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Review

Nicotinic acetylcholine receptor–lipid interactions: Mechanistic insight and biological function☆

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ABSTRACT

Membrane lipids are potent modulators of the nicotinic acetylcholine receptor (nAChR) from *Torpedo*. Lipids influence nAChR function by both conformational selection and kinetic mechanisms, stabilizing varying proportions of activatable versus non-activatable conformations, as well as influencing the transitions between these conformational states. Of note, some membranes stabilize an electrically silent uncoupled conformation that binds agonist but does not undergo agonist-induced conformational transitions. The uncoupled nAChR, however, does transition to activatable conformations in relatively thick lipid bilayers, such as those found in lipid rafts. In this review, we discuss current understanding of lipid–nAChR interactions in the context of increasingly available high resolution structural and functional data. These data highlight different sites of lipid action, including the lipid-exposed M4 transmembrane α-helix. Current evidence suggests that lipids alter nAChR function by modulating interactions between M4 and the adjacent transmembrane α-helices, M1 and M3. These interactions have also been implicated in both the folding and trafficking of nAChRs to the cell surface. We review current mechanistic understanding of lipid–nAChR interactions, and highlight potential biological roles for lipid–nAChR interactions in modulating the synaptic response. This article is part of a Special Issue entitled: Lipid–protein interactions.

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1. Introduction

The nicotinic acetylcholine receptor (nAChR) from *Torpedo* is the prototypic member of a broad family of pentameric ligand-gated ion channels (pLGICs) that are found in pre-, post-, and non-synaptic

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membranes of the central and peripheral nervous systems. These neurotransmitter receptors perform important roles in both synaptic communication and information processing, have been implicated in a variety of neurological processes and diseases, and are targets of numerous pharmaceuticals [1–5].

Early attempts to isolate and reconstitute the *Torpedo* nAChR in model membranes first highlighted the functional sensitivity of the nAChR to lipids. To retain agonist-induced channel flux, the nAChR must be solubilized and purified in the presence of lipid, and then placed in a membrane with an appropriate lipid composition [6–9]. The exquisite lipid sensitivity of the *Torpedo* nAChR is of interest because even subtle changes in human nAChR activity have profound effects on human biology [10,11]. In addition, the lipid environment of the human nAChR changes as the nAChR traffics from intracellular membranes to its pre-, post-, or non-synaptic locations in the plasma membrane, as well as during both aging and neurodegenerative disease [12–14]. These changes in the nAChR lipid micro-environment during both normal and abnormal brain functions likely influence cholinergic biology to alter synaptic communication.

The relative abundance of the nAChR in the electric fish *Torpedo* has made it the ideal model for studies of ligand-gated ion channel structure/function relationships. Biophysical and biochemical studies over the past three decades have led to an extensive literature on lipid–nAChR interactions, which has been summarized in several comprehensive reviews [15–19]. In the past decade, a 4 Å resolution cryo-electron microscopy model of the *Torpedo* nAChR (Fig. 1) [20], as well as X-ray crystal structures of homologous pLGICs [21–29] and water-soluble homologs of the nAChR extracellular agonist-binding domain [30,31], and NMR structures of nAChR transmembrane domains [32,33] have provided an increasingly detailed picture of both nAChR structure and the nature of ligand-induced conformational change. With this structural data in hand, we are now in an unprecedented position to probe the mechanisms underlying lipid–nAChR interactions at a structural/mechanistic level.

This review focuses on our current understanding of lipid–nAChR interactions in the context of these recently solved pLGIC structures. We review the structural properties of the *Torpedo* nAChR, the *Torpedo* nAChR's lipid requirements, and current models of lipid–nAChR interactions. We also highlight potential roles for lipids in the folding, cell-surface trafficking, and domain localization of the nAChRs in mammalian tissues.

2. nAChR structure

There are seventeen homologous nAChR subunits in mammals (α1–α10, β1–β4, γ, ε, and δ) that combine to form a variety of either homo-pentameric or hetero-pentameric structures [34]. The *Torpedo* nAChR is most similar to the muscle-type nAChR found at the neuromuscular junction, being formed from four distinct subunit types organized in an $(\alpha1)_2\beta1\gamma\delta$ pentamer. In the adult muscle, the fetal γ-subunit of the $(\alpha1)_2\beta1\gamma\delta$ pentamer is replaced by the ε-subunit. nAChRs are also an important part of the central nervous system, with both heteromeric α4β2 and homomeric α7 nAChRs abundant throughout the human brain, and less abundant combinations, such as α3β4, α3β2, and α6β2β3 nAChRs, targeted to specific brain regions [1,2].

