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REVIEW

Molecular mechanisms of acetylcholine receptor–lipid interactions: from model membranes to human biology

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Abstract Lipids are potent modulators of the *Torpedo* nicotinic acetylcholine receptor. Lipids influence nicotinic receptor function by allosteric mechanisms, stabilizing varying proportions of pre-existing resting, open, desensitized, and uncoupled conformations. Recent structures reveal that lipids could alter function by modulating transmembrane α-helix/α-helix packing, which in turn could alter the conformation of the allosteric interface that links the agonist-binding and transmembrane pore domains—this interface is essential in the coupling of agonist binding to channel gating. We discuss potential mechanisms by which lipids stabilize different conformational states in the context of the hypothesis that lipid–nicotinic receptor interactions modulate receptor function at biological synapses.

Keywords Lipid–protein interactions · Cys-loop receptors · Pentameric ligand-gated ion channels · Uncoupled state · Lipid rafts · Nicotinic receptor trafficking · Phospholipase activity · Synaptic plasticity

List of abbreviations

ABD	Agonist binding domain
nAChR	Nicotinic acetylcholine receptor
ELIC	*Erwinia* ligand-gated ion channel
GLIC	*Gloebacter* ligand-gated ion channel
PA	Phosphatidic acid
TMD	Transmembrane domain

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Introduction

The activities of many membrane proteins are sensitive to the composition of the lipid bilayers in which they are imbedded, but in most cases the underlying mechanisms remain obscure. The nicotinic acetylcholine receptor (nAChR) from the electric fish, *Torpedo*, is one membrane protein whose activity is exquisitely sensitive to its lipid environment. The nAChR is a ligand-gated ion channel that responds to the neurotransmitter, acetylcholine, by transiently opening a cation-selective ion channel across the post-synaptic membrane. In some reconstituted membranes, the nAChR gates open in response to acetylcholine-binding, while in others they do not. This lipid sensitivity is of interest because the *Torpedo* nAChR is the prototypic member of a large family of acetylcholine receptors that includes both muscle-type and neuronal nAChRs. These receptors mediate inter-cellular communication at the neuromuscular junction and neuronal synapses, and are implicated in a variety of physiological processes including voluntary motion, memory, attention, sleep, wakefulness, reward (nicotine), and pain. While the lipid sensitivities of most mammalian nAChRs are unknown, a genetic mutation that alters the lipid–protein interface of the muscle-type nAChR leads to changes in nAChR gating with profound effects on human biology (Shen et al. 2006). The exquisite lipid sensitivity of the *Torpedo* nAChR and the demonstrated effects of altered nAChR–lipid interactions on human biology suggest that subtle variations in the nAChR–lipid micro-environment could play an important role modulating synaptic communication.

In the past decade, a cryo-electron microscopy model of the *Torpedo* nAChR (Unwin 2005), along with X-ray crystal structures of the homologous prokaryotic pentameric ligand-gated ion channels, GLIC and ELIC (Hilf and Dutzler 2008, 2009; Bocquet et al. 2009), a glutamate-activated chloride channel (Hibbs and Gouaux 2011), and the water-soluble homologs of the nAChR extra-membranous agonist-binding domain (Dellisanti et al. 2007; Brejc et al. 2001; Hansen et al. 2005; Celie et al. 2004; Li et al. 2011), have all been published. With these structures, we are now poised to understand functional data on nAChR–lipid interactions in a structural/mechanistic context. In this review, we discuss current understanding of the molecular mechanisms of *Torpedo* nAChR–lipid interactions, highlighting principles applicable to all membrane proteins. These mechanisms are discussed in the context of the hypothesis that lipids modulate nAChR function at biological synapses.

Lipids as allosteric modulators of function

The lipid sensitivity of the *Torpedo* nAChR has been known since the earliest attempts to isolate and reconstitute nAChR function in model membranes. To recover agonist-induced channel flux, the nAChR must be purified in the presence of lipid and then placed in a membrane with an appropriate lipid composition (Heidmann et al. 1980; Epstein and Racker 1978; Criado et al. 1984; Fong and McNamee 1987). Both anionic lipids, such as phosphatidylserine or phosphatidic acid (PA), and neutral lipids, such as cholesterol or diacylglycerol, appear to be important for activity.

