Journal of Pediatric Biochemistry 1 (2010) 53–73
DOI 10.3233/JPB-2010-0017
IOS Press

Activation and modulation of the nicotinic receptor

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Received 27 May 2010
Accepted 27 May 2010

Abstract. Nicotinic acetylcholine receptors (AChRs) are the best characterized ion channels representing the Cys-loop receptor superfamily. AChRs have all the machinery to recognize the neurotransmitter ACh and other agonists such as nicotine, and to transduce the agonist-induced conformational changes into the opening of the intrinsic cation channel. The gating machinery couples ligand binding, located at the extracellular portion, to the opening of the ion channel, located at the transmembrane region. The interface between the extracellular and the transmembrane domains is considered one of the most important structural and functional features for the process of gating. And finally, in the prolonged presence of agonists, the AChR becomes desensitized. Several drugs affect the functioning of these receptors. Among them, positive allosteric modulators (PAM) have acquired importance since are novel drugs for several neurological diseases. PAMs do not bind to the orthostetic binding sites but allosterically enhance the activity elicited by agonists by increasing the gating process and/or by decreasing desensitization. Instead, negative allosteric modulators (NAMs) produce the opposite effects. Interestingly, this negative effect is similar to that found for another class of allosteric drugs, i.e. non competitive antagonists (NCAs). However, the main difference between both categories of drugs is based on their distinct binding site locations. Although both NAMs and NCAs do not bind to the agonist sites, NACs bind to sites located in the ion channel, whereas NAMs bind to nonluminal sites. Interestingly, PAMs and NAMs might be developed as potential medications for the treatment of several diseases involving AChRs including, dementia-, skin-, and immunological-related diseases, drug addiction, and cancer. More exciting is the potential combination of specific agonists with PAMs. However, we are still in the beginning of understanding how these compounds act and how these drugs can be used therapeutically.

Keywords: Nicotinic receptors, pharmacological properties, gating, allosteric modulators

1. Introduction

Nicotinic acetylcholine receptors (AChRs) are members of the Cys-loop ligand gated ion channel superfamily that are expressed in neuronal and non-neuronal tissues [1–4]. In the nervous system, AChRs mediate rapid transmission by converting a chemical signal into an electric one. Relatively high-resolution structures of the *Torpedo* AChR [5], the 3-D structures of acetylcholine binding proteins (AChBPs) [6], and the recently elucidated prokaryotic ion channels [7–10], have helped in elucidating the functionally relevant structural features of AChRs and their cousins. Structurally, AChRs are pentameric proteins with an extracellular domain that carries the binding sites for the neurotransmitter acetylcholine (ACh) and other agonists such as nicotine. The transmembrane region forms the ion channel that is essential for cation flux, finally producing membrane depolarization. Agonist bind-

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ing at the extracellular domain triggers the opening of the ion channel, an intrinsic process called gating. To determine how the agonist-induced structural changes that start in the agonist binding pocket are propagated through a distance of ~50 Å to the gate is central for the understanding of the receptor function. A large body of experimental evidence has recently shown that the interface between the extracellular and transmembrane domains is essential for functional connection between the binding site and the pore. This is a very unique transitional zone where β-sheets from the extracellular domain merge with α-helices from the transmembrane domain, finally allowing functional communication between both domains [1,11–16]. Different ligands modulate the gating and ion flux processes. Among them, positive and negative allosteric modulators may have therapeutic importance, for example, for the treatment of Alzheimer's disease and schizophrenia. In this review, recent findings on the process of channel gating and its functional modulation by synthetic and endogenous allosteric modulators are discussed.

2. Overall structure of AChRs

AChRs are composed of five identical (homopentamers) or different (heteropentamers) polypeptide chains arranged around an axis perpendicular to the membrane (Fig. 1). Subunits are classified in two types, α and non-α, with the α-type subunits containing a disulphide bridge in the binding site. A large number of α and non-α subunits have been cloned for all members of the superfamily (Ligand-gated ion channel database, http://www.ebi.ac.uk/compneur-srv/LGICdb/cys-loop.php).

All subunits share a basic scaffold composed of: i) a large N-terminal extracellular domain of ~200 amino acids; ii) three transmembrane domains (M1-M3) separated by short loops; iii) a cytoplasmic loop of variable size and amino acid sequence; and iv) a fourth transmembrane domain (M4) with a relatively short and variable extracellular COOH-terminal sequence. The AChRs are therefore built on a modular basis, with the extracellular domain containing the agonist binding sites, and the transmembrane domain containing the pore, the selectivity filter and the channel gate [5] (Fig. 1).

Recent structural studies have provided an insight into the three dimensional structure of AChRs and all members of the superfamily. In particular, a relatively high resolution (4 Å) structural model of the AChR from the marine ray *Torpedo* [5] has revealed important information and has been invaluable in the interpretation of functional and pharmacological data (PDB code 2BG9, Fig. 1). Although no atomic resolution structural information is available for any Cys-loop receptor, the extracellular domain of the AChR α1 subunit has been reported at atomic resolution (1.94 Å) [17]. In addition, high resolution structural information has become available from studies of proteins which show close sequence similarity to AChRs, namely (a) the high-resolution structures of soluble AChBPs from fresh-water snails including, *Lymnaea stagnalis* (2.7 Å; PDB 1I9B) [6] and *Bulinus truncatus* (2.0 Å; PDB 2BJ0) [18], and the sea snail *Aplysia californica* (1.96–3.4 Å; PDB 2BYN) [19]; and (b) X-ray structures of prokaryotic ligand-gated ion channels from the bacteria *Erwinia chrysanthemi* (ELIC) at 3.3 Å resolution (PDB 2VL0) [9] and *Gloebacter violaceus* (GLIC) at 3.1 Å (PDB 3EHZ) [10] and 2.9 Å resolution (PDB 3EAM) [8].

3. The extracellular domain

Our knowledge of the structure of the extracellular domain of AChRs took a giant step forward with the solution of the high-resolution structure of the AChBP from *Lymnaea stagnalis* [6]. This soluble protein is produced and stored in glial cells and is released in an ACh-dependent manner in the synaptic cleft where it regulates synaptic transmission. AChBP lacks the transmembrane region but contains many of the structural cornerstones that give AChRs their unique signature and has therefore become a functional and structural model of the extracellular domain of the Cys-loop receptor superfamily. It contains 210 amino acids and shares ~15–24% sequence identity to aligned sequences of the amino-terminal, extracellular halves of Cys-loop receptor subunits. Each AChBP monomer consists of an N-terminal α-helix, two short 3_{10} helices, and a core of 10 β-strands that form a β-sandwich structure. The inner β-sheet is formed by β1, β2, β3, β5, β6 and β8, and the outer β-sheet by β4, β7, β9 and β10. The N- and C- terminals are located at top and bottom of the pentamer, respectively. In Cys-loop receptors, the end of β10 connects to the start of M1. Located at the bottom of the subunit, the linker between β6 and β7 strands is the signature Cys-loop found in all members of the superfamily.

Agonist binding sites are located at interfaces between subunits. Each binding site is formed by two

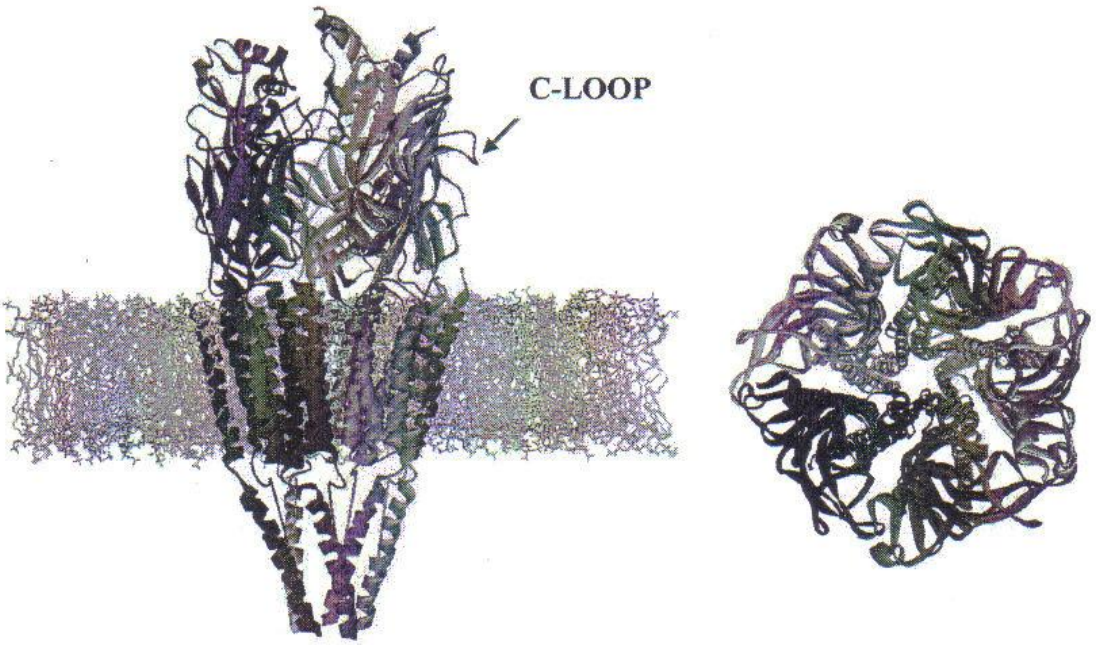

Fig. 1. Structure of the AChR. Cartoon diagrams for the *Torpedo* AChR (2BG9.pdb) [5] viewed parallel to the membrane (left) and from the synaptic cleft (right). (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/JPB-2010-0017)