The five subunits of the *Torpedo* nAChR pentamer are arranged pseudo-symmetrically around a central axis that functions as an ion channel (Fig. 1A) [20]. Each subunit contributes three distinct domains, a roughly 200-residue long N-terminal extracellular domain (ECD) responsible for agonist binding, a roughly 150-residue long transmembrane domain (TMD) responsible for ion channel conductance, and a cytoplasmic domain of variable length that links to the cytoskeleton. The ECD of each subunit consists of 10 β-strands (β1–β10) forming two β-sheets that fold into a classic β-sandwich. The α-subunit contributes the principal face of each agonist site [35], with the complementary face formed by the adjacent γ- and δ-subunits [36,37]. The TMD of each subunit contributes four transmembrane α-helices (M1–M4) organized in a four-helix bundle. The five M2 α-helices line the channel pore, while M1 and M3 from each subunit form a ring of α-helices that shield M2 from the membrane [20,38,39]. The M4 α-helices are located on the periphery of each subunit where they are exposed to the lipid bilayer. The cytoplasmic domain of each subunit contributes an α-helix that extends away from the membrane surface. The cytoplasmic domain is located in a long loop positioned between transmembrane α-helices M3 and M4.

Communication between the ECD and TMD in each subunit is mediated primarily by the covalent link between the C-terminus of β10 in the ECD and the N-terminus of M1 in the TMD, as well as by non-covalent connections between the β1/β2 and β6/β7 loops (the latter is referred to as the Cys-loop in eukaryotic pLGICs) of the ECD and the M2–M3 linker of the TMD (Fig. 2) [40,41]. Although the detailed structural changes that occur when agonist binding couples to channel gating remain controversial, it is thought that concerted movements of the two β-sheets in the ECD lead to changes in structure of both the

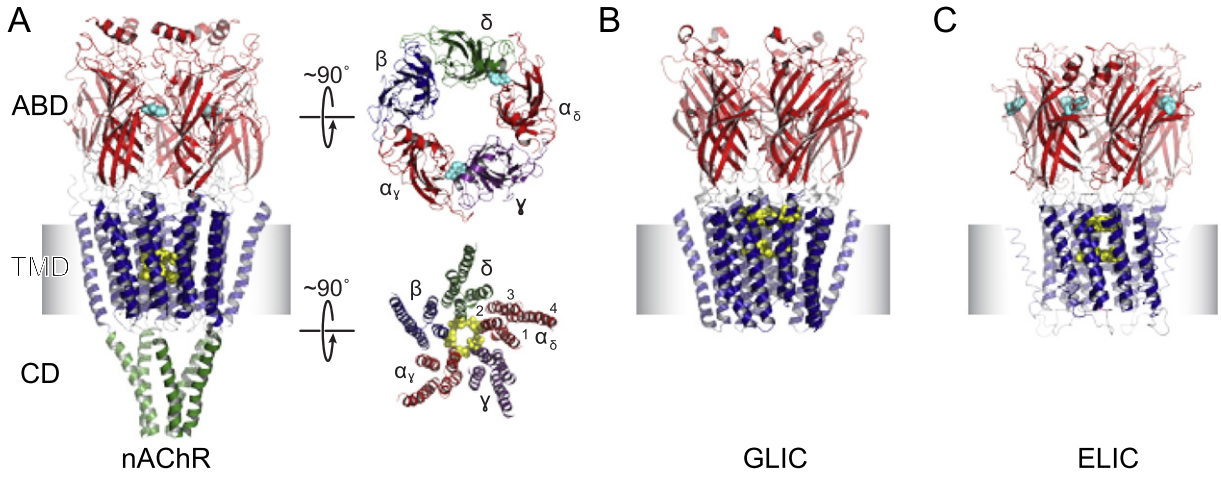

Fig. 1. Structure of the A) nAChR, B) GLIC, and C) ELIC. A) A side view of the *Torpedo* nAChR structure (PDB ID: 2BG9) is shown on the left with the agonist-binding domain (ABD) in red, the transmembrane domain (TMD) in blue, and the cytoplasmic domain (CD) in green. Residues contributing to the proposed channel gate (αLeu251, αVal255 and homologous residues in the β, γ, and δ subunits) are shown as yellow spheres. The agonist binding site αTrp149 is shown as cyan spheres. Top down views of the ECD and TMD are shown on the right. B) Side view of the GLIC structure (PDB ID: 3EAM) with coloring as in A). Residues contributing to the proposed channel gate (I233, I240, L241) are shown as yellow spheres. C) Side view of the ELIC structure (PDB ID: 2VL0) with coloring as in A). Residues contributing to the proposed channel gate (L239 and F246) are shown as yellow spheres. The agonist binding site F187 is shown as cyan spheres.

