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# Structural answers and persistent questions about how nicotinic receptors work

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## TABLE OF CONTENTS



## 1. ABSTRACT

The electron diffraction structure of nicotinic acetylcholine receptor (nAChR) from *Torpedo marmorata* and the X-ray crystallographic structure of acetylcholine binding protein (AChBP) are providing new answers to persistent questions about how nAChRs function as biophysical machines and as participants in cellular and systems physiology. New high-resolution information about nAChR structures might come from advances in crystallography and NMR, from extracellular domain nAChRs as high fidelity models, and from prokaryotic nicotinoid proteins. At the level of biophysics, structures of different nAChRs with different pharmacological profiles and kinetics will help describe how agonists and antagonists bind to orthosteric binding sites, how allosteric modulators affect function by binding outside these sites, how nAChRs control ion flow, and how large cytoplasmic domains affect function. At the level of cellular and systems physiology, structures of nAChRs will help characterize interactions with other cellular components, including lipids and trafficking and signaling proteins, and contribute to understanding the roles of nAChRs in addiction, neurodegeneration, and mental illness. Understanding nAChRs at an atomic level will be important for designing interventions for these pathologies.

## 2. INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are integral-membrane, neurotransmitter-activated ion channels in the central and peripheral nervous systems that participate in signal transmission associated with the physiological release of acetylcholine (1). As cation-selective ion channels, they depolarize transmembrane voltage when they transition from the closed resting state to the open cation-conducting state by binding agonist. Continued exposure to agonist causes transition from the

open state to the closed desensitized state. More than four decades of research have answered many structural questions about nAChRs. Reviews published across these decades reveal the progression of questions and answers (2-15). Current understanding of the structural basis of how nAChRs work is relatively advanced compared to other areas of ion channel biophysics (16-27). Despite this level of understanding, however, questions persist, not only about biophysical properties but also about the structural basis of how nAChRs affect cellular and systems physiology (28). By reviewing research literature published primarily between 2003 and mid-2007, the goals of this review are to describe the current state of understanding of the structures of nAChRs and to describe questions for which atomic-level structural answers still await.

## 3. WHICH STRUCTURAL FEATURES DEFINE NACHRS AND OTHER CYS-LOOP RECEPTORS?

Mammalian genomes contain 16 nAChR subunits ($\alpha1$–$\alpha7$, $\alpha9$, $\alpha10$, $\beta1$–$\beta4$, $\delta$, $\gamma$, $\varepsilon$; $\alpha8$ is found only in chicken). Primary sequence homology and the consensus sequence of the Cys loop (C-x-[LIVMFQ]-x-[LIVMF]-x(2)-[FY]-P-x-D-x(3)-C from reference (29)) are important unifying properties of the Cys-loop superfamily and its component families. In addition, an N-terminal extracellular domain containing the agonist binding site, four transmembrane domains (M1–M4), and an intracellular large cytoplasmic loop between M3 and M4 are unifying topological features. For nAChRs, $\alpha$ subunits contain extracellular tandem cysteine residues near the start of M1. Subunits assemble into pentamers to form homomeric or heteromeric ligand gated ion channels. The nAChRs are selective for cations, typically $Na^+$ and $K^+$ with varying levels of relative permeability for $Ca^{2+}$. For example, the Ca:Na permeability ratio is about 10 for rat $\alpha7$ or $\alpha7$-containing nAChRs (30, 31) and about 0.2 for muscle-type nAChR from frog (*Rana pipiens*) (32).

The presence of two cysteines with a disulfide bond and separated by 13 amino acid residues with various degrees of conservation has been a used as a defining structural characteristic for nAChR subunits from eukaryotes (33, 34) and, more generally, for Cys-loop receptor subunits (20, 22, 26). The Cys-loop receptor and subunit superfamily includes nAChRs and ionotropic 5-$HT_3$ receptors (5-$HT_3$R) (35, 36) (cation selective), ionotropic GABA receptors (37, 38) and glycine receptors (39-41) (anion selective), zinc-activated channels (42, 43), glutamate-gated GluCl chloride channels (44, 45), and serotonin-gated chloride channels (46, 47).

Using the Cys loop as a defining feature of Cys-loop proteins, however, might be overly restrictive for identifying proteins sharing overall structural homology. For example, requiring a Cys loop with 13 amino acid residues between cysteines as a defining feature excludes the acetylcholine binding protein (only 12 residues between cysteines in the Cys loop) (48, 49). Moreover, nicotinoid prokaryotic proteins contain no Cys loop (50, 51), although they are or are likely to be structurally similar to Cys-loop subunits.

The diversity of Cys-loop subunits and potential subunits across vertebrate, invertebrate (52-57), and prokaryotic genomes raises questions about which structural features are common to all Cys-loop subunits and which structural and functional features distinguish subgroups and individual receptors. Comparing structural (primary through quaternary structure) and functional properties can suggest which novel proteins belong to the Cys-loop superfamily regardless of the presence of a true Cys loop. Presently, comparing primary sequences probably is the best starting point for evaluating membership in the Cys-loop receptor family (Figure 1). Structural genomics of membrane and nonmembrane proteins, however, will provide means to identify candidates for membership in the Cys-loop superfamily based on higher order (secondary through quaternary) structural similarities even when primary sequence homology is insignificant (58-61).

## 4. WHAT IS KNOWN ABOUT THE STRUCTURE OF NACHRS?

The highest resolution structural information about nAChRs has come from electron diffraction of helical tubular crystals of nAChRs from *Torpedo marmorata* (4 Å resolution) and from X-ray crystallography of acetylcholine binding protein (AChBP) (about 2 Å resolution). Extending the *Torpedo* nAChR structure to higher resolution with electron diffraction is constrained by disorder of the tubular crystals. Understanding nAChRs based on properties of AChBP is constrained by the absence in AChBP of a transmembrane domain and the absence of ion channel function. Determining atomic-resolution structures of nAChR subtypes beyond *Torpedo* is an important goal for the future. Structural comparisons among different subtypes will yield insights about distinctive patterns of ligand binding, ion channel behavior, and roles in physiology. This information also will guide the design of subtype specific drugs. X-ray crystallography is a promising approach but presently is hampered by the difficulty of obtaining large amounts of nAChRs for crystallization trials and the difficulty of crystallizing integral membrane proteins. NMR can produce information about both protein structure and dynamics but presently is limited by the maximum size of suitable proteins (about 100 kDa).

Model systems can continue to yield important information about structures of nAChRs. Extracellular domain nAChRs, which are high fidelity, pentameric models of full length nAChRs, might be more successful for crystallography if they can be crystallized without detergent and more successful for NMR because of their smaller size. Ion channel homologues from bacteria recently were recognized and might be easier to produce in large quantity for crystallography and NMR.

Static structures provide incomplete pictures of nAChRs. Intramolecular dynamics is essential for starting and stopping ion flow through nAChRs and might be important for intramembranous and cytoplasmic interactions between nAChRs and other proteins.