Recent work has emphasized the role of lipids as allosteric modulators interacting with and stabilizing pre-existing conformational states. Although it was first suggested that lipids influence the natural equilibrium between activatable resting and non-activatable desensitized conformations (McCarthy and Moore 1992; Baenziger et al. 2000; Hamouda et al. 2006b), the membrane-reconstituted nAChR also adopts a non-activatable "uncoupled" conformation (Fig. 1) (daCosta and Baenziger 2009). Lipids influence the activity of membrane-reconstituted nAChR by stabilizing different proportions of resting versus desensitized and uncoupled states (daCosta et al. 2009).

This allosteric model highlights the potentially complex mechanisms by which lipids influence nAChR function. Different membrane physical and/or chemical properties likely interact preferentially with and thus preferentially stabilize different conformational states. To understand the mechanisms by which lipids influence function, we must elucidate how each membrane physical or chemical property preferentially couples with the resting, open, desensitized, and uncoupled conformations.

The M4 lipid-sensor model

The nAChR in phosphatidylcholine membranes lacking cholesterol and anionic lipids is stabilized predominantly (~95%) in an uncoupled conformation that binds agonist with a resting-state-like affinity, but does not undergo agonist-induced allosteric transitions (daCosta and Baenziger 2009). Structural data shed light on the mechanisms by which lipids influence the coupling between binding and gating. The *Torpedo* nAChR is composed of five subunits arranged pseudo-symmetrically around a central axis that functions as the ion channel (Fig. 2). Each subunit consists of a large extracellular agonist-binding domain (ABD) formed mainly from 10 β-strands (β1–β10), a transmembrane domain (TMD) consisting of four transmembrane α-helices (M1–M4), and a large cytoplasmic domain linking transmembrane α-helices M3 and M4 (only one α-helix has so far been resolved in this domain; Unwin 2005). The receptor's agonist-binding site and ion channel gate reside in the extracellular and transmembrane domains, respectively. These domains meet at an interface located close to the bilayer surface. Contact between the two domains is mediated by the covalent link between the C-terminus of β10 in the ABD and the N-terminus of M1 in the TMD, as well as by non-covalent connections between the β1–β2 and β6–β7 loops (in

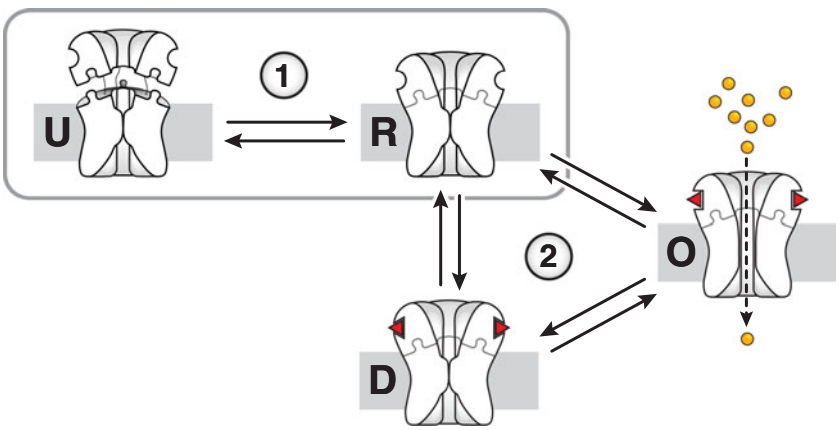

Fig. 1 Lipid composition influences the activatable pool of receptors by controlling the proportion of nAChRs in uncoupled (*U*) versus coupled/resting (*R*) conformations (Scheme *1*, *boxed*). Lipids also influence the equilibrium between R and D (desensitized) states, and may influence the channel open (*O*) conformation (Scheme *2*)

