) which labels the sulfhydryl groups of the ACh-binding site (see above). This led to the identification of a pair of adjacent cysteines (192–193), located in the N-terminal domain of the α-subunit (54). Despite these results, the pharmacological specificity of the ACh binding site remained unknown.

Our group demonstrated that the snake (³H) α-toxin itself, without additional modification, could be used as a photolabel. UV irradiation of the (³H) α-toxin-Torpedo receptor complex not only resulted in the incorporation of covalently bound radioactivity into the α-subunit but also into the γ- and δ-subunits (55). From this observation it was concluded that the ACh-binding sites were located at the interface between subunits (55) and were therefore non-equivalent. This was confirmed in subsequent functional studies.

The use of p-N,N-dimethylammonium benzene diazonium difluoroborate (DDF), an affinity probe similar to TDF (5), provided additional important information (56). The dimethyl ammonium group of DDF created a resonant molecule that could be photoactivated by energy transfer from the protein. Indeed, eight amino acids were found labeled by DDF, six of them with an aromatic side chain, and all of them located in the long hydrophilic NH2 terminal domain of the α-subunit. These amino acids were distributed into three main loops, forming a sort of electronegative aromatic pocket in which the quaternary ammonium group of acetylcholine is lodged (56-58) thus pointing to an analogy with the AChE binding site where pi bonding is exhibited as well. These three loops located on the α-subunit side of the binding site, are referred to as the «principal component» were named A, B, and C (58), a nomenclature that has been adopted by the receptor community. In agreement with the snake (³H) α-toxin photolabeling data, the affinity probe DDF labeled the γ- and δ-subunits in addition to the α-subunit (51, 59). The various groups working on the receptor, including those of Arthur Karlin, Jonathan Cohen and ourselves, further documented this notion and identified additional loops D, E, and F on the non-α side of the subunit interface (ref in 66, 67). These loops form a «complementary» component of the acetylcholine-binding site on the γ- and δ-subunits. These
biochemical data were supported by site-mutagenesis studies of the labeled amino acids identified in these studies (ref in 51, 59).

Confirmation of the binding site organisation has come from the crystal structure of a soluble snail protein that binds ACh, the acetylcholine-binding protein (AChBP), a close homolog of the nAChR extracellular domain (60), and of the full-length eukaryotic GluCl receptor (61) and prokaryotic Erwinia chrysanthemi receptor (ELIC) bound with GABA (62) and ACh (as an antagonist) (63) (rev 1).

**Identification of the ion channel**

By the early 1980s, the biochemical structure of any ion channel was not known. The question was how to chemically identify the amino-acids that line the pore through which ions flow. The quest (1974–1999) proved to be long and difficult (see 51,64). Pharmacological agents, such as local anesthetics, known for decades to block ion currents elicited by nicotinic agonists, in an indirect, noncompetitive, manner, proved to be essential tools for chemical labeling the channel. The first experiments, performed with both *E. electricus* and *T. marmorata* receptor-rich membranes, demonstrated in vitro that, at pharmacologically active concentrations, the local anesthetics do not directly displace nicotinic ligands from the ACh-binding site but reversibly bind to a different allosteric site (65,66). One of these compounds, chlorpromazine, displayed, in addition, the remarkable property of covalently linking to the receptor protein by simple UV irradiation. In receptor-rich membranes from *T. marmorata* chlorpromazine labeled the four types of subunits of the nAChR (67), and precise quantitative measurements demonstrated that it binds to just one high-affinity site per $2\alpha_1.\beta_1.\gamma_1.\delta_1$ oligomer (68). The kinetics of access of chlorpromazine to this site increased 100-fold when rapidly mixed with ACh under conditions expected to generate functional ion channels (69-70). We proposed that chlorpromazine binds to a site located within the ion channel along the pseudosymmetry axis that becomes accessible to chlorpromazine when the ion channel opens. The conditions under which the channel could be specifically labeled were thus established.