faces. One face, called the principal or "positive" face, is formed by three loops of α-type subunits that span β strands and harbor predominantly key aromatic residues [i.e., Loop A ($\beta4\beta5$ loop), Loop B ($\beta7\beta8$ loop), and Loop C ($\beta9\beta10$ loop)]. The complementary or "negative" face contributes with three β strands with residues clustered in segments called Loops D-F. Thus, key residues (corresponding to *Torpedo* α1-subunit) from the principal face come from Loop A (Trp86 and Tyr93), Loop B (Trp149 and Gly153) and Loop C (Tyr190, Cys192, Cys193 and Tyr198). The complementary face is formed by residues from Loop D (Trp55 and Asp57), Loop E (Leu109, Arg111, Thr117 and Leu119), and Loop F (Asp174 and Glu176) (residues from δ or γ *Torpedo* subunits) [6,20–22].

The ancestral Cys-loop receptor was likely homomeric and contained five identical binding sites, similarly to present day homomeric receptors, such as $\alpha7$ and 5-HT_3A receptors [23–25]. Evolution led to the appearance of new subunits which lost the ability to form agonist binding sites, giving rise to heteromeric receptors with fewer than five binding sites. The prototypic heteromeric receptors, such as the muscle ACh and $GABA_A$ receptors contain only two agonist binding sites, which have to be both occupied to allow appropriate gating. Although homomeric receptors contain five identical binding sites, occupancy of only three of the five sites has been shown to be required for optimal activation [26,27].

4. The transmembrane domain

Functional and structural studies have demonstrated that the ion channel is largely lined by the five M2 transmembrane segments provided by each subunit. The first model of the transmembrane region of the *Torpedo* AChR at ~4 Å obtained by cryo-electron microscopy confirmed this long-held view [28]. An outer ring of fifteen α-helices (M1, M3 and M4 segments) shields the inner ring from the lipids. Some years later, the complete picture of the AChR structure was provided by Unwin [5]. Since the model was obtained in the absence of agonist, it is considered to depict the closed resting state, although this remains a matter of controversy. Despite lacking atomic resolution, this model has become a cornerstone for structure-function studies. The membrane-spanning portion, composed of the four α-helical segments from each subunit, is joined covalently to the extracellular domain at the N-terminal end of M1 (Fig. 1). The ion channel is maximally constricted in the middle of the membrane due to side-to-side interactions between hydrophobic residues of neighboring helices at positions 9' and 13'. This tight hydrophobic girdle creates an energetic barrier to ions across the membrane, and it has been suggested that it corresponds to the gate [5,28].

The ion pore contains the filter selectivity, which is the structure that determines which types of ions are

able to pass through the channel. Point mutations in the M2 region supported the role of positions 2' to 2' as main determinants of the selectivity filter of all Cys-loop receptors [29].

The structure of the AChR resembles those of the homologous pentameric ligand-gated ion channels recently identified in bacterial sources [7,25]. The first characterization of a pentameric ion channel from *Gloebacter violaceus* (GLIC) showed that it forms a cation-selective channel that it is activated by protons, and that currents do not decay during activation, suggesting no desensitization [7]. The first X-ray structure at 3.3 Å of a prokaryotic receptor was obtained from *Erwinia chrysanthemi* (ELIC) [9]. This receptor shows 16% sequence identity to α AChR subunits. The extracellular domain is very similar to its eukaryotic counterpart and to AChBPs, but lacks the N-terminal α-helix. However, the putative binding site and several of the aromatic residues found in AChRs are conserved. The central part of the Cys-loop is also conserved but lacks the flanking disulfide-bridge cysteine residues. The transmembrane region is equivalent to that for AChRs, with four α-helices from each subunit, but lacks the long intracellular loop between M3-M4. Unlike in the structure of AChRs, showing a narrow but continuous pore, the hydrophobic residues in ELIC physically obstruct the pore. The structure of GLIC was solved at low pH, and because it is activated by protons and currents do not decay in the presence of protons, it might represent the receptor in a potentially open state [8,10]. Assuming that the ELIC and GLIC structures depict the closed and open conformations, respectively, the comparison of both structures has allowed suggestions of the structural changes occurring during channel opening [8,10].

5. The cytoplasmic domain

The long intracellular region between M3 and M4 contains a short α-helix. It is thought to be associated with cytoskeletal proteins, such as rapsyn [30, 31]. Several proteins allow the clustering of the receptors at appropriate regions of the membrane [32–34]. This intracellular region has been shown to contribute to channel kinetics in muscle AChRs [35,36] and contains determinants of channel conductance in 5-HT_3A receptors [37,38]. It also contains phosphorylation sites, and it has been demonstrated that phosphorylation modulates expression, upregulation, desensitization, and interaction with cytoskeleton proteins of AChRs [39–41].

6. The gating process

The gating reaction couples local structural changes at the binding sites with changes in the ion channel that allow the increase in conductance. The identification of residues that transduce neurotransmitter binding into channel gating is now possible by combining the information of residue locations within functionally crucial regions of the receptors from the high-resolution structures with electrophysiological, pharmacological, and computational studies.

Binding of the agonist and the resulting conformational changes have been well studied in AChRs. Stabilization forces of the agonist at the binding site include π-cation, dipole-cation, hydrogen bonding and van der Waals interactions [42–44]. The superposition of the AChBP crystal structures with a variety of agonists and antagonists shows that Loop C from the principal face is in an "open" conformation in the resting AChR. In the presence of the agonist, Loop C caps the entrance to the binding cavity, trapping the agonist. Molecular dynamics simulation also revealed a time-dependent change of Loop C from uncapped or open to a capped or closed conformation [44–46]. The Loop C is connected directly to M1 *via* β10, and therefore it might propagate conformational changes occurring after agonist binding to the interface between the extracellular and transmembrane domains. The conserved Tyr190 (in AChRs) in Loop C is drawn closer to Lys145 from the β7 strand, breaking or weakening a previous interaction between this Lys and Asp200 in the β10 strand [1, 47]. The movement of this loop seems to be the initial conformational change underlying channel activation after agonist binding in all Cys-loop receptors [48, 49]. By performing cysteine substitutions at Loop C of each binding site and at each of the two juxtaposed subunits of the muscle AChR, and by further recording single-channels from the mutant receptors before and after oxidation, it was shown that capping of the C-loop is involved in the transition of the closed receptor to an activated pre-open intermediate state [50]. It was also shown that simultaneous capping at both agonist binding sites is required to evoke long-lived openings.

Mutational combined with electrophysiological studies have shown that residues from other agonist binding-site Loops in both principal and complementary faces are also involved in binding and gating. The amino acid in position 153 of Loop B from AChRs has been shown to be associated with a slow-channel syndrome [51], to govern the strong activation of nematode AChRs by anthelmintic drugs [52], and to affect gating

Table 1
Subunit sequences at the receptor interface

		$\beta1\beta2$	Cys-loop	$\beta8\beta9$	$\beta10$+ pre-M1	M2M3	post-M4
AChα1	*Torpedo*	DEVNQI	CEIIVTHFPFDQQNC	MESGEW	IMQRIPLYFVVN	SSAVPLIGKY	FAGR–LIELSQ
AChα1	Human	DEVNQI	CEIIVTHFPFDEQNC	MESGEW	VMQRLPLYFIVN	SSAVPLIGKY	FAGR–LIELNQ
AChα7	Human	DEKNQV	CYIDVRWFPFDVQHC	IPNGEW	TMRRRTLYYGLN	SDSVPLIAQY	LMSAPNFVEAVS
5-HT3A	Human	DEKNQV	CSLDIYNFPFDVQNC	MNQGEW	VIRRRPLFYVVS	AIGTPLIGVY	VMLWSIWQYA–
GABAα1	Human	SDHDME	CPMHLEDFPMDAHAC	EDGSRL	HLKRKIGYFVIQ	A-YATAMDWF	WATYLNREPQLK
GABAβ2	Human	SEVNMD	CMMDLRRYPLDEQNC	VTKIEL	KLKRNIGYFILQ	P-YVKAIDMY	WLYYVN——
Glyα1	Human	AETTMD	CPMDLKNFPMDVQTC	ADGLTL	HLERQMGYYLIQ	S-YVKAIDIW	WIIYKIVRREDV

Sequences were aligned with ClustalW. The sequences shown for $\beta8\beta9$ and $\beta10$ correspond to those located at the interface.

by interacting with Loop C in neuronal AChRs [53]. Trp55 in Loop D of the complementary face has been shown to be involved in channel gating and desensitization of muscle and α7 AChRs [54]. In the same loop, Gln57 has been shown to govern the high potency of morantel to activate α7 AChRs [55].