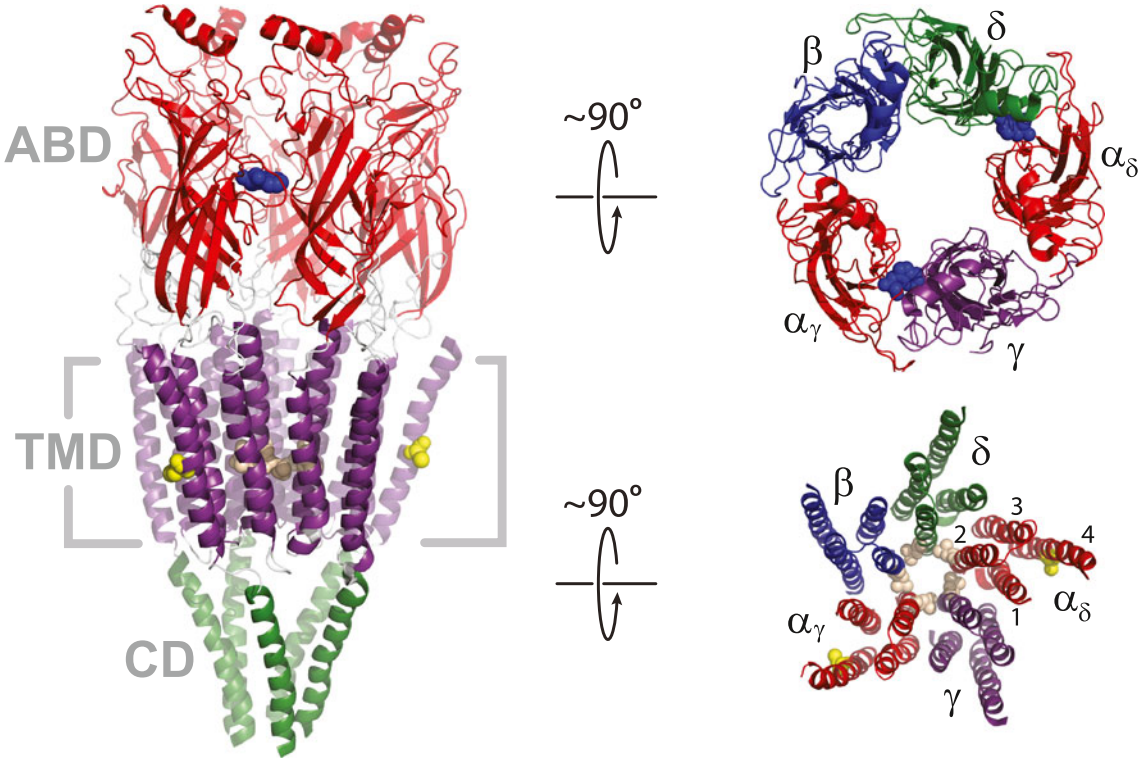

Fig. 2 Structure of the nicotinic acetylcholine receptor (nAChR) from *Torpedo* (PDB: 2BG9). A side view of the nAChR pentamer from the membrane plane (*left*) shows the extra-cellular agonist-binding (*ABD*, *red*), transmembrane pore (*TMD*, *purple*) and cytoplasmic (*CD*, *green*) domains. Side chains of residues forming part of the agonist-binding site (αTrp149) and the channel gate (αLeu251, as well as analogous β-, γ- and δ-subunit leucines) are shown in *blue* and *tan*, respectively. A Cys residue at the lipid-protein interface of α-TM4, whose mutation to Trp in the muscle-type nAChR leads to biological consequences (Shen et al. 2006), is highlighted in *yellow*. Views from the synaptic cleft are shown on the *right* for the agonist-binding (*top*) and transmembrane pore domains (*bottom*) with color coding to highlight the different subunits (α, *red*; β, *blue*; γ, *purple*, and δ, *green*)

eukaryotic nAChRs, the latter is referred to as the Cys-loop) of the ABD and the M2–M3 linker of the TMD (Fig. 3a). *If lipids influence the coupling between binding and gating, it is reasonable to hypothesize that they do so by affecting this interface.*

Of the four transmembrane α-helices in each subunit, *M4 is highly exposed to lipids and therefore in a position to translate bilayer physico-chemical properties into altered nAChR function* (Xu et al. 2005; Blanton and Cohen 1992, 1994). Interestingly, post-αM4 (Q435) extends beyond the lipid bilayer to interact with a conserved residue (F137) in the Cys-loop, which is located at the interface between the ABD and TMD (Fig. 3a). Given that the Cys-loop plays a central role at the interface between the two domains (Lummis et al. 2005; Bouzat et al. 2004), specific interactions between post-M4 and the Cys-loop may be essential for coupling agonist-binding to channel gating. Lipids could influence nAChR function by modulating post-M4 interactions with the Cys-loop. *Altered interactions between post-M4 and the Cys-loop in phosphatidylcholine membranes may lead to weakened interactions between, and thus partial dissociation of the ABD from the TMD, and thus the formation of an uncoupled state* (Fig. 4a) (daCosta and Baenziger 2009). Partial dissociation of the two domains leading to increased solvent accessibility of loops at the coupling interface would account for the increase in polypeptide backbone hydrogen exchange kinetics in the uncoupled versus the resting and desensitized conformations (Methot et al. 1995; daCosta and Baenziger 2009). Note that a similar mechanism of lipid activation involving domain coupling and uncoupling has been elucidated for the PIP2-activated potassium channel (Hansen et al. 2011).