It took more than a year to demonstrate that chlorpromazine labels serine 262, within the second transmembrane segment (TM2) of the $\delta$–subunit (71); a finding rapidly
confirmed by another group using the same protocol, but with a different probe (72). Further identification of the chlorpromazine-labeled amino acids on the other subunits showed that the serines not only form a ring (81), but also revealed the adduct of other amino acids (leucines and threonines) located at a distance of three to four amino acids on both sides of the ring of serines (73). It was concluded that: (a) the TM2 segments contribute to the channel walls; (b) these segments are folded into an \( \alpha \)-helix; (c) the chlorpromazine-binding site is located at a near-equatorial position in the channel’s pseudosymmetry axis; and (d) there exists a positive reciprocal allosteric interaction between ACh and the chlorpromazine-binding sites.

In parallel site-directed mutagenesis experiments in which single channel recordings were carried out after reconstitution in Xenopus oocytes, a region located in the \( \delta \)-subunit was shown to be responsible for a conductance difference between Torpedo and bovine channels that comprises the putative transmembrane segment TM2 and the adjacent bend portion between segments TM2 and TM3 (75). Subsequent analysis (76) identified rings of negatively charged glutamine residues, which were classified as external, intermediate, and cytoplasmic, that beautifully frame the amino acid clusters labeled by chlorpromazine, thus confirming their proposed location within the ion path (68-70). The teams of Henry Lester and Norman Davidson reached a similar conclusion (77).

Further studies, identified amino acids which contribute to the ionic selectivity of the channel (78-80). A group of three residues was found to drive the conversion of the cationic selectivity of the ion channel into one of anionic selectivity (79-80). For the first time, an excitatory receptor could be transformed into an inhibitory one. This finding, as well as the converse operation (from anionic to cationic) was reproduced with other receptors: GABA\(_{A}\), glycine, GluCl, and 5HT3 (51 and this volume). A functional chimera was successfully constructed that joined the synaptic domain of \( \alpha7 \)-nAChR and the transmembrane domain of 5HT3 receptor (81). Even combinations of prokaryotic and eukaryotic receptor domains were found functional (82). This unambiguously demonstrates a conservation of tertiary organization between members of the receptor superfamily. Lastly, the high resolution X-ray data from prokaryotic ELIC and GLIC (83-85) are consistent with the
biochemical data and EM structure (rev 28, 51) of the nAChR ion channel (1). They demonstrate further that the channel domain is topographically distinct from the neurotransmitter-binding domain and that the interaction between the neurotransmitter and the ion transport mechanism is an allosteric interaction (1, 64, 86).

Allosteric transitions of the nicotinic receptor: the quaternary twist mechanism

Direct evidence for the conformational changes that mediate this interaction was still unavailable. Early rapid mixing experiments using snake 3H α−toxin as a probe and receptor-rich membranes from T. marmorata revealed changes of conformation that took seconds to reach a high-affinity state, possibly desensitized, from a low-affinity resting state (87). Consistent findings were subsequently reported using muscle cells (88) and Torpedo membranes (89-90). A refined kinetic analysis of the binding interaction of the fluorescent nicotinic agonist, dansyl-C6-choline with receptor-rich membranes (91-92), and correlation with the in vitro measurement of ion transport through the ion channel (93), resulted in the demonstration of allosteric transitions between several conformational states: a resting closed-channel state stabilized by snake α-toxin and nicotinic antagonists, an active, transient, open-channel state with low affinity for acetylcholine and nicotinic agonists; and, at least one desensitized, slowly accessible, refractory state, with a high affinity for both agonists and antagonists (Fig 4).

Moreover, under resting conditions a sizeable fraction (approximately 20%) of the receptor was found to be present in the high-affinity, desensitized state, and spontaneous channel openings of the muscle nAChR were recorded in the absence of ACh (94). This ruled out the induced-fit mechanism to the benefit of the conformational selection (MWC) scheme (see 86). Still the situation appeared more complex than for regulatory enzymes. There exists not only one but a cascade of discrete transitions between open and closed conformational states (see 1, 51) (Fig 4).