The endpoint of the activation process is the transient removal of the barrier to ion flow. Various molecular rearrangements have been proposed to underlie channel opening but the fundamental motion of M2 that opens the pore remains unknown. It was first proposed that channel opening proceeds by a rotation of the pore-lining helices around their helix axis [28,45] whereas subsequent studies suggested rigid-body tilting of M2 [56], a subtle rearrangement of transmembrane segments [57], or a mixed picture. For example, the rotation of M2 was not supported by single-channel electrophysiological experiments in which residues that line the pore in the open state were detected by measuring the ability of protons to block the channel after Lys substitution [57,58]. These studies suggested that the pore dilation that underlies channel opening involves only a subtle rearrangement of M2, M3, and M1 transmembrane helices and that the rotation of M2, if any, is minimal. A subtle movement is fully consistent with the concept of "hydrophobic gating" proposed for the AChR [28,59,60]. In this model, the large hydrophobic residues located at positions 9', 13' and 17' of M2 [61, 62] act as a desolvation barrier for ions instead of a steric one and the gate is so narrow that an ion has to shed at least some water molecules from its hydration shell to pass the constriction. Simulations of the AChR have suggested that increasing the radius of this hydrophobic girdle of the closed-state pore by as little as ~1.5 Å is enough to increase the computed conductance to values similar to the experimental ones [59]. However, the hydrophobic girdle hypothesis is still a matter of controversy. Computational simulations using homology models also suggested different mechanisms by which the channel is opened, such as twisting motion of the extracellular domain combined with tilting of the M2 which, in turn, disrupts the hydrophobic girdle [63], rotation of M2 [64], rotation with bending motions of M2 [65], and a quaternary twist motion [22,66].

6.1. *The extracellular-transmembrane interface: the coupling region*

The agonist binding site projects into the synaptic cleft, whereas the region that gates ion flow localizes within the membrane domain. Communication over the 50 Å separating the two regions is thus essential to the function of Cys-loop receptors. The binding-pore interface is a structural transition zone where β-sheets from the binding domain merge with α-helices from the pore (Fig. 2). Within this zone several regions form a network that relays structural changes from the binding site towards the pore. Structures at the interface include: $\beta1\beta2$ loop, Cys-loop, $\beta8\beta9$ loop, and the end of $\beta10$, all from the extracellular region, and the pre-M1 region, M2-M3 linker, and the C-terminal end of M4 from the transmembrane region (Fig. 2 and Table 1).

A structural interplay between loops at the interface required for coupling agonist binding to channel gating was demonstrated by generating a chimeric receptor composed of the AChBP protein, which presumably evolved without the constraint of functional coupling to an ion pore, and the pore domain from the 5-HT_3A receptor [11]. Although the chimeric receptor expressed on the cell surface and showed high affinity for ACh, it was not functional. However, when amino-acid sequences of three loops ($\beta1\beta2$-, Cys-, and $\beta8\beta9$-loops) in AChBP were changed to their 5-HT_3A counterparts, ACh bound with low affinity characteristic of activatable receptors and ACh was capable of triggering opening of the ion pore. The findings revealed that this region mediates a bi-directional allosteric interaction between the binding sites and the pore domain and that the functional coupling process is mediated by a network of loops from both domains [11]. Further insights into the global role of the interface in synaptic responses mediated by homomeric Cys-loop

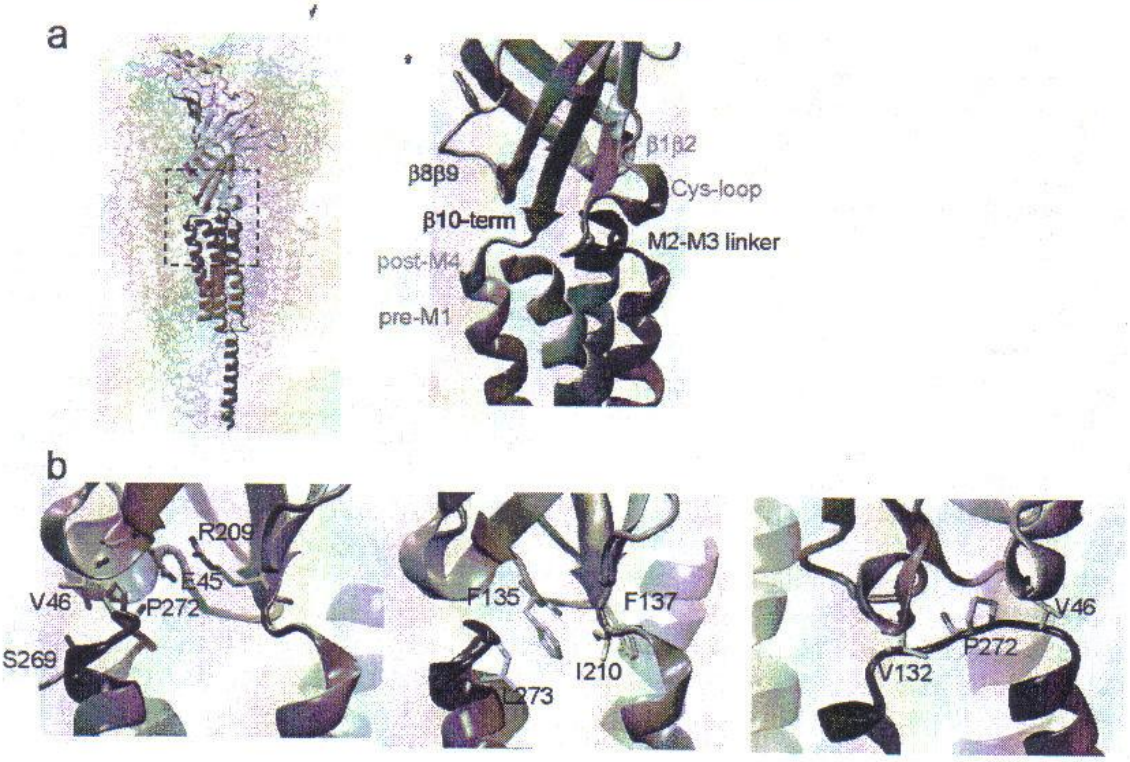

Fig. 2. The interface between the extracellular and transmembrane domains. (a) Left: Structure of the *Torpedo* AChR with one of its subunits highlighted with the extracellular domain in yellow and the transmembrane and intracellular domains in red. The interface is shown in the dashed square. Right: View of the structures at the interface. The different segments are colored as follows: orange ($\beta1\beta2$ loop), ice blue ($\beta8\beta9$ loop), green (Cys-loop), purple ($\beta10$-terminal), pink (pre-M1), blue (M2-M3 linker), and cyan (post-M4). (b) Different views of the interface with key residues labeled. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/JPB-2010-0017)

receptors emerged from studies of a chimeric receptor containing the extracellular region of $\alpha7$ and the transmembrane region of 5-HT_3A ($\alpha7$-5HT_{3A}). This chimeric receptor has served as a model for studying the pharmacology of $\alpha7$ receptors because it shows high expression in mammalian cells [38]. Interestingly, although it carries a mixed $\alpha7$/5-HT_3A interface, it functions as an ACh-gated ion channel. The chimeric $\alpha7$-5HT_{3A} receptor shows an intermediate kinetic profile between that of $\alpha7$ receptors, which show fast desensitization and brief openings, and 5-HT_{3A} receptors, which show slow desensitization and long opening events [67,68]. The functional signatures of each parent receptor could be reconstituted after substituting in the original $\alpha7$-5HT_{3A} chimera the major loops within the extracellular-transmembrane interface with sequences from the corresponding parent receptor. The overall results showed that the interface is not only involved in channel opening, but also in determining channel lifetime and desensitization in homomeric receptors. The replacement of individual loops one at a time revealed that the kinetics depends on the interplay between all loops, giving further support to the idea that the interface is a complex network of loops which couples conformational changes from the binding sites to the ion pore [67].

The finding that the extracellular-transmembrane interface is involved in the rate of fast desensitization is of relevant significance. Desensitization seems not to affect normal muscular transmission through AChRs, but it has a role in synaptic transmission in pathologies underlying gain-of-function mutations of the muscle AChR [69]. In addition, desensitization seems to be very important in the process of nicotine addiction, and it may be important under the presence of certain ligands or enhanced phosphorylation [70–72]. For mode details on AChR desensitization in nicotine addiction see the minireview by Ortells and Arias in this special issue. Despite its relevance in controlling synaptic efficacy, understanding the structural movements underlying desensitization has lagged behind. This is due to the fact that the extent of desensitization results not only from its onset rate but also from the kinetics of recovery from desensitization and gating. Also, the accurate measurement of desensitization rate can be achieved only under optimal time-resolution systems,

and finally, desensitization probably involves many different conformational states.