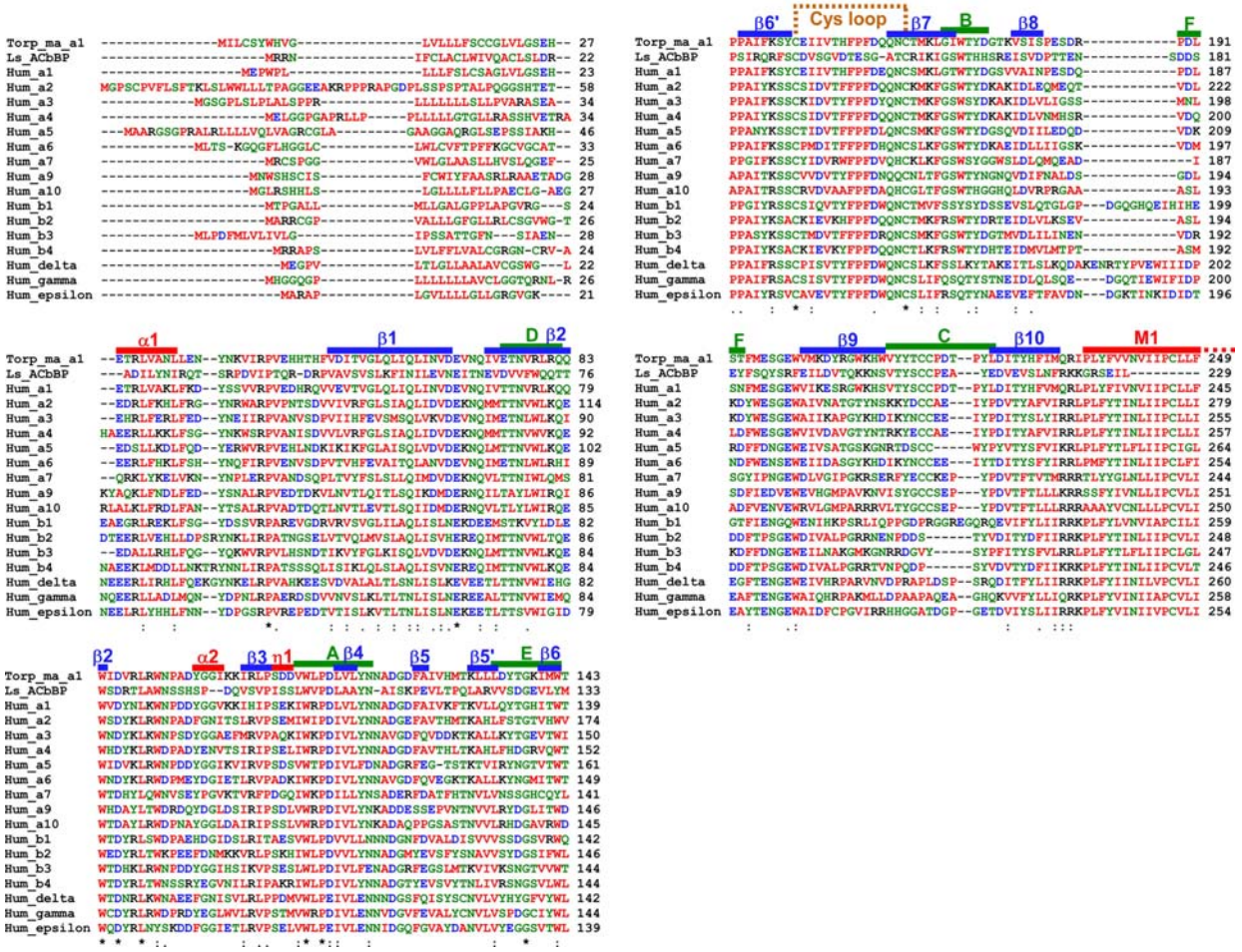


**Figure 1**. Multiple sequence alignment by ClustalW of the N-terminal extracellular domains from the α1 subunit from *Torpedo marmorata*, the AChBP subunit from *Lymnaea stagnalis*, and all 16 human nAChR subunits. The residue numbers shown at the ends of the lines include the residues of the N-terminal signal sequences. The alignment includes a portion of the N-terminal end of transmembrane domain M1. Annotations above the blocks of sequences show structural features of the extracellular domain. The red labels α1 and α2 show α-helices; η1 shows a $3_{10}$-helix, which is slightly more tightly coiled than a α-helix (a $3.6_{13}$-helix in this nomenclature) (107). The blue labels β1 through β10 show β-sheets. The green labels A through F show loops of the principal face (A, B, and C) and complementary face (D, E, and F) of the orthosteric binding site of *Torpedo marmorata* (14, 24). The Cys loop is labeled with an orange dotted line. Color code for amino acid residues: red for small and hydrophobic residues, excluding tyrosine (AVFPMILW); blue for acidic residues (DE); black for basic residues (RK); green for hydroxyl and amine residues and others (STYHCNGQ).

Understanding such dynamics will come not only from physical methods but also from computational methods like molecular dynamics simulations. The challenge for the future is to integrate structure and dynamics to explain how nAChRs work.

### 4.1. Electron microscopy and electron diffraction

Electron microscopy has contributed much information about the overall shape and internal structure of nAChRs (62-76). The most comprehensive and highest resolution structures have come from electron diffraction studies of muscle-type nAChRs in helical tubular crystals grown from membrane vesicles of *Torpedo marmorata*, an electric fish (77-88). The *Torpedo* nAChR structure at 4 Å resolution in the resting state is the best structure to date (Figure 2) and often is the foundation for homology modeling of other nAChRs (89, 90).

In this structure, the N-terminal extracellular domain of each subunit contains ten β-strands and one major α-helix, similar to the structure of the acetylcholine binding protein (AChBP) (Figure 3). Based on the AChBP structure, the subunit order in muscle-type receptors based on the locations of the principal and complementary faces of the binding sites is anticlockwise (α1)γ(α1)δ(β1) when viewed from the extracellular compartment (Figure 2). Adjacent subunits interact through contacts in the N-terminal extracellular domains (mainly polar contacts), α-helical transmembrane domains (mainly hydrophobic contacts), and loops between transmembrane domains and

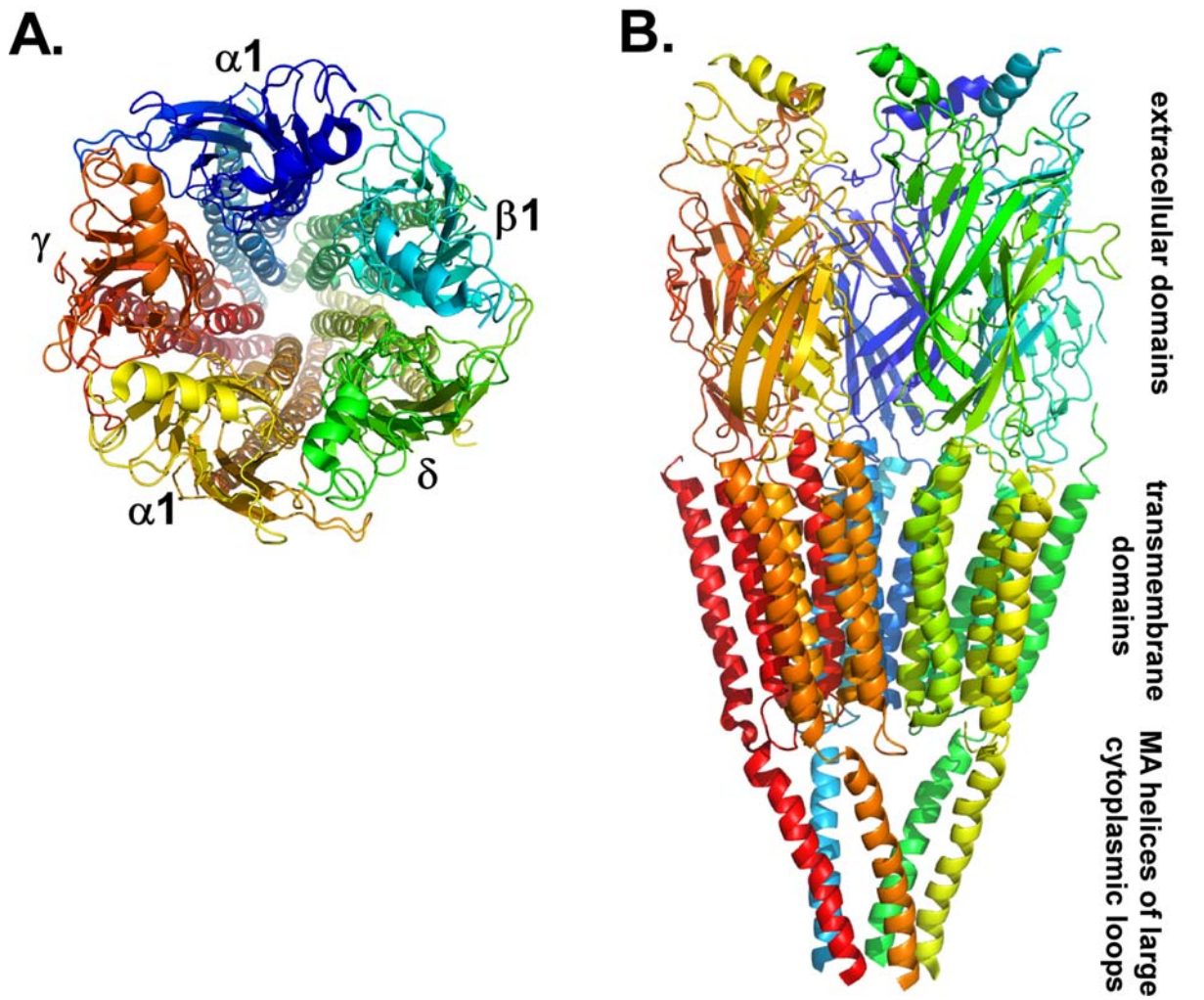


**Figure 2**. Ribbon diagram of the pentameric structure of the nAChR from *Torpedo marmorata*. Color scheme of the five subunits from N terminus through C terminus of each subunit: α1-blue to cyan; β1-cyan to green; δ-green to yellow; α1-yellow to orange; γ-orange to red. A. View from the extracellular side of the nAChR and looking along the path of ion travel into a cell. The N terminal α-helix of the extracellular domain of each subunit is closest to the viewer. The five subunits are labeled. The subunit order is anticlockwise (α1)γ(α1)δ(β1) when viewed from the extracellular compartment. B. View in the plane of the transmembrane domains. The adjacent α1 and δ subunits at the bottom of panel A are in front in panel B. The top third of panel B shows the five extracellular domains. The middle third of panel B is the plane of the membrane and shows the twenty α-helical transmembrane domains (four from each subunit). The bottom third of panel B shows the five amphipathic intracellular MA helices from the large cytoplasmic loops of the five subunits. Electron diffraction did not identify the structure of the portion of the large cytoplasmic loop connecting M3 to amphipathic intracellular helix MA for any of the five subunits. Except for the MA helices, therefore, the large cytoplasmic loops do not appear in this figure. The MA helices precede the M4 helices and are below the plane of the transmembrane domains. These images were created from Protein Data Bank accession 2bg9 (90).

the amphipathic MA helix (91) (containing both hydrophobic and hydrophilic properties) in the large cytoplasmic loop just before M4. The Cys loop and the β1–β2 loop of the N-terminal extracellular domain of a subunit interact with the short M2–M3 loop of the transmembrane domain. This interaction across domains might help open the channel in the agonist-bound conformation. In addition, the conformation of the two α1 subunits is different from the conformation of the β1, γ, and δ subunits. A change in the conformation of the α1 subunits to match the conformation of β1, γ, and δ subunits might be a feature of the gating mechanism that opens the pore. AChBP with a HEPES buffer molecule at the binding site (48) and the open channel of the *Torpedo* receptor after rapid spray-freezing with ACh (85) led to a model of structural transitions from the closed to open state (90). A rearrangement within an α1 subunit potentially is associated with ligand binding and gating and closes its B and C loops onto the ligand binding site.