The M4 lipid-sensor model proposes that lipids influence nAChR function by modulating interactions between M4 and the M1+M3 transmembrane α-helices (Fig. 4a), which in turn influences post-M4/Cys-loop interactions leading to altered coupling of binding and gating. A central feature of this model is that the conformation of M4, particularly the C-terminus, is critical for nAChR function, a postulate that is supported by considerable biochemical data. Mutations of lipid-exposed residues on M4 alter channel gating showing that there is a direct role for M4 in nAChR function. ((Li et al. 1992; Lee et al. 1994; Lasalde et al. 1996; Bouzat et al. 1998; Tamamizu et al. 2000; Mitra et al. 2004). As noted, mutation of a lipid facing residue on M4 (αC418W) in the muscle type receptor leads to pathological consequences, demonstrating that M4-lipid interactions are important in

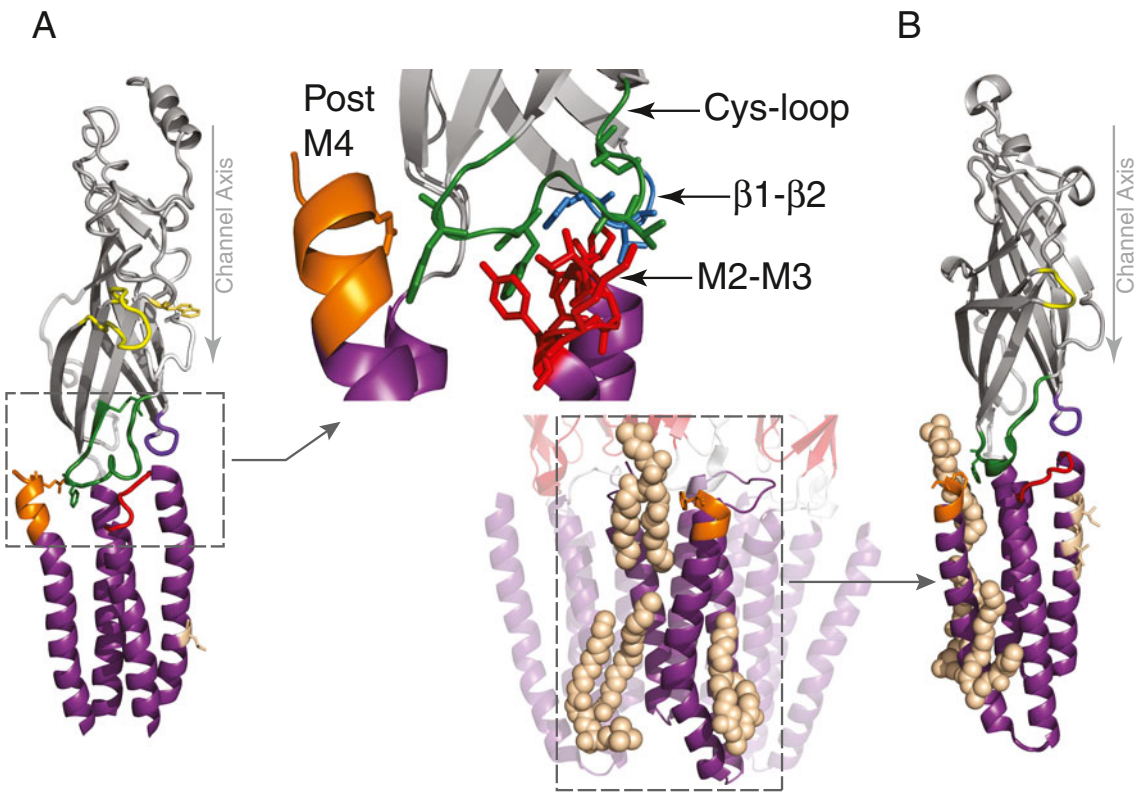

Fig. 3 **a** Structure of the *Torpedo* nAChR α-subunit (*left*) showing the ABD and TMD, highlighting structures at the interface between the ABD and TMD (*top right*). Post-M4, *orange*; β6–β7 (Cys) loop, *green*; M2–M3 linker, *red*; β1–β2 loop, *blue*. The C-loop of the agonist binding site is in *yellow*. The orientation of the close-up view on the *right* is slightly rotated relative to the view on the left to highlight the close interactions between the ABD (β6–β7 and β1–β2 loops) and the TMD (M2-M3 linker), **b** Structure of a single subunit of the prokaryotic homolog, GLIC, showing bound lipids (*tan*, space filling), one of which links post M4 with the β6–β7 loop (*right*). A close-up view of the TMD (*bottom left*, view rotated ~90° about channel axis relative to the single subunit on the *right*) highlights the three lipid molecules (*tan*, space filling) bound to a single subunit at the interface between M4 and M1+M3. Only the three front subunits are shown. In both (**a**) and (**b**), hydrophobic residues thought to form the channel gate are shown as *tan sticks*