The important role of the interface loops, $\beta1\beta2$, Cys, $\beta8\beta9$, the end of $\beta10$, pre-M1 region, M2-M3 linker, and post-M4, in coupling agonist binding to channel gating has been shown in numerous studies for all members of the Cys-loop superfamily [13,15,55]. A large number of studies identified residues within these regions with crucial roles in channel gating. The $\beta1\beta2$ loop has been shown to be essential for gating. Val46 in this loop has been initially proposed to be the key "pin" residue in the "pin-into-socket" mechanism for channel gating [28]. A great number of different reports have shown that several other residues of this loop are essential for gating in different family members [12,73,74]. The Cys-loop, which is the signature of the family, inserts between the pre-M1 region (close to its C-terminal half) and the M2-M3 domain (close to its N-terminal half). It is essential for AChR assembly [75,76]. Its crucial role in channel gating has been widely demonstrated for all family members [12,53,73, 77–82].

The role of the $\beta8\beta9$ loop is less understood, but computational and experimental evidence confirm that it is involved in channel gating. This loop is long and relatively unstructured, and it is the region of most sequence variation among family members [45] (see Table 1). The residue Glu172 in the $\beta8\beta9$ loop was found to undergo agonist-dependent movements during receptor activation [1,83]. By using fluorescence anisotropy decay to study the segmental motion of side chains in AChBPs, Hibbs et al. [84] demonstrated that agonists (but not antagonists) induced changes in conformational dynamics in the $\beta8\beta9$ linker. Simulations of a homology model of $\alpha7$ showed that $\beta8\beta9$ moves inward toward its subunit. This motion occurs in all of the subunits, but it occurs to the greatest degree in the subunits adjacent to those whose Loops C move out the most [45].

The pre-M1 region, which connects $\beta10$ to M1, contains several cationic residues, including several Arg that are conserved in several Cys-loop receptors. Arg209, which is present in all family members, has been shown to have a fundamental role in gating of the human muscle AChR [85].

Several lines of experimental evidence reveal that the M2-M3 linker and the flanking regions play a key role in channel gating in AChRs [80,86,87], 5-HT_3 [88], GABA [89], and glycine receptors [90]. The importance of this region is also supported by the identification of mutations that lead to human diseases [1,91, 92].

The M4 domain is the least conserved among the transmembrane domains, is the most hydrophobic and has been extensively labeled by hydrophobic probes [93,94]. The C-terminal region of M4 (post-M4) is located at the interface between the extracellular and transmembrane domains (see Fig. 2). Potential interactions between residues in this portion of M4 and residues in the extracellular domain, including the Cys-loop, have been determined by computational studies of the $\alpha7$ AChR [95], in agreement with large experimental evidence showing that this segment contributes to gating kinetics [68,96–98] and that it moves during channel gating [99].

By combining mutagenesis, single channel kinetic analyses, and thermodynamic mutant cycle analyses Lee and Sine [85] identified an important transduction pathway in which the pre-M1 domain is coupled to the M2-M3 linker through the $\beta1\beta2$ loop in the human muscle AChR. The authors proposed that agonist binding leads to the disruption of a salt bridge between Arg209, located at the end of $\beta10$ in the pre-M1 region, and Glu45 in $\beta1\beta2$ of the $\alpha1$ subunit (Fig. 2). The key Glu45 and flanking Val46 residues energetically couple to the conserved Pro272 and Ser269 residues at the top of M2, and this may be a main point at which the binding domain triggers ion channel opening. The positioning of key elements of this pathway, such as the buried salt bridge formed by Arg209 and Glu45, has been later verified by high-resolution structures of the isolated α-subunit extracellular domain [17] and the bacterial channel GLIC [9]. The salt bridge between residues equivalent to Glu45 and Arg209 has also been found to be important in $GABA_A$ and $GABA_C$ receptors [100]. Another pathway in which the pre-M1 region is also coupled to the M2-M3 linker through the Cys-loop was further identified in the human muscle AChR [101]. The studies revealed energetic coupling among $\alpha1$-Leu210 from the pre-M1 region, $\alpha1$-Phe135 and $\alpha1$-Phe137 from the Cys-loop, and $\alpha1$-Leu273 from the M2-M3 linker (Fig. 2). Thus, studies in human muscle AChR showed that the extracellular $\beta1\beta2$- and Cys-loops bridge the pre-M1 region and the M2-M3 linker, transducing agonist binding into channel gating. A highly conserved Pro in M2-M3 linker (Pro272) has also an important role in channel gating on muscle AChRs. By single-channel kinetic analysis of mutant muscle AChRs, Lee et al. [82] showed that $\alpha1$-Pro272 functionally couples to the flanking Val46 residue from the $\beta1\beta2$ loop and Val132 from the Cys-loop, serving as an anchor that joins the hydrophobic residues from both loops. The functional contributions

Table 2
Classification of positive and negative allosteric modulators

Allosteric modulator (Type)	Synthetic or endogenous	Name	AChR specificity	References
Positive (Type I)	Synthetic	Ivermectin	Not specific	108
		Genistein	α7 AChR	110
		NS-1738	Not specific	111
		Compound 6 (also called CCMI or XY4083)	Not specific	113
		LY-2087101	Not specific	114
		SB-206553	α7 AChR	115
		Morantel	$\alpha3\beta2$ AChR (but also a potent agonist for α7 AChRs)	55,109
Positive (Type II)		PNU-120596	Highly specific for α7 AChRs (but also pontentiates chicken α8 AChRs)	122,123
		A-867744	α7 AChR	124
		TQS	α7 AChR	110
		Galantamine	Not specific	125,126
		Memogain	Not specific	132
		Physostigmine	Not specific	125,126
		Codeine	Not specific	125,126
Positive	Synthetic	A-998679	$\alpha4\beta2$ AChR	116
		A-969933	$\alpha4\beta2$ AChR (also called NS-9283)	117
		JNJ1930942	α7 AChR	118
Positive (Type I)	Endogenous	5-Hydroxyindole	α7 AChR	141
(Type I)		Albumin derivatives	α7 AChR	142
(Type I)		SLURP-1	α7 AChR	152
(Type I)		Lypd6	Not specific	144
		17β-Estradiol	$\alpha4\beta2$ AChR	145
		CGRP1-4/5/6	Not specific	149,150
		14 residues from the C-terminus of acetylcholinesterase	Not specific	147,148,151
Negative	Synthetic	COB-3	Not specific	174
		UCI-30002	Not specific	175
	Endogenous	Lynx-1 and -2	Not specific	177-180
		Prostate stem cell antigen	Not specific	183

of the three residues may depend jointly on proper steric fit and hydrophobicity [82] (Fig. 2).

Rate-equilibrium free energy relationship (REFER) analysis of hundreds of residues of the mouse muscle AChR allowed the construction of a Φ map, which may represent the sequence of movements of different domains [87,102]. Φ is the slope of a log-log plot of the changes in the forward closed–open rate to changes in the gating equilibrium constant between closed and open states, when a specific amino acid residue is mutated to several types. The map suggests that during channel opening the presence of the agonist triggers motions of the binding site, which, next, moves the Cys- and $\beta1\beta2$-loops and then, the M2-M3 linker, some M2 residues, and finally the gate [79,81,103].

A variety of functional and computational evidence over the last years suggests that movements around the binding site propagate through the β-strands to cause rearrangements of the interface. The emerging view indicates that the $\beta1\beta2$ loop, Cys-loop, M2-M3 linker and pre-M1 region act jointly to allow the increase in ion conductance that follows the binding of the agonist. It is less known how other interface regions, such as the post-M4 region and the $\beta8\beta9$ loop, participate in this mechanism.