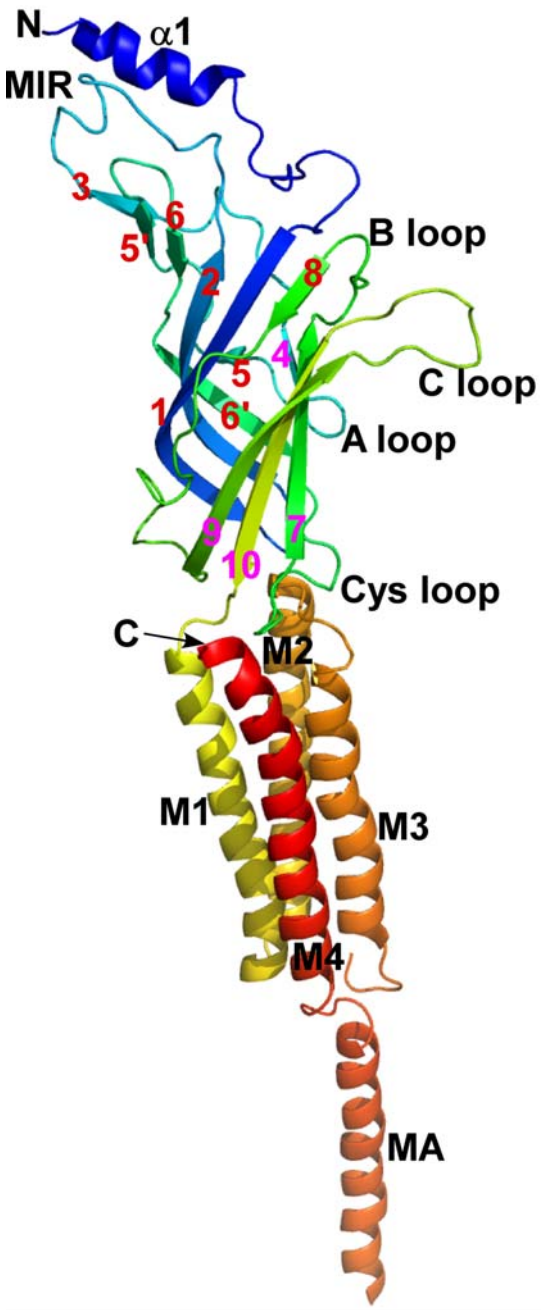


**Figure 3**. Ribbon diagram of a single α1 subunit of the nAChR from *Torpedo marmorata* viewed in the plane of the transmembrane domains. Starting with violet at the N-terminus of the ribbon, the color of the ribbon proceeds through the colors of the rainbow ending with red at the C-terminus. The extracellular α-helix is labeled α1. The β-strands 1, 2, 3, 5, 5′, 6, 6′, and 8 (labels in red) form the inner sheet of the β-sandwich core; β-strands 4, 7, 9, and 10 (labels in pink) form the outer sheet of the β-sandwich core. The transmembrane helices are labeled M1, M2, M3, and M4. MA is the amphipathic intracellular helix in the large cytoplasmic loop near M4. The N-terminus and C-terminus are labeled N and C, respectively. The loops labeled A loop, B loop, and C loop belong to the structure of the principal face of the orthosteric binding site (14, 24). Strand 2, the loop between strands 5′ and 6, and the loop between strands 8 and 9 form the complementary face components D, E, and F, respectively, of the orthosteric binding site (Figure 1). The Cys loop is the 15-residue sequence beginning and ending with disulfide-bonded cysteines that gives the name to the Cys-loop superfamily of receptors. The main immunogenic region (MIR) is a major focus of the autoimmune response leading to myasthenia gravis (482). The structure of the large cytoplasmic loop connecting M3 to MA, which precedes M4, was not identified by electron diffraction and does not appear in this figure. This image was created from Protein Data Bank accession 2bg9 (90).

Comparing the closed and open structures suggests that rotational movements within subunits contribute to channel opening. The extracellular and cytoplasmic vestibules (central spaces ringed by the subunits on the extracellular side and cytoplasmic side, respectively, of the transmembrane domain) of the *Torpedo* receptor are strongly electronegative, which likely contributes to cation selectivity of the open channel. Narrow windows in the walls of the cytoplasmic vestibule also appear to contribute to selectivity for ion charge and size and to regulation of the amount of current allowed through the pore. This structure combined with AChBP structures form the foundation for homology modeling of other nAChRs and modeling Cys-loop receptors, in general.

### 4.2. X-ray crystallography and X-ray diffraction

Although the supply of muscle-type nAChRs from natural sources is abundant, crystallizing nAChRs and most other integral membrane proteins is more difficult than crystallizing water-soluble proteins because of the presence of transmembrane domains (92, 93). X-ray diffraction of membranes from *Torpedo californica* electric organ provided estimates of the dimensions of the nAChRs (66). More recently, three-dimensional microcrystals of detergent-solubilized nAChRs from *Torpedo marmorata* and bound with α-bungarotoxin were grown in a lipidic matrix (94), a technique that has successfully crystallized other membrane proteins (95-97). These crystals can serve as seeds for growing larger crystals. In contrast to muscle-type nAChRs, obtaining sufficient quantities of native neuronal nAChRs for crystallization trials is considerably more difficult. Heterologous expression systems operated at laboratory scale produce only microgram quantities of nAChRs under favorable circumstances. Hundreds of milligrams of protein often are needed for successful crystallization trials, although nanotechnology techniques applied to protein crystallography might considerably reduce that amount of protein (98). Heterologous expression, however, probably is essential if site-specific mutations are to be studied in muscle-type or neuronal nAChRs. Such mutations, impossible to obtain from natural sources of nAChRs, likely will be useful to facilitate crystallization or test hypotheses about nAChR function.

The crystallographic structure at 1.94 Å of the extracellular domain of the mouse α1 subunit bound to α-bungarotoxin provided the first X-ray crystallography of a mammalian nAChR subunit and demonstrated advantages of recombinant production of nAChRs for crystallography (99). Important findings include structures of the main immunogenic region (MIR; an important target of autoantibodies in myasthenia gravis), the Cys loop, and the carbohydrate chain interacting with α-bungarotoxin. A hydrophilic domain with a water molecule forming a pocket within the subunit contrasted with a hydrophobic domain within the acetylcholine binding protein (see next section). This difference might be important for ion channel function of α1 in pentameric nAChRs.

### 4.3. AChBP structural studies

High resolution X-ray crystallographic information about the structure of nAChRs started to unfold in 2001 from an unexpected source: acetylcholine binding protein (AChBP) (100-104). Lacking transmembrane domains, this pentameric water-soluble protein was identified during a study of the effects of glia on synaptic transmission in the freshwater mollusk *Lymnaea stagnalis* (49). Its primary sequence homology with Cys-loop receptor subunits, formation of homomeric pentamers, and binding of nicotinic ligands in a pattern similar to α7 nAChRs supported its use as a water-soluble structural model for the N-terminal extracellular domain of nAChRs (48, 105). Each subunit contains an N-terminal α-helix, ten β-strands forming a β sandwich and an immunoglobulin fold (106), and two $3_{10}$ helices (107) (Figure 4). Ligand binding sites (occupied by HEPES in the crystal) are formed between adjacent subunits by loops from one subunit (the principal face or plus side; Figure 4) and β-strands from the other subunit (complementary face or minus side; Figure 4). Loops from the plus side and β-strands and loop β8–β9 from the minus side form interfaces between subunits. The Cys loop contains 14 residues instead of the usual 15 residues (48). The location of the Cys loop suggests a potential role for interaction between the Cys loop and transmembrane domains in nAChRs that might contribute to gating of the transmembrane domain. Residues of the subunit interfaces are not well conserved in the Cys-loop superfamily, a feature that might contribute to the selective association between subunits when forming pentamers.

Crystal structures of nicotinic ligands instead of HEPES bound to AChBP have provided more detail about the ligand binding site. The crystal structures of nicotine and carbamylcholine bound to AChBP suggest how nicotinic agonists bind to nAChRs. They also help explain the results of previously reported biochemical and electrophysiological experiments that probed the interactions of specific side chains with ligands (108, 109). These structures answered a long-standing question about the negatively charged site that interacted with the positive charge at the tertiary nitrogen of nicotine and quaternary nitrogen of carbamylcholine (110-112). The negatively charged site is formed by interaction with π electrons from the aromatic side chains of Trp143, Tyr192, Tyr185, and Trp53 and the backbone carbonyl of Trp143. Compared to the HEPES-bound structure, loop C of the principal binding site contracted around each ligand. Structures of AChBP with peptides that competitively inhibit nAChRs might resemble the resting state of nAChRs. AChBP with α-cobratoxin (113) showed movement of loop C away from the binding site compared to the AChBP structures with HEPES, nicotine, or carbamylcholine. Conformational changes of loop C might be important distinguishing features of resting, open, and desensitized states of nAChRs and might be an important target for designing drugs to affect different nAChR states.