humans (Shen et al. 2006). The non-competitive antagonist, promegestone likely interacts at the interface between M4 and M1+M3 (Blanton et al. 1999). Interestingly, M4 is the least conserved among the nAChR transmembrane α-helices. While the hydrophobic transmembrane portion of M4 can be replaced with transmembrane α-helices from other proteins with minimal functional consequences, the extended length of M4 appears to be critical. The C-terminal of M4 (referred to as post-M4) is essential for trafficking of both *Torpedo* nAChR and an α7nAChR-5HT$_{3A}$ chimera to the cell surface to form functional ion channels (Tobimatsu et al. 1987; Pons et al. 2004). Post-M4 is also the site for neurosteroid-induced potentiation of neuronal α4β2 nAChRs (Paradiso et al. 2001). The essential role for the C-terminus of M4 in nAChR function is consistent with the M4 lipid-sensor model.

Another feature of the M4 lipid-sensor model is that the ABD adopts an independent structure that can partially dissociate from the TMD to form the uncoupled conformation. The structural independence of the ABD is supported by the existence of stable pentameric water-soluble acetylcholine binding proteins, which are homologous to the ABD of the nAChR (Brejc et al. 2001; Hansen et al. 2005). Folded, stable, and water-soluble ABDs of the nAChR α1- and α7-subunits, as well as the prokaryotic homolog, GLIC, can be expressed independently of their TMDs (Dellisanti et al. 2007; Nury et al. 2010; Li et al. 2011).

The M4 lipid-sensor model of uncoupling provides a framework for understanding general mechanisms of nAChR–lipid interactions. Lipid-dependent changes in the packing of M4 against M1+M3 could more broadly influence the TMD structure leading to functional consequences. For example, altered M4–(M1+M3) interactions could influence the orientation of M3 relative to the pore lining M2 α-helix and thus the conformation of the M2–M3 linker. Given that the M2–M3 linker, which interacts with the β6–β7 and β1–β2 loops of the ABD, is involved in gating, lipid-dependent change in M2–M3 conformation should influence nAChR function. In general, the conformation of structures at the coupling interface (i.e. the β6–β7 and β1–β2 loop of the ABD, and the M2–M3 linker of the TMD) must differ between the resting, open, desensitized, and uncoupled states. Lipid-dependent changes in TMD α-helix packing that favor