7. Allosteric modulators

The activity of different AChRs can be modulated positively or negatively by exogenous and endogenous ligands by binding to sites different from the agonist/competitive antagonist binding sites (i.e., the orthosteric sites) (Table 2). This section explains the

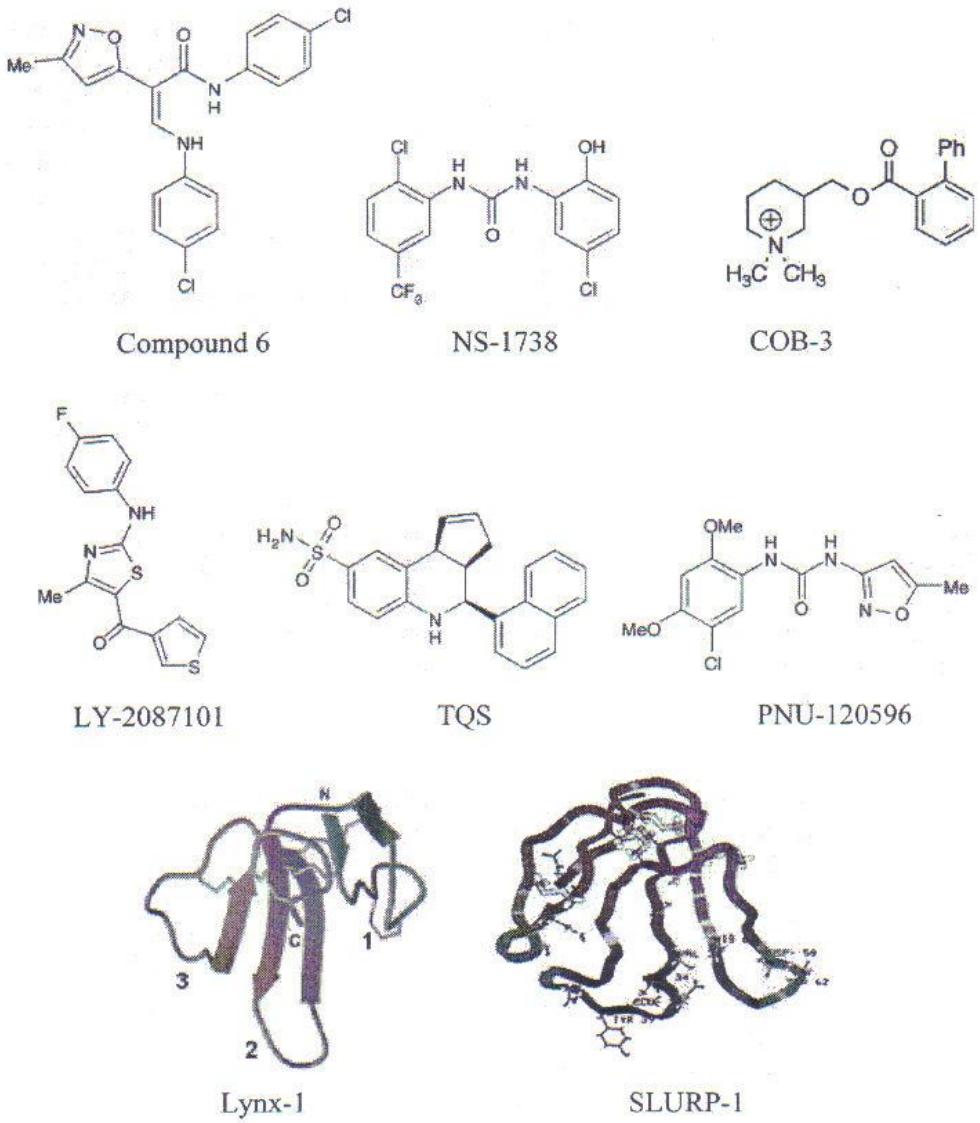

Fig. 3. Molecular structures of the most important allosteric modulators including, the synthetic type I (e.g., Compound 6 and NS-1738) and type II (e.g., PNU-120596, LY-2087101, and TQS) PAMs, the endogenous PAM SLURP-1, and the synthetic (e.g., COB-3) and endogenous (e.g., Lynx-1) NAMs. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/JPB-2010-0017)

pharmacological basis for the classification between positive and negative modulators, and between type I and II positive modulators. Figure 3 shows the molecular structures of several of the most important AChR modulators.

7.1. *Positive allosteric modulators*

Positive allosteric modulators (PAMs) bind to sites different from that for agonists and competitive antagonists and enhance gating of the receptor in the presence of agonists. This is why these compounds are also called co-agonists or noncompetitive agonists. This pharmacological activity resembles to that produced by benzodiazepines on $GABA_A$ receptors [104]. PAMs can be functionally divided in two main classes [105–107]: type I modulators, that enhance agonist-induced AChR activation but do not alter AChR desensitization, and type II modulators, that enhance agonist-induced AChR activation by stabilizing the open channel state and slow additional desensitization. Decreasing the process of AChR desensitization could be very important for subtypes that present very fast desensitization rates as in the case of $\alpha 7$ AChRs.

The anthelminthic agents ivermectin [108] and morantel [109], the tyrosine kinase inhibitor genistein [110], the urea derivative NS-1738 [1-(5-chloro-2-hydroxyphenyl)-3-(2-chloro-5-trifluoromethylphenyl)

urea] [111,112], the $GABA_A$ receptor positive modulator compound 6 (also called CCMI or XY4083) [*N*-(4-chlorophenyl)-α-[[(4-chloro-phenyl)amino]methylene] -3-methyl-5-isoxazoleacet-amide] [113], (2-amino-5-keto)thiazole compounds (e.g., LY-2087101) [114], and the G-protein-coupled serotonin receptor 2B/2C antagonist SB-206553 (3,5-dihydro-5-methyl -*N*-3-pyridinylbenzo [1, 2-*b*:4,5 -*b*']-di pyrrole-1(2*H*)-carboxamide) [115], are synthetic type I modulators that increase AChR activation without changing the desensitizing properties. There are several PMAs that in principle are type I modulators, but we do not have enough information to determine if they can behave as type II modulators. For example, A-998679 [116] and A-969933 (also called NS-9283) [117] are novel positive allosteric modulators of $\alpha4\beta2$ AChRs, whereas JNJ-1930942 is more specific for the $\alpha7$ AChR [118]. Although the majority of type I modulators do not change the desensitization kinetics, SB-206553 increases the slow component of the desensitization process in a concentration-dependent manner, indicating that this compound has certain characteristic of type II modulator, although less profound. Some compounds present certain pharmacological characteristics that make them potential candidates for future drug development. For instance, SB-206553 has higher specificity for the $\alpha7$ AChR compared with that for $\alpha4\beta2$, $\alpha1$-, and $\alpha3$-containing AChRs [115]. However, the pharmacological properties of some of these compounds unfortunately preclude their potential therapeutic use. For instance, although ivermectin presents specificity for the $\alpha7$ subtype among AChRs, it also acts on glycine [119] and purinergic 2X receptors [120]. LY-2087101 increases both the potency and the efficacy of agonists for the $\alpha7$, $\alpha2\beta4$, $\alpha4\beta2$, and $\alpha4\beta4$, but not for $\alpha3$- or $\alpha1$-containing AChRs [114]. The PAM effect elicited by genistein seems to be produced by a combination of direct (co-activation of $\alpha7$ AChRs) and indirect mechanisms (i.e., inhibition of tyrosine kinases) [121]. Although morantel behaves as a PAM on the $\alpha3\beta2$ AChR [109], it also acts as a potent agonist on the $\alpha7$ AChR and a low-efficacy agonist in muscle AChRs [55]. The lack of receptor specificity, complex pharmacology, and combined mechanisms of action of some of these compounds limit its appeal for potential therapeutic uses.

Using different recombinant $\alpha7$-$5HT_3$ chimeras, Bertrand et al. [111] demonstrated that NS-1738 binds to the M2-M3 extracellular loop. This loop has been proved to be very important in the process of gating (see Section 6.1.). Nevertheless, considering that LY-2087101 does not potentiate agonist-induced responses in chimeric $\alpha7$-5HT receptors, it was suggested that its binding site resides in regions downstream of the N-terminal and M2-M3 extracellular loop [114]. More specifically, LY-2087101 binds to residues Ala225 and Met253 located in the transmembrane segments [122]. On the other hand, using the substituted cysteine accessibility method, Seo et al. [109] determined that the amino acids Ala106 and Thr115 at the $\alpha3$ subunit, and Ser192 and Thr150 at both $\alpha3$ and $\beta2$ subunits are important for morantel interaction with the $\alpha3\beta2$ AChR. These amino acids are located at the $\alpha(-)/\beta(+)$ interface, an opposed location from the canonical $\alpha(+)/\beta(-)$ interface for agonists. These results indicate that although these compounds are classified as type I modulators, they differ in their binding site locations. Interestingly, morantel has potent agonistic activity on $\alpha7$, but not on muscle, AChRs, and the amino acid Gln57 was found to be very important for this specific activity [55]. These results highlight the importance of non-canonical subunit interfaces in the intramolecular transfer of information from ligand binding to channel gating.

PNU-120596 [*N*-(5-chloro-2,4-dimethoxyphenyl)-*N*'-(5-methyl-3-isoxazolyl)-urea] (115,122,123), the pyrrole-sulfonamide derivative A-867744 [4-(5-(4-chlorophenyl)-2-methyl-3-propionyl-1H-pyrrol-1-yl) benzenesulfonamide] [124], TQS (4-naphthalene-1-yl-3*a*,4,5,9*b*-tetrahydro-3-*H*-cyclopenta[*c*]quinoline-8-sulfonic acid amide) [110], and the morphine derivative codeine [125], are synthetic type II modulators that exert a much greater effect on AChR activation than agonists alone and decrease desensitization. The plant alkaloids galantamine and physostigmine are also type II modulators [125,126]. An interesting pharmacological property of some type II modulators (e.g., TQS and PNU-120596) is that they can reactivate desensitized AChRs [110]. Several of these compounds increase the Hill coefficient, suggesting an increase in ligand cooperativity and the existence of more than one binding site, which supports the view of at least two different binding sites, one for agonists and another for PAMs. These mechanisms are also part of the potentiating activity elicited by these compounds. In general, type II modulators have higher specificity for the $\alpha7$ AChR. However, PNU-120596 also acts as a potent PAM on $\alpha8$ AChRs, but this receptor subtype has not been detected in any mammalian species yet. In addition, galantamine, codeine, and physostigmine are unspecific ligands [125,126]. Interesting, PNU-120596 and type I PAMs do not produce co-agonistic action but

attenuate the amplitude of the ACh response in ACR-16, the homolog of the α7 AChR in *Caenorhabditis elegans* [127].