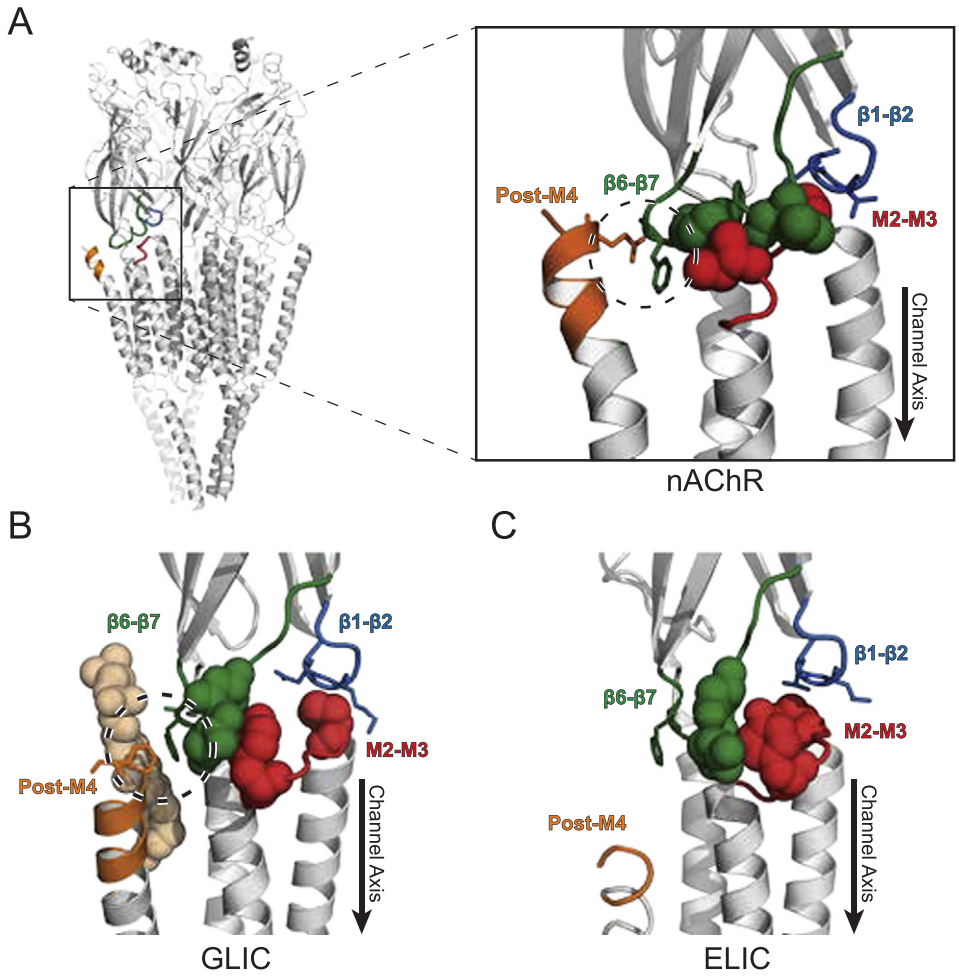

Fig. 2. Conformations of M4 at the ECD/TMD interface of the nAChR, GLIC and ELIC. Structures are for the A) nAChR (PDB ID: 2BG9), B) GLIC (PDB ID: 3EAM), and C) ELIC (PDB ID: 2VL0). In each case, post-M4, the β1–β2 loop, the β6–β7 loop (Cys-loop), and the M2–M3 linker are highlighted in orange, light blue, green, and red, respectively. For the nAChR, direct interactions between post-M4 (Gln435) and the Cys-loop (Phe137) are circled. For GLIC, a lipid molecule (beige space filling model) bridges interactions between post-M4 (Phe315) and the β6–β7 loop (Phe121).

β1–β2 and β6–β7 loops, which are then proposed to translate into movement of the pore-lining M2 α-helices via direct interactions with the M2–M3 linker [22–24,41–44]. In the closed resting conformation, the ion channel is occluded by hydrophobic residues located near the center of the bilayer that provide a barrier to the flow of a solvated ion (Fig. 1) [45]. Upon gating to the open state, a proposed twisting and tilting of the M2 α-helices widen the pore by ~3 Å, allowing the flow of solvated ions down their electrochemical gradient. Prolonged exposure to agonist leads to the formation of one or more poorly-defined channel inactive desensitized conformations, which are characterized by a relatively high affinity for agonist [46].