The structures of AChBPs from different species and with different pharmacological properties have been the foundation for proposals about how nAChRs differ in


**Figure 4**. Ribbon diagram of nicotine bound to two subunits of the pentameric AChBP from *Lymnaea stagnalis*. Nicotine is shown as an orange-colored, space-filling model. The loops labeled A, B, and C from the green subunit contribute to the principal face of the orthosteric binding site hosting nicotine. β-strands 2, 5, 5′, 6, 6′ and the β8–β9 loop from the blue subunit contribute to the complementary face of the orthosteric binding site hosting nicotine. This image was created from Protein Data Bank accession 1uw6 (108).

pharmacology and how the channel gates. The second example of an AChBP was obtained from *Aplysia californica*, a saltwater mollusk (114). Comparison of the structures of the *Aplysia* AChBP without ligand, with nicotinic agonists (+)-epibatidine and lobeline, and with nicotinic antagonists α-conotoxin ImI and methyllycaconitine showed binding of the two agonists was associated with closing of loop C. In contrast, binding of the two antagonists was associated with an extended or open loop C (115). These results support the participation of loop C in the differing effects of agonists compared to competitive antagonists. The α-conotoxin PnIA (A10L D14K) (116) binds nonselectively to AChBP homologs from different mollusks. By comparison, α-conotoxin ImI (117) binds selectively to *Aplysia* AChBP and to α7 and α3β2 nAChRs. The difference in structures and binding properties of these two conotoxins with AChBP homologs suggested protein contacts that might be important for ligands that are selective for specific nAChR subtypes. Galantamine and cocaine, positive allosteric modulators of nAChRs, bound deeply into subunit interfaces of *Aplysia* AChBP in the crystal structure without contacting the tip of loop C (118). These structures from homomeric *Aplysia* AChBP suggested these positive allosteric modulators bind at non-α subunit interfaces in heteromeric neuronal nAChRs. The third AChBP was obtained from *Bulinus truncates*, a freshwater mollusk, and was crystallized with a CAPS buffer molecule in four of the five ligand binding sites (119). The backbone structures of AChBPs from *B. truncates*, *L. stagnalis*, and *A. californica* were similar although pairwise comparisons show only 20% to 40% sequence identity. Differences in affinity for nicotinic ligands by the AChBP from *B. truncates* compared to AChBP from *L. stagnalis* arose primarily from differences at three homologous positions in the primary sequences (119).

### 4.4. AChBP with methods other than X-ray crystallography

Biophysical methods other than X-ray crystallography have been applied to AChBP to understand energetics and dynamics of ligand binding and channel

gating of nAChRs. The large amount of properly folded and water-soluble AChBP that can be heterologously expressed makes applying these methods to AChBP often easier than applying the methods to native or heterologously expressed neuronal nAChRs in the presence of membranes or detergents. Changes of intrinsic tryptophan fluorescence (five Trp residues on each subunit) were associated with ligand binding, making fluorescence spectroscopy a valuable tool for probing kinetics and structural changes of ligand binding (120). Fluorescent labels attached to AChBP through native and nonnative cysteine residues signaled the environments around the cysteines and the dynamics of distinctive changes in those environments caused by binding of different ligands (121, 122). Frictional coefficients and fluorescence anisotropy decay that measured hydrodynamic properties of the AChBP:cobratoxin complex in the presence of bound and specifically labeled α-cobratoxin suggested that this ligand was not rigidly bound but retained segmental flexibility (123). Absorption and fluorescence spectroscopy of benzylidine-ring substituted anabaseines bound to AChBPs from *L. stagnalis*, *A. californica*, and *B. truncates* showed the extent of proton dissociation and the reduced flexibility of the bound ligand compared to free ligand (124). Molecular dynamics simulations and intrinsic tryptophan fluorescence in solution suggested changes in relative tryptophan conformations on loops C and D on adjacent subunits when ACh binds. These studies also identified closing of loop C over the opening to the binding site as another potentially important change with ligand binding. These effects of binding acetylcholine might be important for channel gating (125). Molecular dynamics and ligand docking simulations of AChBP and either *d*-tubocurarine or metocurine suggested two different orientations for these curare derivatives at the ligand binding site. This conclusion, supported by binding measurements with binding site mutations, indicated that apparently minor differences in ligand structure could significantly alter binding interactions between ligand and AChBP (126). Hydrogen-deuterium exchange mass spectrometry (127) and solution NMR (128) provided evidence that binding agonists induced conformation changes in loop C and loop F at the binding site that were different from the conformation changes induced by antagonists. Molecular dynamics modeling of AChBP with bound nicotine and carbamylcholine potentially augments conclusions from X-ray crystallographic structures and suggested that water molecules are present at discrete locations in the binding site and may participate in ligand binding (129).

### 4.5. Homology modeling of nAChRs based on AChBP and *Torpedo* nAChR

A frequently applied method to link findings about the known structure and dynamics of AChBP to undetermined structural and dynamic features of neuronal nAChRs is homology modeling (130). This method starts with alignment of primary sequences of AChBP and nAChR and then develops structural models of pentameric nAChR receptors based on structures of AChBP and nAChR from *Torpedo*. Models of nAChR subtypes α7 (131-135), α4β2 (131, 132), muscle-type $(\alpha1)_2(\beta1)\gamma\delta$ (131, 135), α4β4 (132), and α3β2 (132) have been reported. Homology models of nAChRs help generate hypotheses about ligand binding (131-133, 135), channel gating (134), and ion conduction (134) that can be tested experimentally and that guide interpretations of experiments. Homology models based on AChBP and *Torpedo* nAChR also have been developed for $GABA_A$ (136, 137), $GABA_C$ (138), $5\text{-}HT_3$ (139, 140), and glycine (141-144) members of the Cys-loop receptor superfamily.

### 4.6. NMR studies

Solution NMR as a method of determining protein structure has the advantages that crystals are not needed and protein structure and dynamics can be determined under a range of conditions, including physiological conditions. A major obstacle for applying solution NMR to neuronal nAChRs is the limitation of about 100 kDa in the molecular mass suitable for solution NMR (145). Other obstacles are the relatively large amount of protein needed (in the range of milligrams) and the need for $^{13}C$ and $^{15}N$ isotopic labeling of amino acids in a heterologous expression system. Peptide models of the primary face of ligand binding site of α1 subunits (146-149) and α7 subunits (150) have overcome these obstacles. Studying transmembrane domains in micelles instead of an entire nAChR also overcomes these obstacles while providing information relevant to the ion conduction path (151-153). The extracellular domain of α1 combines native-like structure and a size small enough (31 kDa) to be compatible with solution NMR (154).

Advances in protein NMR techniques in areas of isotopic labeling (155) and analysis of large (e.g., 900 kDa) proteins (156-159) promise to make solution NMR in the future more relevant to full length nAChRs. Solid-state NMR overcomes some of the size limitations of solution NMR and can be applied to nAChRs in their native membrane environment, which potentially maintains structural and dynamic features that depend on that environment (160). A solid-state NMR study of isotopically labeled antagonist neurotoxin II bound to *Torpedo* nAChR showed the toxin structure likely is similar in the free and bound state with the exception of an isoleucine (161). A study with solid-state NMR of isolated M1 peptides suggested strong protein-lipid interaction involving M1 from α1 in model membranes (162).

### 4.7. Extracellular domain nAChRs

The large N-terminal extracellular domain of nAChRs is an attractive candidate for structural studies. Conceptually similar to the AChBP, extracellular domains of nAChR subunits might form pentameric, water-soluble, high fidelity models for the extracellular domain of full length nAChRs. The size of extracellular domain nAChRs (120–150 kDa), although still beyond current NMR technology, is more compatible for NMR than is the size of full length nAChRs. Their potential for water solubility without detergents makes them better candidates than full length nAChRs for crystallization and biophysical methods intolerant of detergents or membranes.

The feasibility of extracellular domain nAChRs was supported by studies with muscle-type subunits (163-

167), α7 subunits (168-171), and α4 and β2 subunits (172). Extracellular domain muscle-type subunits without any transmembrane domains formed pentamers detected by electron microscopy (165). Extracellular domain α7 and α4β2 nAChRs likely were pentamers and showed affinities for small and large ligands equal to the ligand affinities of the respective full length nAChRs (169, 172). Retaining M1 on the extracellular domain, however, was required for efficient expression of extracellular domain α7 and α4β2 nAChRs. Based on this observation, preparing large amounts of water-soluble extracellular domain nAChRs might require a strategy that includes *in vitro* removal of M1 from extracellular domain nAChRs. The feasibility of such a strategy was supported by inserting a specific proteolysis site in extracellular domain α7 subunits between the extracellular domain and the N-terminus of M1 (169). This extracellular domain α7 subunit with M1 and a specific proteolysis site outside M1 formed pentamers and had ligand affinities equal to the ligand affinities of full length α7 nAChRs. Concatamers of extracellular domains with or without a C-terminal M1 domain offer another potential approach to water-soluble extracellular domain nAChRs that has been successful with full length α4 and β2 subunits (173) and other Cys-loop receptors (174).