Using the substituted cysteine accessibility method, Barron et al. [128] determined that PNU-120596 causes conformational changes in the extracellular ligand binding domain of the α7 Leu247Thr mutant. More specifically, at the inner β sheet composed of the β1, β2, and β6 strands, at the transition zone, and at the agonist binding sites. Nevertheless, these changes are similar but not identical to that produced by ACh and agonists. For example, PNU-120596 induced changes at Asn170Cys located in the transition zone that were different to that evoked by ACh. Additional studies using subunit chimeras have identified the binding site for PNU-120596 in the transmembrane domain of the α7 AChR [122]. More specifically, at amino acids Ser222 and Ala225 from M1, Met253 from M2, and Phe455 and Cys459 from M4, where the side chains of all five amino acids point toward an intrasubunit cavity located between the four transmembrane domains. This potential location was supported by additional studies using α7-5HT chimeras [111] and by contrasting the structure between the α7 AChR and ACR-16, its *C. elegans* homologous [127]. Figure 4 depicts the location for the PNU-120596 binding site and five of the most important amino acids. Interestingly, M2-Met253 and M4-Cys459 lie in positions exactly analogous to amino acids involved in the binding of neurosteroids and volatile anesthetics on $GABA_A$ and glycine receptors [122]. A location different from the N-terminal extracellular domain was also determined for A-867744 [129]. However, this compound showed pharmacological properties different to that for PNU-120596 and TQS. For example, low concentrations of A-867744 do not activate a distinct secondary component, and unlike other PAMs, it displaces the binding of the α7 AChR agonist [^{3}H]A-585539. These results suggest different modes of positive allosterism among structurally distinct PAMs.

Galantamine does not offer the characteristics of an ideal PAM including, high selectivity and potency, and modulation accompanied by the maintenance of rapid receptor desensitization kinetics, a fundamental characteristic of these ligand-gated ion channels. For example, galantamine is nonselective and evokes very low efficacy over a narrow concentration range, whereas it behaves as a channel blocker at slightly higher concentrations [126,130]. Although galantamine is an actylcholinesterase inhibitor, increasing finally the synaptic concentration of ACh, the positive allosteric property of this drug on AChRs could be also important for its clinical efficacy in the treatment of Alzheimer's disease [130]. Nevertheless, the competitive effect of galantamine on the inhibitory action elicited by the endogenous compound kynurenic acid on the α7 AChR could be another mechanism underlying its clinical efficacy [131]. In addition, memogain, a galantamine pro-drug, has higher activity than galantamine and produces less side effects [132].

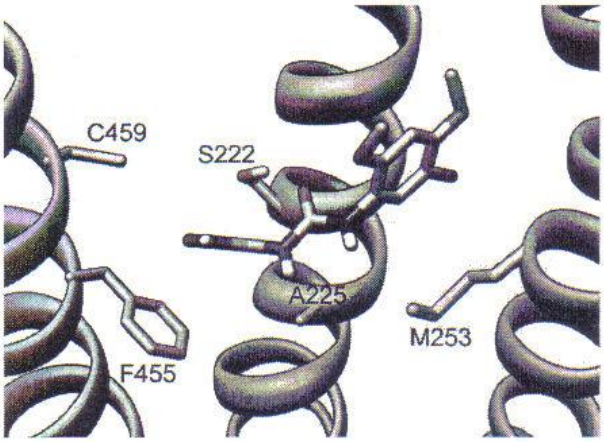

Fig. 4. Molecular docking of PNU-120596, a synthetic type II PAM, to the α7 AChR model [modified from [122]]. The positions of the five amino acids within the transmembrane domain identified by site-directed mutagenesis as being important in the pharmacological action of PNU-120596 are also included. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/JPB-2010-0017)

Based on docking experiments with galantamine, physostigmine, codeine, and the neurotransmitter 5-HT on homology models of the ligand binding domain from α7 and $\alpha4\beta2$ AChRs, as well as on site-directed mutagenesis and electrophysiological results, the residue α7-Thr197 (and to a smaller extent α7-Ile196 and α7-Phe198), at the outer surface of the ligand binding domain, was identified as an essential element for the binding of these compounds [133]. These residues, located in the β10 strand, are directly connected to Asp195, which together with Tyr186 and Lys143 (in the β7 strand) are involved in ACh-induced conformational changes [47], and in the particular case of Lys143, in agonist-induced gating [134]. Using the AChBP, galantamine was docked in the complementary (non-α) face of the subunit interface [135]. More specifically, the amine nitrogen of galantamine was located between Trp147 and either Tyr93 or Tyr55, and the polar oxygen atoms of galantamine were situated towards loop C. Photoaffiniy labeling studies indicated that galantamine and physostigmine also binds to α1-Lys125 in *Torpedo* AChRs (or the correspond-

ing α7-Lys123) [136,130]. Additional docking results indicate that α7-Lys123 is located in the inner funnel surface of the receptor [137]. Using the α3β4 AChR model physostigmine was docked close to α3-Lys122 [138]. In more detail, the hydrophobic ring of physostigmine lines β5 from the α3 subunit, the carbamate moiety is located in proximity of α3-Glu48 (at β2) and α3-Lys122 (at β6), and the charged group is stabilized by either water molecules or β4-Glu100 (at β5). Nevertheless, the orientation of physostigmine in the α4β2 AChR is slightly different. Although these results suggest a role of α-Lys in the allosteric binding of physostigmine and galantamine, the mutation α7-Lys123Gly perturbs neither nicotine activation nor galantamine potentiation [133], whereas mutations on α1-Lys125 reduce the direct stimulatory effect elicited by physostigmine, but not by carbachol, on muscle AChRs [139]. It has been postulated that this site, although it is not located within the ion channel, is involved in the noncompetitive inhibition mediated by physostigmine (and galantamine) at high concentrations (>10 μM) [133]. However, this contradicts the evidence suggesting that this site is involved in the agonistic action of physostigmine, and it is more likely that the noncompetitive inhibition is elicited by ion channel blocking [139].

Taking into account all these results, we can generalize that there are several loci for structurally different PAMs and probably distinct mechanisms of positive allosterism. At least three different allosteric sites have been proposed so far: (1) close to the extracellular portion of the transmembrane segments, (2) close, but in the opposite face from the agonist binding sites, and (3) in the linker region.

In animal models, PNU-120596 [123], A-867744 [124], compound 6 [113], SB-206553 [115], and NS-1738 [112], can partially restore auditory gating deficits in mutant mice or those induced by drugs. In this regard, these PAMs can be used to treat the symptoms of schizophrenia. Some of these compounds as well as NS-1738 [112] can also improve cognition at different effective doses. For instance, the minimal effective dose to improve cognition for NS-1738 was 30 mg/kg, producing a brain concentration of ~1 μM, whereas the minimal nootropic dose for PNU-120596 was 0.3 (i.p.) or 0.1 (I.V.) mg/mL with a brain concentration of ~80 nM. This latter brain concentration corresponds to that producing ~25% increase in ACh-induced currents in hippocampal neurons or in α7 AChR expressing cells, suggesting a very high *in vivo* efficacy. Taking into advantage of these nootropic effects, future medications can be developed for the treatment of dementia-related disorders. It was postulated that the retardation of the desensitization kinetics elicited by type II modulators may produce Ca^{2+}-induced toxicity, whereas type I modulators that increase α7 AChR activation without changing the desensitizing properties are less prone to producing toxic effects [113]. However, both type II (e.g., PNU-120596 and A-867744) and type I (e.g., compound 6) modulators increase α7 AChR activation without having toxic effects [140]. In addition, these drugs did not demonstrate adverse effects on animals. These results suggest, in principle, that both types of modulators can potentially be used clinically. It will be very interesting to determine the synergistic effects of specific agonists and PMAs in the treatment of neurological diseases.

In addition to synthetic PAMs, several endogenous molecules or derivatives from endogenous sources have been identified to possess the same basic pharmacological properties as those for synthetic PAMs. Among them, we can name type I PAMs including, the tryptophan metabolite 5-hydroxyindole [141], selected serum albumin derivatives [142], SLURP-1 (secreted mammalian Ly-6/uroquinase plasminogen activated receptor related protein 1) [143] and the structurally related protein Lypd6 [144], as well as other PAMs where the current information does not permit to differentiate them into type I or type II modulators yet, including 17β-estradiol [145,146], and peptides derived from the C-terminus of acetylcholinesterase [147,148] and from the N-terminal region of calcitonin gene related peptide (CGRP) (i.e., CGRP1-6, CGRP1-5, and CGRP1-4) [140,150].