Up until recently, little new information became available to help explain the structural transitions of the nAChR, except for in situ electron microscopy studies of Torpedo receptor (95). In silico modeling from the available structural data brought
novel insight into the conformational transitions of the receptor protein (96-97). Normal mode analysis performed on a 3D model of the α7 AChR gave a breakdown of the protein movements into discrete modes. Among the first 10 lowest frequency modes, the first mode produced a structural reorganization that caused a wide opening of the channel pore resulting from a concerted and symmetrical transition—a quaternary twist motion of the protein—with opposing rotations of the upper (extracellular) and lower (transmembrane) domains, and significant tertiary reorganizations within each subunit in particular at the domain interface. The global quaternary twist motion accounted reasonably for the available experimental data on the gating process (97). Strong evidence emerged from the comparison studies of the X-ray structure of the prokayotic receptors GLIC (from Gloeobacter violaceus) that showed an open channel conformation and ELIC which displayed a closed channel (83-85). Comparison of the two structures indicated that, at least 29% of the quaternary twist transition model accounts for channel opening. Future developments include the molecular dynamics of the transition in the microsec time scale (98).

Allosteric modulatory sites.

The signal transduction process mediated by nAChR is regulated by at least three main categories of allosteric « modulators » which bind to sites distinct from the neurotransmitter site and the ion channel. These modulators are thought to selectively shift the allosteric equilibrium in favor of either an active (positive modulators) or a resting/desensitized conformation (negative modulators) without competing with the neurotransmitter binding to the orthosteric sites (64, 86 ref in 1).

One category of modulator is Ca\(^{2+}\), which potentiates most neuronal nAChRs (99-100), binds to the extracellular domain below the ACh site at residues contributed from both sides of the subunit interface (95). Another is Zn\(^{2+}\).

A second important category of modulators that includes galantamine bind at « non-agonist » interfaces which, in hetero-pentameric nAChRs, differ from the neurotransmitter binding site, and appears to be homolog of the benzodiazepines site on GABA\(_A\) receptors (see R Olsen this volume).

Another group of allosteric modulators interact with the
transmembrane domain. The antihelminthic ivermectin was originally discovered to behave as a strong positive modulator of α7 nAChR. Its action was altered by mutations within the transmembrane domain TM2 (101). General anesthetics (both intravenous and volatile) negatively modulate excitatory nAChRs but positively enhance inhibitory GABA receptors. Photolabelling studies with GABA A receptors (see R Olsen & J Cohen this volume) and X–ray structures of GLIC complexes with propofol or desfurane reveal a site within the upper part of the transmembrane domain of each subunit (102) to which nicotinic allosteric modulators may also interact in neuronal nAChRs (103) (AM in Fig 4).

Allosteric modulatory sites have also been identified in the cytoplasmic loop that links TM3 and TM4 in all eukaryotic (but not prokaryotic) pentameric receptors. Including in nAChRs several phosphorylation sites (104) that control desensitization in muscle and α7 nAChR and contribute to endplate localization by agrin-induced tyrosine phosphorylation of the cytoskeletal protein 43K-rapsyn (22, ref 51). The cytoplasmic domain of the α4 nAChR subunit also binds a variety of scaffold protein that interacts with cytoskeletal proteins, and with G protein systems that are involved in intracellular signalling pathways (105).

Conclusion

Since the isolation of the nAChR and the discovery that GABA-A and glycine receptor subunits are close orthologs of the nAChR thereby founding the superfamily of pentameric ligand-gated ion channels, the whole field of pentameric receptors for neurotransmitter has blossomed, including the discovery of homologous receptors in prokaryotes. Several of them are the target of most commonly used drugs such as benzodiazepines, barbiturates, curare and general anesthetics. The recent advances in the X-ray structure of several of these receptors (1) open new avenues for the rational design of pharmacological agents acting on the brain, in parallel with the abundant studies on the GPCRs which were initiated later.

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Figure legends.

Figure 1. Top: Binding method by equilibrium dialysis used for the identification of the nicotinic receptor. Bottom: effect of the snake toxin $\alpha$-bungarotoxin on the nicotinic agonist $^3$H decamethonium binding (from Changeux et al 1970 (12)).
Figure 2. Purification of the nicotinic acetylcholine receptor by affinity chromatography (from Olsen et al 1972 (17)).
Figure 3. First structural observation of the purified nicotinic receptor protein from Electrophorus electricus (Top) and from purified subsynaptic membrane fragments from Torpedo marmorata (Bottom) (from Cartaud et al. 1973 (26)).
Figure 4. Minimal four states model for the allosteric transitions of the nicotinic receptor (from Changeux 1990 (64))

ACh, acetylcholine, CB competitive (orthosteric) blocker, NCB non-competitive (channel) blocker, AM allosteric modulator, P, phosphorylation site.