### 4.8. nAChRs and unnatural amino acids

Incorporating unnatural amino acids into ion channels as they are synthesized *in vivo* provides a powerful method beyond the spectrum of twenty natural amino acids to test hypotheses about structure and function of nAChRs (175-178). Unnatural amino acids include side chains with chemical properties beyond properties of natural amino acids such as fluorescence and photochemical reactivity. Unnatural amino acids first were incorporated into muscle-type nAChRs expressed in *Xenopus* oocytes (179). Carboxyl, amino, and fluoro derivatives of Tyr190, Tyr198, and Phe198 at the agonist binding site changed the dose-response behavior for acetylcholine and the inhibition constant for *d*-tubocurarine. The advantages of fine-tuning chemical properties through unnatural amino acids was evident in a study of the role of hydrogen bonds involving Asp89 near the binding site in the mouse embryonic muscle-type nAChR (180). Substitution of nitro or keto groups in place of the carboxyl of aspartate suggested that Asp89 contributes not only hydrogen bonds and electrostatic charge (181) but also a favorable arrangement of dipoles. Fine-tuning of side chain properties at sites of tyrosine residues with tyrosine derivatives (e.g., fluoro, bromo, *p*-methyl, *p*-methyoxy, and *m*-hydroxy derivatives) in 5-$HT_3$ receptors helped develop a model combining ligand binding and channel gating based on rearrangements of hydrogen bonds (182). Structure of the peptide backbone also can be altered by incorporating components beyond the natural amino acids. Replacing amino acids in M1 and M2 with α-hydroxy acids changed specific bonds in the backbone from peptide to ester bonds (183). The changes in electrophysiological behavior associated with these changes suggested that backbone hydrogen bonds in M1 and backbone conformation of M2 contribute to gating. Frameshift suppression (184) promises to open a route to incorporating *in vivo* multiple, different unnatural amino acids in a nAChR subunit (185). Reassigning sense codons to unnatural amino acids instead of depending on nonsense codons to introduce unnatural amino acids into proteins is an alternate approach that might lead to multiple different unnatural amino acids in proteins (186).

## 5. HOW DO LIGANDS BIND TO NACHRS?

The general structure of the agonist binding site, also called the orthosteric binding site (in contrast to an allosteric binding site), in nAChRs has been outlined by homology with AChBP (187-190). General principles, however, do not explain the diversity of ligand interactions and functional behaviors observed with the diversity of subtypes of nAChRs. Because the extent of structural homology between AChBP and specific types of nAChRs is uncertain at the atomic level, the structural information from AChBP is a starting point and not the final answer for questions about how nAChRs work. These questions will continue to be answered by combining biochemical, electrophysiological, pharmacological, and structural methods to nAChRs. Some of the questions are: Why does a given agonist show different affinities and different potencies for different nAChRs? Why do antagonists show different affinities for different nAChRs? Is a unique set of interactions between agonist and nAChR required for opening the channel? Which structural and chemical properties are required in a nicotinic agonist? How do competitive antagonists bind tightly to nAChRs and keep the channel from opening? How can a specific type of nAChR be maintained pharmacologically in its closed, opened, or desensitized states without affecting other types of nAChRs? How does the binding of small molecules at sites on nAChRs other than the agonist binding site affect function of nAChRs? Such sites are called allosteric binding sites.

This section concentrates on answers and persistent questions associated with ligand binding at the orthosteric binding site and at allosteric binding sites and with distant effects associated with ligand binding, namely, channel gating. The next section will consider in more detail how nAChRs gate.

### 5.1. Muscle nAChRs

Recent studies about how nicotinic ligands bind to muscle-type nAChRs have been based on curare derivatives, nicotine, epibatidine, choline derivatives, and trimethylammonium derivatives. A ligand binding study of interactions between functional groups of *d*-tubocurarine and the γ subunit of fetal mouse muscle ($(\alpha 1)_2\beta\gamma\delta$) nAChRs was interpreted with single model of *d*-tubocurarine in the ligand binding site (191). By comparison, modeling of ligand binding focused on the ε subunit of adult human muscle ($(\alpha 1)_2\beta\varepsilon\delta$) nAChRs produced a different orientation of *d*-tubocurarine in the ligand binding site and showed that methylation of *d*-tubocurarine could dramatically change predicted interactions between ligand and binding site (192, 189). Descriptions of ligand–nAChR interactions might need to consider differences arising from seemingly minor changes

in ligand structure and in subtype and species of nAChRs.

The wide range of chemical modifications possible with unnatural amino acids and electrophysiological measurements indicated a potential range of interactions available to agonists at the ligand binding site of fetal muscle ($(\alpha1)_2\beta\gamma\delta$) nAChRs (193). Cation-π interaction with Trp α149 was important for ACh binding, hydrogen bonding to a backbone carbonyl was important for nicotine binding, and both cation-π interaction and hydrogen bonding to the backbone were important for epibatidine binding. Additional interactions between epibatidine and the desensitized ligand binding site were suggested from ligand binding to chimeras between γ and δ subunits (the binding site at the αγ interface binds epibatidine more tightly than does the site at the αδ interface) (194). Photochemical labeling of *Torpedo* nAChRs (195, 196) with a benzoylcholine partial agonist might lead to understanding the basis for low efficacy of partial agonists and the development of photochemically-active full agonists with well-defined photoreactive intermediates (197).

Long, bisquaternary dicholine agonists like suberyldicholine (18.7 Å between quaternary nitrogens) appeared to bind to multiple sites on muscle-type nAChRs, suggesting that the extent of an agonist binding site depends on the agonist (198, 199). For the simpler alkyltrimethylammonium functional group of choline, length requirements of alkylthiol derivatives of the minimalist agonist tetramethylammonium tethered to cysteines supported the sufficiency of the single agonist binding site formed by aromatic side chains in the AChBP structure (200). This interpretation presumed that the AChBP structure described the open conformation of nAChRs. Identifying residues from muscle-type nAChRs potentially interacting with peptide inhibitors waglerin-1 (201), α-cobratoxin (202), a short-chain α-neurotoxin from *Naja oxiana* (203), and a short-chain α-neurotoxin from *Naja nigricollis* (204) was aided by comparisons with AChBP or *Torpedo* nAChR structures. Focusing on the dynamics of bound α-cobratoxin, the C-terminal domain of fluorescein derivatives of the antagonist appeared to be mobile and not bound to *Torpedo* nAChR by time-resolved fluorescence anisotropy. This result suggested that only the N-terminus interacts with the nAChR surface (205).

Besides structural descriptions of ligand binding, dissociation constants of low affinity agonists (206) and electrostatic effects important for ligand binding to *Torpedo* nAChRs (207) provided thermodynamic and energetic descriptions of ligand binding to muscle-type nAChRs. A model combining acetylcholine diffusion and binding, protein structure, reaction kinetics, and synaptic geometry at the neuromuscular junction incorporated the complex three-dimensional physiological environment for nAChRs (208). Continued development of such unifying models will help interpret and reveal physiological roles for atomic level details of nAChR structure and dynamics.

### 5.2. Neuronal nAChRs

Understanding ligand binding to α4β2 and α7-containing nAChRs is important because of the prominence of these subtypes in the human brain. Results from computer simulated docking and molecular modeling of nicotinic ligands suggested structure-activity relationships to explain how nicotine and deschloroepibatidine bind to α4β2 nAChR (209). Similar methods were applied to a variety of nicotinic ligands that distinguish between α4β2 and α3β4 nAChRs (210, 211). Understanding binding to α4β2 nAChRs is complicated by the existence of multiphasic binding or dose-response curves suggesting receptor populations with different relative α4:β2 stoichiometries (212, 213, 173, 214, 215). In contrast to the multiphasic concentration-response curves of ACh, nicotine, and cytisine with α4β2 nAChRs, binding of TC-02559 was monophasic (216). Similar to muscle-type nAChRs, comparing ligand binding to α4β2 and α7-containing nAChRs suggested the importance of subunit-specific local interactions at the binding site (217) as well as long-range electrostatic interactions (218). Residues of α-bungarotoxin, a long α-neurotoxin, that interact with α7 nAChRs are identical to or overlapping with the toxin residues that interact with muscle-type nAChRs (219). Chimeras of short α-neurotoxins, long α-neurotoxins, and κ-neurotoxins have helped identify regions of these toxins important for interaction with α7 nAChRs and for selectivity of κ-neurotoxins with α3β2 nAChRs (220). The agonist properties of antihelminthic drug pyrantel with α7 nAChRs depend on interaction with Gln57 of the complementary face (221). Molecular dynamics simulations of the extracellular domain of α7 nAChRs have allowed more global assessment of predicted structure and dynamics induced by ligand binding (222, 223). High resolution X-ray crystal structures and physicochemical characterization of nicotinic ligands are important components of molecular dynamics and docking computations (224).