Based on the functional activity of several of these PAMs, there is a small margin for potential clinical uses. For instance, although 5-hydroxyindole produces robust positive allosteric modulation of α7 AChRs enhancing ACh-induced glutamate release in cerebellar slices, its potency is in the millimolar range [141]. Certain bovine serum albumin derivatives are specific for the α7 AChR compared with other ligand-gated ion channels, and restore the inhibition mediated by β-amyloid in ganglion neurons [142]. However, rat and chicken albumins failed to potentiate significantly the respective rat and chicken α7 AChR responses, raising questions on the potential physiological importance of these peptides in human beings. The positive allosteric effect on human α4β2 AChRs mediated by 17β-estradiol is rather weak compared with other PAMs. The binding site for 17β-estradiol corresponding to its positive allosteric effect was found to be lo-

cated at the C-terminal of the $\alpha 4$ subunit, more specifically in a short segment formed by Trp-Leu-Ala-Gly-Met-Ile [145,146]. This location adds another potential site for PAMs.

The CGRP-derived peptides produced mild potentiating effects (~30–40%) on rat chromaffin cell AChRs, probably $\alpha 3\alpha 5\beta 4$ AChRs, but not on muscle AChRs [149]. The PAM binding site for these peptides was postulated to be different to that for physostigmine. Interestingly, the change in just one single amino acid transforms a PAM (e.g., CGRP1-6) into a competitive antagonist (e.g., CGRP1-7) [150]. This deletion disrupts the Cys2-Cys7 bridge responsible for the closed ring structure of CGRP1-7, producing a more flexible peptide that can form a helical configuration. This configuration seems to be important for the PAM effect. More information about the pharmacological and functional effects of these peptides on other important neuronal AChRs are necessary to obtain a clearer picture of their potential for therapy.

Although initially a 14 amino acid peptide derived from the C-terminus of acetylcholinesterase (i.e., AEFHRWSSYMVHWK) was found to behave as a PAM [147,148], new evidence indicates the this peptide and another of 30 amino acids partially inhibit [^{125}I] α-BTx binding in live cells with two different components, suggesting that they interact with two binding sites of different affinities, whereas they decrease choline affinity when cell membranes were used instead [151]. Chronic incubation with these peptides increases the number of $\alpha 7$ AChRs in a MLA-specific manner, and decreases [^{125}I] α-BTx affinity. It seems that these peptides can act as PAMs at low concentrations (i.e., nM concentration regime) and as competitive antagonists in the μM concentration range. Since activation of $\alpha 7$ AChRs reciprocally up-regulates acetylcholinesterase expression, these two proteins may be potentially coordinated by a positive feedback mechanism. The physichochemical properties of these peptides (e.g., low blood-brain barrier permeability) preclude their potential use in pharmacotherapy.

SLURP-1 is a 9 kDa secreted protein that resembles the structure of elapid neurotoxins α- and κ-bungarotoxin, high affinity competitive antagonists of several AChRs. SLURP-1 is a positive allosteric modulator at the human $\alpha 7$ AChR, where it increases ACh potency and the Hill coefficient without alteration of desensitization kinetics, closely resembling type I modulators [152]. Mutation and sequence homology studies suggest that the C-terminal of the toxin, located in the third loop, could be involved in the binding to the $\alpha 7$ AChR [153]. However, we need more direct evidence to support this molecular interaction. In fact, we do not have any evidence indicating where SLURPs bind in the $\alpha 7$ AChR or other AChR subtype.

From the physiological point of view, SLURP-1 and -2 are considered autocrine and paracrine ligands that regulate keratinocyte proliferation, apoptosis, and differentiation. In addition to skin, SLURPs are expressed in other tissues including, uterus, trachea, lung, stomach, esophagus, immune cells, and spinal cord [154–156]. Thus, SLURPs as well as ACh can regulate lymphocyte function *via* AChR-mediated pathways [155]. Additional evidence indicates that SLURP-1 participates in the regulation of gut immune functions and motility [157]. Mutations in the gene encoding SLURP-1 produces the Mal de Meleda, a rare autosomal recessive palmoplantar keratoderma characterized by an inflammatory, malodorous, sharply demarcated hyperkeratosis of the palms and soles [152, 153,143]. Palmoplantar pustulosis, a psoriatic inflammatory skin disease where the expression of subunits $\alpha 3$ and $\alpha 7$ are abnormal [158], produces symptoms very similar to that observed in the Mal de Meleda, and is observed in smokers with higher frequency than in non-smokers [159]. SLURP-2 was also found to be upregulated in psoriatic nonlesional skin [143]. Interestingly, SLURP-1 and -2 are downregulated in cancer cells and they can alleviate tobacco nitrosamines-induced tumor [143]. The expression of SLURPs was also decreased due to silencing the gene coding for the $\alpha 9$ subunit, indicating that AChR signaling upregulates SLURPs. SLURP-2 increases the number of keratinocytes in culture and their resistance to apoptosis, and this effect is inhibited by mecamylamine with higher efficiency than that produced by α-BTx [160]. Although SLURP-2 is structurally similar to SLURP-1, the evidence found so far indicates that SLURP-2 binds to the [3H] epibatidine sites with higher affinity compared to that for the [3H] nicotine sites. These results suggest that SLURP-2 actually behaves as a competitive antagonist with high specificity for $\alpha 3$-containing AChRs. Taking together, the mutual SLURP-AChR modulation may have a role in skin-related diseases and cancer development. However, there is little information of the potential role of SLURPs in the central nervous system. Nevertheless, another member of this family of three-fingered proteins, Lypd6, has neuronal function. In mice overexpressing Lypd6, the nicotine-evoked Ca^{2+} signals in trigeminal ganglia neurons are enhanced compared to that in wild-type and knock-down mice [144]. Pharmacological results revealed

that this process is mediated by heteromeric AChRs. Interstingly, mutant mice showed an augmented cholinergic tone including, higher locomotor arousal, increased prepulse-inhibition, and hypoalgesia, as well as higher sensitivity to the analgesic effects mediated by nicotine. Although this evidence suggests that Lypd6 is another type I PAM with potential neurochemical activity.

Since PAMs exhibit little or no intrinsic activity because their mode of action is to enhance the action of the neurotransmitter, this mode of action provides greater physiological selectivity compared to agonists or antagonists that act upon receptors selectively but indiscriminately with regard to ongoing physiological activity. As explained above, the binding sites for PAMs are located at domains distinct from the orthosteric site. Thus, molecules with superior subunit selectivity, especially between related family members can be potentially identified. In this regard, understanding the molecular mechanisms and structural determinants of PAM actions could lead to the development of drugs for the treatment of a wide variety of neuropsychiatric disorders, including schizophrenia and Alzheimer's disease [161].

7.2. *Negative allosteric modulators*

In addition to PAMs, several synthetic and endogenous negative allosteric modulators (NAMs) have been characterized. NAMs can be pharmacologically differentiated from noncompetitive antagonists (NCAs) on the basis of their binding site locations and mechanisms of action.

Different approaches have helped uncover the diverse molecular mechanisms of noncompetitive inhibition. However, NCA-induced AChR inhibition seems to be very complex since many compounds inhibit receptor function by more than one mechanism. In addition, for many of these compounds both the mechanism of action and their sites of interaction remain unknown. The noncompetitive inhibition of AChR functions likely occurs *via* several different molecular mechanisms:

(1) Open-channel blocking mechanisms: several NCAs may inhibit AChRs by binding within the pore when the receptor is in the open state, thereby physically blocking ion permeation. While some NCAs act only as pure open-channel blockers, others block both open- and closed-channels. A wide number of compounds have been shown to act as channel blockers of AChRs [145,162–164]. Electrophysiological studies have allowed the detailed characterization of the blocking mechanism for many of these compounds such as local and general anesthetics, antidepressants, ephedrine and amphetamine [165,166], alcohols [167], and barbiturates [168].

(2) Allosteric mechanisms, including: (a) binding to and stabilizing a nonconducting conformational state of the AChR (e.g., resting or desensitized state), and/or (b) increase AChR desensitization rate, decreasing subsequently the probability of channel opening. Unfortunately, differentiation between slow open-channel blocking and increasing desensitization mechanisms is technically very difficult [169]. Within this category, we can mention some examples including, antidepressants (e.g., tricyclic antidepressants, bupropion, and serotonin specific reuptake inhibitors), which in addition to act as open channel blockers they may also increase desensitization of muscle AChRs [169–171], quinacrine, which modifies the desensitization kinetics of muscle AChR [172], and the local anesthetic proadifen, which acts on the resting state of the muscle AChR to induce a desensitized state whose kinetics of recovery resemble those of ACh-induced desensitization [173].