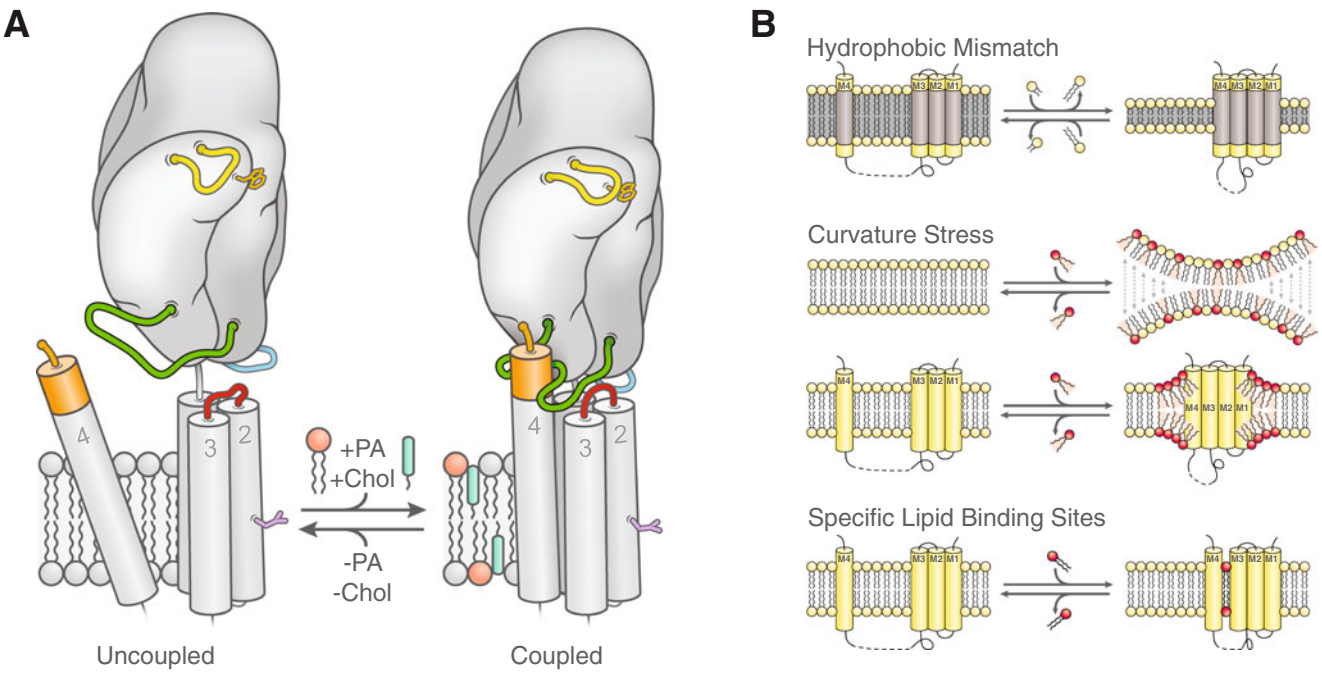

Fig. 4 **a** Schematic depicting the proposed role of M4 as a lipid-sensor modulating the coupling of agonist-binding to channel-gating in the *Torpedo* nAChR. Lipid-dependent structural rearrangements of the transmembrane α-helices leads to a loss of interactions between the C-terminal end of M4 and the β6–β7 (Cys) loop. **b** Possible mechanisms by which lipids allosterically influence packing of M4 relative to M1+M3. Hydrophobic mismatch (*top*), curvature stress (*middle*), and specific lipid binding sites (*bottom*) could all influence how M4 interacts with the remaining transmembrane α-helices

the resting, open, desensitized, or uncoupled conformations of structures at the coupling interface will influence the ability of the nAChR to respond to agonist.

Note that the ability of membrane physical properties, such as hydrophobic mismatch, curvature stress, etc. (Fig. 4b), to modulate transmembrane α-helix–α-helix interactions is well documented (Lee 2004). In fact, hydrophobic thickness influences interactions between membrane-reconstituted γM4 α-helices of the nAChR (Antollini et al. 2005). The M4 lipid-sensor model supports the general hypothesis that lipids influence membrane protein function by altering transmembrane α-helix/α-helix packing.

Sites of lipid-nAChR interactions

Lipids can allosterically influence protein conformational equilibria by (1) binding directly to conformationally sensitive protein binding sites, by (2) altering bulk membrane physical properties (which indirectly couple with different conformational states), or by (3) a combination of both (Lee 2004). With regards to the nAChR, M4 is the most lipid-exposed transmembrane α-helix and is thus a likely candidate for mediating both specific and non-specific nAChR-lipid interactions.