Understanding ligand binding to α4β2 and α7-containing nAChRs also helps develop subtype-specific drugs and, more narrowly, state-specific (e.g., the open state) drugs for a given subtype (225-233). Epibatidine has been the starting point for synthesizing derivatives with high affinity for α4β2 nAChRs and low affinity for α7-containing nAChRs (234-236) or low affinity for other β2-containing or β4-containing nAChRs (237-240). Epibatidine also has been the starting point for fluorescent derivatives that potentially will be useful for high-throughput screening and single-molecule analysis of binding (241). Derivatives of pyridine (242, 243); piperazines, diazepanes, diazocanes, diazabicyclononanes, diazabicyclodecanes (244); and cytisine (245) also have shown promise for selectivity for α4β2 nAChRs. For finer discrimination of subunit composition, a piperidine derivative selectively and noncompetitively blocked α3- and α4-containing nAChRs when only β2 or β4 subunits also were present. The presence of additional nonessential subunits α5, α6, or β3, however, decreased inhibition by this blocker (246). Pyridine derivatives are the basis for α4β2-selective radioligands for detection by positron emission tomography (PET) (247). Drugs with high affinity for α7-containing nAChRs (248, 249) and with

minimal cross-reactivity with 5-$HT_3$ receptors (250) are targets for medicinal chemistry that might become therapies for Alzheimer disease and schizophrenia. In other contexts, insecticides for example, low affinity for human nAChRs is the goal. Understanding the structural basis of binding by the neonicotinoids class of compounds (e.g., imidacloprid and thiacloprid) specifically to insect nAChRs will lead to better insecticides with lower human toxicity (251, 252).

Conotoxins are an especially rich source of tools for achieving and analyzing subtype-specific interactions (253-259). Marine snails of the *Conus* genus produce these peptide toxins in their venom that target ligand-gated and voltage-gated ion channels. Members of the family of α- and αA-conotoxins contain multiple disulfide bonds and are competitive antagonists for nAChRs. Many α- and αA-conotoxins are selective for muscle-type and/or α3- and α7-containing nAChRs. With an estimated 50 to 200 peptide toxins produced by each *Conus* species (260), many useful toxins for nAChR research have yet to be characterized. Recently identified conotoxins are specific for different types of nAChRs: muscle-type nAChRs (261-263), α3β4 nAChRs (264), α3β2 and α7 nAChRs (265), α7-containing nAChRs (266), and α9α10 nAChRs (267). Conotoxin antagonism against α9α10 nAChRs showed both a role of α9α10 nAChRs in neuropathic pain and the potential therapeutic role of conotoxins (268). With α3-containing nAChRs, α-conotoxin BuIA kinetically distinguished β2 subunits from β4 subunits because of much slower off-rates with β4 subunits (269). Understanding structural features of conotoxins and of conotoxin interactions with muscle-type (270), α7 (271), and α3β2 (271, 272) nAChRs might lead to targeted modifications of conotoxins for enhanced subunit selectivity or novel activities. For example, a benzoylphenylalanine derivative of a α-conotoxin GI was designed as a photoaffinity label for *Torpedo* nAChRs (273).

### 5.3. Allosteric binding

Beyond the orthosteric binding sites of nAChRs are allosteric binding sites (274-277). The concept of allosteric binding sites that modulate channel activity arises from observations that some compounds affect channel function stimulated by agonists. These compounds themselves do not have agonist activity nor are they competitive inhibitors of agonist binding at the orthosteric binding site. The interpretation of these observations is that binding of allosteric modulators to allosteric binding sites on nAChRs modifies the function of nAChRs. These binding sites, in principle, can be within the ion pore or can be within the extracellular, cytoplasmic, or transmembrane domains. Positive allosteric modulators increase agonist-induced activity; negative allosteric modulators decrease such activity. Because allosteric binding sites are outside the orthosteric binding site, allosteric modulators extend beyond the constraints of the orthosteric site the opportunities for developing subunit and subtype specific modulation.

Defining allosteric binding sites and the activity of allosteric modulators is important for understanding nAChR function and for drug development. Galantamine, a positive allosteric modulator of human α7 and α4β2 nAChRs important for treatment of Alzheimer disease (278-280), binds at the subunit interface in AChBP without interacting with loop C. This result suggests this drug binds at non-α subunit interfaces in heteromeric neuronal nAChRs (118). In contrast, this drug was a partial agonist without allosteric activity on mouse muscle nAChRs (281). It was not a competitive inhibitor of nicotinic agonists, so the binding site of its agonist activity likely is distinct from the binding site of the typical nicotinic agonists ACh and carbachol. The negative allosteric effects of kynurenic acid might attenuate the positive allosteric effects of galantamine on α7-containing nAChRs (282). A collection of derivatives of methyllycaconitine with negative allosteric modulator activity on α3β4 nAChRs produced quantitative structure-activity relationship and pharmacophore models useful for detecting other negative allosteric modulators (283). Derivatives of (2-amino-5-keto)thiazole were positive allosteric modulators of α2β4, α4β2, α4β4, and α7 nAChRs but did not potentiate $(\alpha1)_2(\beta1)\gamma\delta$, α3β2, or α3β4 nAChRs (284). Desformylflustrabromine showed promise as a positive allosteric modulator of α4β2 nAChRs (285, 286). Four positive allosteric modulators of α7 nAChRs showed two classes of effects, raising the possibility that combinations of modulators could produce additional options for therapeutic effects on α7-containing nAChRs *in vivo* (287). Crystal violet, a negative allosteric modulator, bound at a site overlapping the phencyclidine binding region in the resting and desensitized states of *Torpedo californica* nAChRs (288). Docking computations can suggest allosteric binding sites on nAChRs as was tested with positive allosteric modulators galanthamine, codeine, and serine and with α7, α3β4, and α4β2 nAChRs (289). Neurosteroids had allosteric effects on nAChRs (290) and $GABA_A$ receptors (291) in the transmembrane domain. Mutations leading to autosomal dominant nocturnal frontal lobe epilepsy reduced the positive allosteric effect of $Ca^{2+}$ on α4β2 nAChRs (292, 293). $Zn^{2+}$ also modulated the function of neuronal nAChRs (294, 295). Residues in α4 at the interface with β2 or β4 subunits might mediate this modulation by $Zn^{2+}$ (296). This proposed location of the $Zn^{2+}$ allosteric site might be structurally and functionally analogous to the site for benzodiazepine binding on $GABA_A$ receptors.

### 5.4. Effects of post-translational modifications

*N*-linked glycosylation of the extracellular domain affects nAChRs during subunit assembly into mature nAChRs and during transport to the cell surface. The importance of glycosylation for these processes was demonstrated for *Torpedo* nAChRs (297-301) and α7 nAChRs (302). Glycosylation also affected desensitization and conductance of *Torpedo* nAChRs (303). Partial deglycosylation of purified *Torpedo californica* nAChRs did not appear to affect structure, internal dynamics as measured by $^1H/^2H$ exchange, or ability to desensitize with agonist (304). In contrast, deglycosylation adversely

affected binding of α-bungarotoxin to the extracellular domain of human α1 (166). Establishing the cellular machinery for adding mammalian patterns of glycosylation to nAChRs produced in high-level expression systems like yeast (305-307) and baculovirus (308, 309) might be important for efficiently producing large amounts of human nAChRs for structural studies. Nitrosylation of tyrosines is another post-translational modification potentially affecting nAChRs (310).

## 6. WHAT ARE THE FUNCTIONALLY IMPORTANT DYNAMICS OF NACHRS?

### 6.1. How does the channel open and close?

One of the most important actions induced by agonist binding to nAChRs is opening the channel (i.e., the transmembrane domains) thus permitting the flow of cations. How does the channel open? A second question involving structure and dynamics of desensitization is, How does the channel close? The first question is being answered in terms of residues, secondary structures, and dynamics of nAChRs. Rotational movements within α1 subunits of muscle-type nAChRs that serve to open the channel were suggested by electron microscopy of resting and open *Torpedo* nAChRs (85, 90). As another possibility, tilting of the pore-lining M2 segments was suggested as a mechanism for opening the channel of a homomeric α7–5-$HT_{3A}$ chimeric receptor. This mechanism was based on $Zn^{2+}$ binding to metal ion binding sites created by substituting histidine residues into M2 (311). Molecular dynamics simulations (312) suggested both types of motions might be contributing to channel opening in α7 nAChRs (313-318). Transient photoreactive accessibility of residues in M1, M2, and the M2–M3 loop of the δ subunit of *Torpedo* during gating was evidence of gating-specific and possibly subunit-specific transmembrane domain motions (319). A computational analysis of *Torpedo* nAChR using elastic networks theory suggested that symmetric quaternary twisting and asymmetric tilting of M2 combine to open the channel and that loops A and F and the large cytoplasmic loop also affected channel gating (320).