Although NAMs also allosterically inhibit AChRs, these compounds bind to sites different to that located within the ion channel as for NCAs. Among synthetic NAMs, we can name the methyllycaconitine derivative COB-3 [174], and UCI-30002 [*N*-(1,2,3,4-tetrahydro-1-naphthyl)-4-nitroaniline] with selectivity for the $\alpha4\beta2$, $\alpha7$, and $\alpha3\beta4$ AChRs over muscle AChRs [175]. A mode of negative allosteric mechanism that has been demonstrated for COB-3 and derivatives includes the increase in AChR desensitization [174].

The binding site for COB-3 determined by homology modeling and docking was considered to be located ~7 Å from the agonist binding site at the $\alpha3/\beta4$ interface on the pore-side of the channel [174]. Figure 5 depicts the binding site location of COB-3 in the $\alpha3\beta4$ AChR. More specifically, the ester group of COB-3 forms a hydrogen bond with Asn108, and the positively charged piperidine moiety of the ligand interacts electrostatically with Asp88. Interestingly, this site location is similar to that determined for the PAM galantamine using molecular docking [137]. This suggests that subtle differences in the binding site can produce a positive or negative allosteric effect on the AChR.

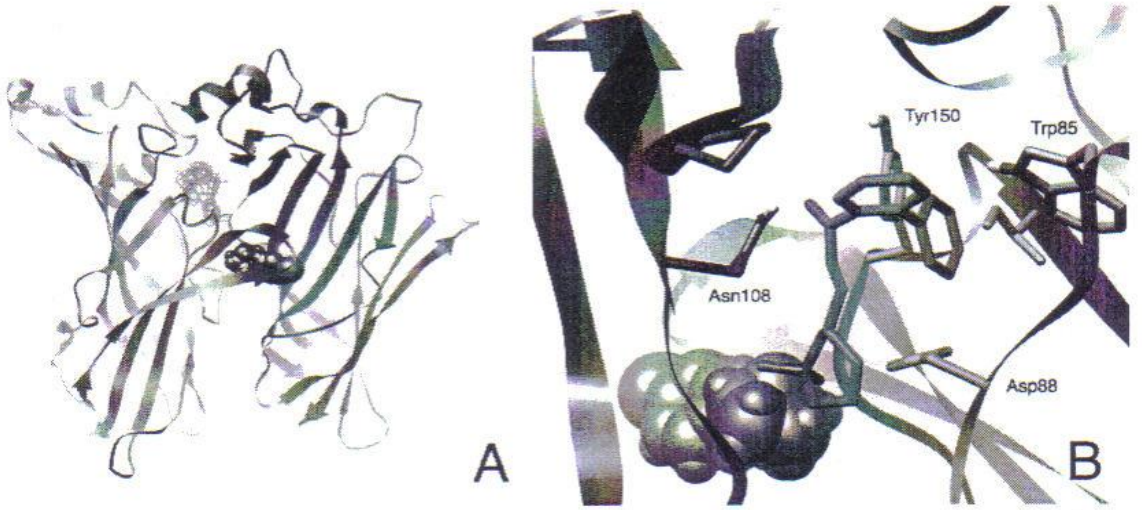

Fig. 5. Molecular docking of COB-3, a synthetic NAM, to the rat $\alpha3\beta4$ AChR model (modified from [174]). (A) Position of the COB-3 (pink) binding site at the α/β interface on the pore-side of the channel. Three of the five subunits are not shown for clarity. (B) Detailed interactions of COB-3 at the α/β interface as viewed from inside the pore. The $\alpha3$ subunit is shown in cyan and the $\beta4$ subunit is in blue. For comparison purposes, epibatidine is shown in gray at the agonist binding site. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/JPB-2010-0017)

From the clinical point of view, UCI-30002 significantly reduces nicotine self-administration in rats [175], suggesting the NAMs can be used for the treatment of drug addiction. Unfortunately, UCI-30002 is a nitro aryl compound that may form reactive oxygen species, leading consequently to cellular toxicity.

In addition to synthetic NAMs, several endogenous molecules have been identified to possess the same basic pharmacological properties as synthetic NAMs. Among them, we can name progesterone and neurosteroids [146,163], fatty acids [176,146], and the prototoxins Lynx-1 and -2 [177–180].

Progesterone and neurosteroids, but not 17β-estradiol (see section 7.1.), inhibit $\alpha4\beta2$ AChRs in a noncompetitive manner. The results obtained using different approaches including, patch-clamp, fluorescence, EPR, photoaffinity labeling, and single mutations have not arrived to a consensus, but include both extracellular and transmembrane domains as binding sites for steroids [146,163]. Fatty acids of different lengths inhibit distinct AChR subtypes by two main mechanisms: by blocking the proper ion channel or by modifying the properties of the lipid-protein interface [146].

Like SLURPs, Lynx proteins resemble the structure of the α- and κ-bungarotoxins, but in this case, they have a glycosylphosphatidylinositol anchor to the lipid membrane. Lynx proteins bind to the most abundant brain AChRs, the $\alpha4\beta2$ and $\alpha7$ subtypes, decreasing the agonist potency, increasing the rate and extent of receptor desensitization, and recovering more slowly from desensitization [177–180]. The effect on desensitization can also change agonist sensitivity. For example, Lynx-2 increased ~20-fold the EC_{50} value for ACh on $\alpha4\beta2$ AChRs [180].

Lynx-2 is expressed in brain areas associated with anxiety disorders including, prefrontal cortex, amygdala, hippocampus, mediodorsal thalamus, dentate gyrus, and specific brainstem nuclei, whereas Lynx-1 is expressed in hippocampus, cortex, and cerebellum. Lynx proteins are also expressed in normal and cancer cells from non-neuronal tissues [181,182]. Prostate stem cell antigen, a Lynx1-like protein, is highly expressed in telencephalon and peripheral ganglia, correlates with the expression of $\alpha7$ AChRs, and prevents programmed cell death of neurons by antagonizing $\alpha7$ AChRs [183]. Although prostate stem cell antigen has similar structure as Lynx proteins, there is no current information regarding the intrinsic mechanism of inhibiton (competitive vs allosteric) elicited by this peptide yet.

Using Lynx-2 null mutant mice, Miwa et al. [179] demonstrated that the lack of this protein increases AChR signaling *in vivo* with the consequent enhancement of the synaptic efficacy. For example, it exacerbates nicotine action on glutamatergic signaling in the prefrontal cortex and induces anxiety-like behaviors in mutant mice [180]. In addition, Lynx-1 is expressed at significantly lower levels in lung tumors than adjacent normal tissue, where it inhibits $\alpha7$ and non-$\alpha7$ AChRs, suggesting that the combination of lower levels

of Lynx-1 and exogenous nicotine may enhance cancer development [182]. The fact that Lynx proteins are well conserved across species, in terms of structure and function [184], and considering the above results, it is very clear that these proteins are endogenous AChR modulators. In particular, Lynx-2 might have clinical importance in the treatment of anxiety-related disorders, whereas Lynx-1 could be a target for lung cancer therapy. An interesting subject would be to determine how positive (e.g., SLURP-1) and negative (e.g., Lynx peptides) endogenous modulators can finally modify AChR function homeostasis in the brain of healthy patients versus patients with neurological problems.

Since NAMs can reduce the gain of the physiological signal without completely inhibiting transmission, they can be therapeutically used to modulate transmission in conditions where the cholinergic system is overstimulated. For example, increased cholinergic stimulation over the norepinephrine system has been hypothesized as the cause of depression [164]. On the other hand, PAMs can increase the gain of the physiological signal without causing an undesired tonic signal. Thus, these compounds can be therapeutically used to boost transmission in conditions where the cholinergic system is less active (e.g., Alzheimer's disease). However, one of the problems in advanced stages of these degenerative diseases is that the release of ACh becomes so low that it decreases the efficacy of these drugs. To advance in the characterization of neuronal circuits, "tethered toxins" were recently developed based on the selectivity of venom peptide toxins and endogenous peptide ligands, such as Lynx-1 [184].

8. Summary

AChRs mediate rapid transmission throughout the nervous system by converting a chemical signal into an electric one. AChRs also present functional roles in non-neuronal tissues. The agonist, after binding to the extracellular region, triggers the opening of the transmembrane ion channel. Structural and functional studies have permitted to highlight the importance of the extracellular–transmembrane interface in the functional communication of both events. Modulation of the gating process opens the door for the development of therapeutic drugs. For example, PAMs or NAMs alone or in combination with specific agonists might be used for the treatment of several diseases involving AChRs including, dementia-, skin-, and immunological-related diseases, drug addiction, and cancer.

Acknowledgements

This work was supported by grants from CONICET, Universidad Nacional del Sur, Agencia Nacional de Promoción Científica y Tecnológica (to C.B.), and from the College of Pharmacy, Midwestern University, USA (to H.R.A.).

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