M4 is a potential site for the effects of cholesterol and anionic lipids. Both lipids show a particular affinity for the lipid annulus that surrounds the nAChR transmembrane domain (Ellena et al. 1983; Marsh and Barrantes 1978). At any moment in time, cholesterol and anionic lipids in this annulus are "bound" to the surface of the nAChR, and thus interact extensively with M4. A photoactivatable cholesterol analog labels sites on the lipid-exposed surface of the nAChR, including predominantly residues on M4 (Hamouda et al. 2006a). Another cholesterol analog covalently linked to phosphatidylcholine, which presumably resides within the bulk membrane environment, is as effective as cholesterol in supporting nAChR function, consistent with a lipid-exposed surface-site for cholesterol action (Addona et al. 1998). In fact, the entire lipid-exposed surface of the nAChR may serve as an "allosteric site" that is sensitive to the mechanical properties of the membrane.

In addition to annular sites of action, "non-annular" cholesterol binding sites located between transmembrane α-helices have been proposed based on fluorescence quenching studies with brominated lipids (Jones and McNamee 1988). Simulations suggest that by binding to such cavities cholesterol could stabilize the nAChR transmembrane domain and facilitate interactions with the ABD (Brannigan et al. 2008).

How do cholesterol and anionic lipids allosterically influence nAChR function in the context of the M4 lipid sensor model? Cholesterol, via effects on bulk membrane properties, could drive interactions between M4 and the transmembrane α-helices, M1+M3, thereby positioning post-M4 at the coupling interface to interact with the Cys-loop (Fig. 4b). In the absence of appropriate membrane

properties (i.e. in membranes lacking cholesterol), post-M4 may dissociate from the Cys-loop leading to formation of the uncoupled state. In another scenario, cholesterol could interact with post-M4 in a manner similar to neurosteroids, which bind to post-M4 and potentiate α4β2 nAChR activity (Paradiso et al. 2001). Alternatively, non-annular cholesterol sites could be important for stabilizing the structure of the entire TMD, thus modulating the position of post-M4 relative to the Cys-loop and/or altering the conformation of the M2-M3 linker.

Anionic lipids could influence nAChR activity via similar mechanisms. The net pattern of electrostatic residues at the interface between the agonist-binding and transmembrane domains plays an important role in gating (Xiu et al. 2005). The structure of this interface is thought to be different between resting, desensitized, and uncoupled conformations, which presumably leads to alterations in charge distribution. Changes in this charge distribution could enhance interactions with anionic lipids in a conformationally-specific manner. Anionic lipids could also interact directly with post-M4 leading to attractive (R429) or repulsive (E432, E426) forces that modulate the position of post-M4 in relation to the Cys-loop (Fig. 4b).

Allosteric regulation of multimeric proteins tends to occur at the interfaces between subunits. For example, the agonist-binding sites of the nAChR are located at the α–δ and α–γ subunit interfaces of the ABD. In terms of lipid-dependent modulation of nAChR function, the above discussion suggests that the lipid allosteric sites are at the interfaces between transmembrane α-helices, particularly between M4 and the M1+M3 α-helices. In agreement, one of the two crystal structures of GLIC exhibits three lipids bound directly to each M4 α-helix (Bocquet et al. 2009). One of the three lipids is located near post-M4 where it bridges interactions between post-M4 (F315) and the β6–β7 loop (F121), the loop analogous to the Cys-loop in eukaryotic nAChRs (Fig. 3b). The lipid-bridged F121 in the β6–β7 loop is analogous to the conserved αF137 in the Cys-loop of the *Torpedo* nAChR, which in the latter structure directly contacts post-M4. The observed lipid bound to both the β6–β7 loop and post-M4 highlight this region as a potential site for lipid-dependent allosteric modulation of pentameric ligand-gated ion channels.

Role of nAChR-lipid interactions in biology?

The identification of a lipid-dependent uncoupled conformation is of potential biological interest. Several studies with heterologously expressed neuronal nAChRs have suggested the existence of a functional equivalent of the lipid-dependent uncoupled state. For example, less than 10% of the agonist-binding neuronal $\alpha_4\beta_2$ nAChRs expressed in HEK cells are agonist-activatable (Li and Steinbach 2010). Other nAChR subtypes, including those with α6 subunits, exist in oligomeric structures on the surfaces of heterologous expression systems, but do not flux cations in response to acetylcholine binding (Drenan et al. 2008; Broadbent et al. 2006). In fact, the β3 subunit is important for the biogenesis of α6α4β2β3 nAChRs, yet β3 suppresses acetylcholine-evoked currents in this and other receptor subtypes (Broadbent et al. 2006). Are these effects due to the expression system, or could they underlie biologically relevant phenomena? Could the presence of the β3 subunit favor an uncoupled state? Neurosteroids potentiate acetylcholine-induced responses and apparently awaken "non-activatable" nAChRs (Paradiso et al. 2001). Could this population of "non-activatable" receptors be uncoupled? Chronic nicotine exposure upregulates nAChR trafficking to the cell surface, but there appears to be a role for inactive nAChRs in this process (Nashmi et al. 2007). Chronic nicotine exposure may lead to the activation of previously assembled but inactive nAChRs (Vallejo et al. 2005). Could the activation of previously non-activatable receptors reflect a transition from uncoupled to coupled states? While in some cases the lack of apparent agonist-induced activity is likely due to other phenomena, the above observations collectively raise the possibility that uncoupling could be more than a membrane-reconstitution-related phenomenon.