Different regions and residues of nAChR subunits have been implicated in channel gating. Loops β1–β2, β6–β7, and β8–β9 at the interface between the extracellular domain and transmembrane domain have attracted much attention as potential transducers of the opening signal from the agonist binding site to the transmembrane domain. The successful construction of a chimeric ion channel gated by ACh and containing the AChBP extracellularly and the 5-$HT_{3A}$ receptor sequence starting at M1 demonstrated the importance of the loops β1–β2, β6–β7, and β8–β9 (321). Residues in loop β6–β7 (Cys loop) and the M2–M3 loop influenced fast opening (322). Conserved proline and serine residues of the M2–M3 loop were coupled to the agonist binding site through a conserved glutamate on loop β1–β2 and a conserved arginine on the C-terminal end of the β10 strand of the α1 subunit (323, 324). Unnatural amino acid substitutions supported the importance of the conserved proline residue in the M2–M3 loop for channel gating in mouse 5-$HT_{3A}$ receptors (325, 326). Adjacent residues within the β2 strand of the β4 subunit affected the coupling of binding and gating for the species selective agonist 5-(trifluoromethyl)-6-(1-methyl-azepan-4-yl)methyl-1*H*-quinolin-2-one (TMAQ) (327). Electrostatic interactions among three residues (αK145, αD200, and αY190) in the human α1 subunit at the periphery of the agonist binding site (328) and interaction of two residue pairs at the α–δ (αY127 and δN41) and α–ε (αY127 and εN39) subunit interfaces (329) in muscle nAChRs affected gating.

The link between the agonist binding site and channel gating might not be restricted to a few residues. An investigation of charged residues within loops β1–β2, β6–β7, and β8–β9 of muscle nAChRs and $GABA_A$ and glycine receptors concluded the overall distribution of electrostatic interactions rather than interactions between specific ion pairs was important at the gating interface between the extracellular domain and transmembrane domain (330). Analysis of covariance of amino acid residues in the multiple primary sequence alignment of Cys-loop receptors suggested highly coupled positions form a three-dimensional network connecting the agonist binding site to the transmembrane domain and to channel gating (331).

In α7 nAChRs, a network of electrostatic interactions involving residues in loops β1–β2 and β6–β7 and the M2–M3 linker linked the agonist binding site to gating of the channel (332). The inner and outer layers of β sheets in the β-sandwich (Figure 3) of the extracellular domain participated in coupling agonist binding and channel gating in α7 nAChRs (333, 334). Accessibility of inner layer residues to cysteine modification suggested that motions in addition to rotational motion within the extracellular domain might be important in the gating of α7 nAChRs. The dynamics of the transition between closed and open states of muscle-type nAChRs has been characterized by rate equilibrium linear free energy relationship (REFER) analysis. This method compares changes in the opening rate to changes in the equilibrium between closed and open states when a specific position is mutated to several different amino acid types (335). The REFER analysis is interpreted to measure to what extent a given position in the primary sequence is in an open-like state when the nAChR reaches its transition state between closed and open. Positions near the agonist binding site were in an open-like state; positions in the transmembrane domain were in a closed-like state. Positions located between the agonist binding site and the transmembrane domain in the nAChR in three-dimensional space were in states with a gradient of contributions of open and closed states (336, 337). This spatial variation of open state character in the transition state suggested that, during the transition to an open channel, parts of the nAChR near the agonist binding site move before parts near the transmembrane domain. The apparently asynchronous movement of different parts of the nAChR during opening suggested a broad potential energy surface without sharp features (338, 339). Investigations involving REFER

analysis have focused on the extracellular domain (340); on M2 (341, 342), M3 (343), and M4 (344) domains; and on the theoretical underpinnings of the interpretation (345-347).

Structural answers to the second question, about how the channel closes, have come from investigating where ion conduction is blocked along M2 in the resting and desensitized states. A site of such blocking of ion conduction, called a gate, was identified in the resting state in the middle of M2 in $GABA_A$ (348) and $5HT_3$ (349) receptors by scanning cysteine accessibility mutagenesis (SCAM) (350, 351). A gate located near the cytoplasmic end of M2 was suggested for nAChRs by SCAM (352, 353). Additional understanding of how to interpret SCAM experiments might reconcile these gate locations for different Cys-loop receptors (348). Different gates might act in different structural states of the channel. For example, ion channel block by choline affected a gate for the resting state in nAChRs but did not affect a gate for the desensitized state, implying the gate of the resting state is different from the gate of the desensitized state (354).

### 6.2. Pore structure and dynamics: How do ions traverse the membrane through nAChRs?

Experimental and computational studies focusing on the transmembrane domains, especially M2, are contributing information about the structure, dynamics, and ion permeation of the pore region (355). Molecular and Brownian dynamics computations of the transmembrane domain of *Torpedo* nAChR suggested that a hydrophobic region of the channel prevents cation flow in the closed state. For the open state, small changes in channel structure led to large changes in conductance (356-358). Molecular dynamics of M2 domains with spatially fixed M1, M3, and M4 domains showed asymmetrical dynamics in M2 and bending motions at this central hydrophobic region of M2 that could correspond to the hydrophobic gate (359). A hydrophobic region of M2 also might be the gate for ion flow for α4β2 nAChRs (360) and α7 nAChRs (361), based on profiles of pore radius and water density from molecular dynamics calculations of the transmembrane domains. A model of a pore composed solely of M2 peptides from the δ subunit of *Torpedo marmorata* (362, 363) or rat (364) agreed with results from solid-state NMR and other experimental methods and might represent the closed form of the pore. Lysine substitutions at a single homologous position within the central hydrophobic region of M2 of the four subunits of human muscle-type nAChRs caused differing effects on open channel conductance. This finding suggested that the subunits at this position experience substantially different microenvironments and are unequally affected by transmembrane voltage (365). Substitutions of lysines at all positions and histidine and arginine at some positions of M2 in the δ subunit of mouse muscle-type nAChRs detected individual proton binding events of single open channels (366). These substitutions also identified the positions of δM2 facing into the pore in closed and open states. These results suggested that rotation of M2 during opening is minimal. A point mutation at a conserved leucine near the middle of M2 of α3 affected gating in α3β4 and α3β2 nAChRs (367). Rhodamine labeling of a Cys residue substituted into the extracellular end of M2 of β1 demonstrated the feasibility of time-resolved fluorescence for investigating the dynamics of channel opening (368).

In addition to understanding ion flow through the transmembrane domain, understanding how channel blockers interact with the transmembrane domain and with nAChR dynamics is important. For example, such understanding could guide the development of new and more specific channel blockers. The noncompetitive channel blocker chlorpromazine interacted with several M2 residues of muscle-type nAChR according to molecular dynamics simulations with the whole nAChR embedded in a lipid bilayer (369). Moreover, chlorpromazine also inhibited conformational changes in the nAChR that might be important for transitions between resting, open, and desensitized states.

Although M2 lines the ion conduction path, the other transmembrane domains indirectly might affect nAChR gating and conductance even though they do not directly interact with ion flow. For example, mutations at position 15′ in the middle of M1 of different subunits in mouse muscle-type nAChRs affected kinetic parameters of gating in a subunit-specific manner. This finding suggested M1 contributes to gating (370). M1 peptide from *Torpedo californica* showed unexpected conformational flexibility in solid-state NMR experiments with phospholipids, possibly because of the effect of a conserved proline residue near the middle of M1 (371). Interaction between the transmembrane segments M1 and M2 in muscle nAChRs was found to affect gating (372). Molecular dynamics simulations of the transmembrane domain of muscle nAChR suggested that M4 plays a role in communicating effects from the membrane environment to M2 and then to channel gating (373). In this modeling, conformations of side chains of M2 contributed to channel gating. M4 appeared to link the lipid environment to nAChR gating (374). Electrostatic forces facilitating ion flow through the pore likely arise from the transmembrane domain and to a much lesser extent from longer range interactions from the extracellular domain or large cytoplasmic loop (375, 376).

Beyond gating and ion flow, the pore also participates in the structure of the nonconducting desensitized state. According to infrared spectroscopy, the α-helices of the transmembrane domain of *Torpedo californica* nAChRs were preferentially perpendicular to the membrane surface and showed no reorientation with desensitization (377). In general, however, the structure of the desensitized state is poorly understood.

## 7. WHAT IS THE STRUCTURAL BASIS FOR FUNCTIONS OF THE LARGE CYTOPLASMIC LOOP?

The amino acid sequence of the large cytoplasmic loop between M3 and M4 shows considerable diversity among different nAChR subunits. The functions of the large cytoplasmic loop, however, have not been

studied as extensively as the functions of the extracellular domain or transmembrane domain. The structure of this loop was not seen in the 4 Å structure of *Torpedo* nAChR (90) and was presumed to be disordered.

Structural disorder is important to the functions of some proteins (378) and might contribute to various functions of large cytoplasmic loops in the resting, open, and desensitized states. Studies of the large cytoplasmic loop from the δ subunit from *Torpedo californica* and rat suggested the possibility of structural studies of this domain isolated from the remainder of the subunit or nAChR (379, 380). Residues in the large cytoplasmic loop near the intracellular end of M4 affected assembly of subunits and electrophysiological properties of nAChRs (381-384). Because of the sequence diversity of the large cytoplasmic loop, this region might be important for subunit-specific behavior and interactions with cellular components and physiology. The large cytoplasmic loop interacts with cytoplasmic transport machinery for trafficking nAChRs to synapses (385-389). Residues in M1 (390) and at the cytoplasmic and extracellular ends of M4 (391) of α1 affected assembly and targeting to the cell surface. At the cell membrane, the large cytoplasmic loop interacted with cytoskeleton (392-394). Phosphorylation of the large cytoplasmic loop (395, 396) affected desensitization (397, 398), expression (399, 400), and cytoskeletal interaction (401). Palmitoylation (402, 403) affected expression of α7 nAChRs (404). Future development of the structural description of how nAChRs work will need to include the dynamic structure of the large cytoplasmic loop, including posttranslational modifications and interactions with other proteins.

## 8. HOW DO CELLS REGULATE BIOSYNTHESIS AND FUNCTION OF NACHRS?

Considered broadly, elements of structural biology contribute to the function of nAChRs during their whole life cycle. Extending beyond nAChRs in isolation, these elements include the structural basis of genomic regulation and allelic variations; structures and dynamics important to subunit translation and assembly; and nAChR interactions with cellular elements, including other proteins and lipids.

### 8.1. Genomic regulation

Components of gene regulation for nAChRs (405, 406) include activation and repression elements (407), long range regulatory elements coordinating expression of nAChR gene clusters (408), cell-specific promoter activity (409), and transcription factors controlling nAChR expression (410). RNA splicing is known to affect transcripts for ε (411), α7 (412-415), insect α6 (416), and the glycine receptor β subunit (417). A functionally significant allelic variation of the *CHRNA7* gene for α7 was identified in a Japanese population (418).

### 8.2. Subunit assembly

Diverse combinations of neuronal subunits can assemble into diverse types of nAChRs with functional implications (419-423). Some details about the process of subunit assembly are known, but much remains to be discovered. Ultimately, a set of five stable subunit-subunit interfaces must form. What structural intermediates exist between primary sequences of amino acid residues and the final quaternary structures? What conformational changes convert one intermediate to the next? The process of subunit assembly is best understood for muscle-type nAChRs (424-426), where $(\alpha1)_2(\beta1)\delta\gamma$ and $(\alpha1)_2(\beta1)\delta\varepsilon$ are endpoints. For neuronal nAChRs, the diversity of endpoints remains a subject of investigation (427, 428). Which subunits assemble together (429-431)? What is the stoichiometry of assembly for heteromeric nAChRs (432, 213, 433, 434)? Knowing structures of subunit-subunit interfaces might help decode rules that regulate which subunits can assemble to form nAChRs.

Subunit composition of nAChRs changes during development (435) and affects where the nAChRs are positioned within spatially complex neurons (436). Because neurons often produce several types of subunits that potentially can produce a diverse set of nAChRs, what factors influence the types of nAChRs that are produced from a diverse pool of subunits? Signals in the cellular environment can affect the outcome. For example, the inflammatory cytokines interleukin-1β and tumor necrosis factor α affected the relative production of α4β2 and α4β4 nAChR from a pool of α4, β2, and β4 subunits (437).

### 8.3. Interactions with cellular components

In cells, nAChRs interact with other cellular components, notably proteins and lipids. These interactions affect expression, targeting, electrophysiological function, signaling, and survival (438). Rapsyn, originally identified as a 43 kDa protein associated with *Torpedo* nAChRs, mediated the effect of agrin and helps cluster muscle-type nAChRs (439-442). The tumor suppressor protein adenomatous polyposis coli (APC) participated in the signaling path downstream of agrin and the muscle-specific kinase MuSK for clustering nAChRs in muscle (443).

In neurons, proteins in the PSD-95/SAP90 family (444), splice variants of PSD93 (445), and APC (446) promoted the organization of nAChRs at synapses. The chaperone protein 14-3-3η (447) and possibly other 14-3-3 isoforms (448) modified expression of α4β2 nAChRs and changed the proportions of nAChRs with high and low agonist sensitivity (448). These effects possibly were mediated by interactions with a phosphorylated site in the large cytoplasmic loop of α4 subunits. Visinin-like protein-1 (VILIP-1), a protein sensor of intracellular calcium, also appeared to interact with the large cytoplasmic loop of α4 subunits and increased the surface expression of α4β2 nAChRs (449). PICK1 likely bound to the large cytoplasmic loop of α7 and decreased clustering of α7 nAChRs (450).

RIC-3 is a transmembrane protein of the endoplasmic reticulum produced from the resistance to inhibitors of cholinesterase (*ric-3*) gene in *Caenorhabditis elegans* and found in humans, mice, and *Xenopus*. It has

varying effects on expression of nAChRs. In *Xenopus* oocytes, RIC-3 increases the functional expression of α7 nAChRs but reduces the whole-cell currents from α4β2 and α3β4 nAChRs and abolishes current from 5-$HT_3$ receptors (451). It had little effect on current from α1 glycine receptors (452). RIC-3 appears to interact with an isoleucine residue near the extracellular end of M1 to stop transport to the cell surface and with residues in an amphipathic helix (residues 410–427) in the large cytoplasmic loop of α7. The outcome is to increase the total number and the surface expression of α7 nAChRs (451, 453). In contrast to the mixed effects of RIC-3 on nAChRs expressed in *Xenopus* oocytes, co-expression of nAChR subunits and RIC-3 in human kidney tsA201 cells increased functional expression of α7, α8, α3β4, α4β2, α3β2, and α4β4 nAChRs. RIC-3, however, did not increase functional expression of α9 or α9α10 nAChRs (454). Association between RIC-3 and unassembled α4 subunits and β2 subunits suggested RIC-3 interacts with nAChR subunits during assembly and maturation into nAChRs (454). Calnexin (455), a chaperone protein in the endoplasmic reticulum, also interacted with nAChR subunits during subunit folding and assembly (456-459). The ubiquitin-proteasome system helped regulate the number of active nAChRs by degrading subunits removed from the endoplasmic reticulum by the process of ER-associated degradation (ERAD) (460).

The gene *lynx1* originally was found in mice (461) and produces the protein lynx1 with sequence and structural similarity to snake neurotoxins like α-bungarotoxin. The protein lynx1 physically associated with α7 and α4β2 nAChRs and modified the current through and enhanced desensitization of α4β2 nAChRs (462). Deletion of *lynx1* in mice was expected to increase cholinergic function and did improve learning and memory (463). Deletion of *lynx1*, however, led to a vacuolar degeneration of the brain that was consistent with the presence of hypersensitive nAChRs. These results suggested that lynx1 helps modulate neuronal activity *in vivo*. Another member of the *ly-6/neurotoxin* gene superfamily, *lynx2*, was expressed at specific locations in the mouse nervous system during development (464). This gene superfamily has twenty-seven members in the human genome (465), some of which beyond *lynx1* might have roles in the nervous system.

Lipids directly interact with the transmembrane domain of nAChRs. These lipid-protein interactions not only affected ion conduction (466) but also might affect nAChR trafficking (467, 468).

## 9. WHY IS UNDERSTANDING NACHRS AS BIOPHYSICAL MACHINES RELEVANT TO QUESTIONS ABOUT NORMAL AND PATHOLOGICAL FUNCTION OF THE NERVOUS SYSTEM?

Understanding structures of nAChRs when interacting with other cellular components is important for understanding structural aspects of normal function and pathological function of nAChRs in addiction, neurodegeneration, and mental illness. For example, features of the structural mechanism of upregulating nAChRs by nicotine and other nicotinic ligands are beginning to emerge. Nicotine might interact as a protein folding chaperone with the extracellular domain leading to enhanced expression of α4β2 nAChRs and other nAChRs (469-473, 421). Thermodynamics of ligand interaction at the binding site of α4-containing nAChRs might explain increased assembly and upregulation of these nAChRs (474). Other proposed mechanisms of upregulation of α4β2 nAChRs by nicotine include effects on nAChR trafficking (475) and stabilizing by nicotine of a form of the receptor that is more sensitive to activation (476). Understanding the mechanism of nAChR upregulation by nicotine will lead to methods for preventing and treating nicotine addiction. Interactions between nAChRs and 14-3-3η might help control the activity of nAChRs, a potentially important feature in schizophrenia and neurodegeneration (447). Designing new drugs for diseases related to the function of nAChRs will benefit from a comprehensive and detailed understanding of the structural basis for nAChR function in cellular environments.

## 10. WHAT CAN WE LEARN FROM NACHRS ABOUT PROTEIN FOLDING AND DE NOVO DESIGN OF PROTEINS?

Lessons relating structure and function for nAChRs will contribute other pieces to the protein folding puzzle for integral membrane proteins (477, 478). The combined presence of aqueous phase, lipid phase, and surface charge on the lipid bilayer brings greater complexity to protein folding for integral membrane proteins than is encountered with water-soluble proteins. Creating nAChRs containing unnatural amino acids (section 4.8) illustrates the potential of nAChRs with properties tailored by human imagination. Foreshadowed by nAChRs as biosensors (479-481), understanding how nAChRs are produced and work as biophysical machines under controlled conditions of single channel recording and in a complex cellular milieu will guide the development of proteins with novel functions.

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**Abbreviations:** ACh, acetylcholine; AChBP, acetylcholine binding protein; APC, adenomatous polyposis coli; 5-$HT_3$, 5-hydroxytryptamine (serotonin); GABA, γ-aminobutyric acid; $GABA_A$, γ-aminobutyric acid type A receptor; $GABA_C$, γ-aminobutyric acid type C receptor; M1–M4, transmembrane domains M1 through M4; MIR, main immunogenic region; nAChR, nicotinic acetylcholine receptor; NMR, nuclear magnetic resonance

**Key Words:** Acetylcholine, Addiction, Neurodegeneration, Nicotine, Protein Design, Protein Folding, Protein Structure, Cys-Loop Receptors, Review

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