One mechanism by which lipids could influence the proportion of coupled versus uncoupled nAChRs in a biological context is via partitioning between lipid raft and non-raft environments. Both muscle-type and neuronal nAChRs require lipid rafts for trafficking to the plasma membrane, with the raft-forming lipids cholesterol, sphingolipids, and ceramides being important (Baier and Barrantes 2007; Gallegos et al. 2008; Baier et al. 2010; Bruses et al. 2001; Marchand et al. 2002). Both the clustering of nAChRs on the plasma membrane and the internalization of cytoplasmic membrane-located nAChRs are raft dependent. Lowering cholesterol levels to disrupt lipid rafts leads to altered nAChR clustering and mobility on cell surfaces, rapid internalization, and ultimately enhanced function of the surface-retained nAChRs (Borroni et al. 2007; Borroni and Barrantes 2011). Could altered nAChR–raft associations that lead to modified nAChR–lipid interactions have an impact on synaptic activity?

Although the molecular mechanisms underlying nAChR–lipid raft associations remain obscure, it has been shown that incorporation of the nAChR into model membranes containing anionic lipids, particularly PA, leads to a change in the packing properties of the surrounding bilayer (daCosta et al. 2002). The nAChR concentrates cations, including protons, at the bilayer surface facilitating the ordering of nearby anionic lipids (Sturgeon and Baenziger 2010; daCosta et al. 2004). There appears to be favorable interactions between the nAChR and ordered lipids, such as those found in lipid rafts.

Another intriguing observation is that affinity-purified and detergent-solubilized *Torpedo* nAChR exhibits a PA-specific

phospholipase C activity. The hydrolysis product of PA, diacylglycerol, is a potent nAChR activator (Labriola et al. 2010). An nAChR-associated phospholipase activity would allow the nAChR itself to alter its own lipid micro-environment, which could have two effects. In the context of a lipid raft-associated receptor, nAChR-induced changes in the lipid micro-environment could lead to relatively long-term changes in nAChR activity and thus synaptic strength. Secondly, PA hydrolysis would reduce the number of negatively charged PA headgroups in the nAChR micro-environment. Given the nAChR's ability to concentrate cations and interact preferentially with negative lipids, particularly PA, a loss of PA may create less favorable interactions between the nAChR and its surrounding lipids, which may ultimately lead to nAChR trafficking from a less favorable to a more favorable lipid micro-environment. While the existence of this phospholipase activity in native membranes and its relation to nAChR activity in vivo both remain to be defined, these speculative hypotheses highlight the fact that we are just beginning to understand the mechanisms underlying nAChR–lipid interactions and the potential importance of these interactions in vivo.

Conclusions

Both the exquisite lipid sensitivity of the *Torpedo* nAChR and the demonstrated effects of altered nAChR-lipid interactions on human biology suggest that even subtle variations in the nAChR lipid micro-environment in vivo could play an important role modulating synaptic communication. With recent structural data, we are now formulating testable hypotheses describing the mechanisms of nAChR–lipid interactions. We are just beginning to understand the nature of the lipid micro-domains that surround the nAChR, as well as the influence of these micro-domains on nAChR trafficking . Ultimately, these micro-domains may modulate nAChR function under a variety of biologically relevant conditions. Further work will provide a better understanding of the roles of lipid–receptor interactions in the function of other members of the nAChR family, and possibly even other members of the broader Cys-loop receptor super-family of ligand-gated ion channels.

Conflict of interest statement We declare no conflict of interest

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