3. Lipid-dependent modulation of nAChR function

3.1. The nAChR's lipid requirements

Studies in the 1980s established that the ability of the *Torpedo* nAChR to both undergo agonist-induced state transitions and flux cations across the membrane depends on the nAChR's surrounding lipid environment [8,9,47–49]. Although a variety of lipids and membrane properties were found to influence the nAChR, both cholesterol and anionic lipids quickly emerged as being critical for channel function. The role of cholesterol in promoting nAChR function is not surprising given that cholesterol is a major component of native *Torpedo* membranes (~35 mol%). Anionic lipids are also common (~15 mol%), with phosphatidylserine (PS) being the dominant anionic lipid (~10 mol%) and phosphatidylinositol (PI), phosphatidic acid (PA), and cardiolipin found in lesser amounts [50]. The lipid requirements of the nAChR in reconstituted membranes thus reflect the natural lipid composition of *Torpedo* membranes.

Many of the original assays designed to survey lipid requirements, however, simply probed whether the inclusion of a particular lipid in a PC membrane stabilized a measurable pool of functional nAChRs. As these assays could not accurately quantify the relative proportions of activatable versus non-activatable conformations, membranes were typically characterized as either supporting channel function or stabilizing a non-functional nAChR. For example, PC membranes containing cholesterol and an anionic lipid at a 3:1:1 molar ratio were found to stabilize a large pool of agonist-responsive nAChRs, while 3:1 molar ratios of PC/cholesterol, PC/PS, PC/PA, or PC alone did not [8]. Subsequent studies revealed that lipids influence nAChR function primarily via a conformational selection mechanism, modulating the equilibria between functional and non-functional conformations (see Section 3.3) [51,52]. Increasing levels of either cholesterol *or* PA alone in a PC membrane were found to stabilize an increasing proportion of agonist-activatable resting state nAChRs, with even low dosages of either lipid having measurable effects [52]. Even though the levels of cholesterol or PA in a PC membrane that are required to *optimally* support nAChR

channel function are beyond those found in native membranes, these findings suggest that neither cholesterol nor anionic lipids are absolutely essential for the nAChR to undergo agonist-induced state transitions.

The lack of a specific requirement for cholesterol is supported further by the observation that several cholesterol analogs and other neutral lipids, such as diacylglycerol, effectively replace cholesterol in PC/anionic lipid bilayers to support channel function [53,54]. On the other hand, while anionic lipids are broadly effective at promoting agonist-induced flux in the presence of PC and cholesterol [53], distinct efficacies emerge in the absence of cholesterol. Binary mixtures of PC and PA stabilize a large pool of agonist-responsive nAChRs, while mixtures of PC and PS stabilize predominantly non-activatable conformations [55,56]. There is clearly more to the effects of anionic lipids on nAChR function than the net anionic lipid charge.

The observed complexities in lipid–nAChR interactions may stem from the fact that lipids exert their effects on channel function via both specific and non-specific mechanisms. Cholesterol may bind to sites within the nAChR TMD (see Section 3.2). It may also influence nAChR function by increasing the order of PC bilayers, thus influencing other bulk membrane properties, such as bilayer thickness [15,57]. Bulk membrane effects would explain the ability of cholesterol analogs and other neutral lipids to substitute for cholesterol in promoting channel function, as well as why a cholesterol analog covalently linked to PC, which presumably resides within the bulk membrane environment, is as effective as free cholesterol in supporting nAChR function [58].

The distinct efficacies of PA and PS in supporting nAChR function in the absence of cholesterol can also be rationalized in terms of both specific and non-specific lipid–nAChR interactions. The anionic lipid charge may be required to promote effective electrostatic interactions within the nAChR (see, for example [59]), but such interactions may only be effective when the nAChR is immersed in an appropriate bulk membrane physical environment — such as that created in the presence of cholesterol. High levels of the anionic lipid PA in a PC membrane may be uniquely effective at stabilizing a functional nAChR because PA has both an anionic charge and a small head group. Given the small head group, incorporation of PA into a PC bilayer leads to an ordering of the membrane and an increase in its gel-to-liquid phase transitions [55], which may mimic the ordering effects of cholesterol. In contrast, high levels of PS, which has a larger head group, in a PC membrane, have little effect on reconstituted membrane physical properties [56].

The role of bulk membrane physical properties in modulating nAChR function has been debated in numerous publications, with some studies concluding that bulk membrane effects are the key modulators of channel function, and others concluding that lipid chemistry, and thus presumably the binding of lipids to specific sites on the nAChR, is more important [8,56,60]. Part of the controversy likely stems from the fact that membrane physical properties have typically been assessed using bilayer gel-to-liquid crystal phase transition temperatures and/or either fluorescent or spin-labeled probes, the latter providing a numerical measure of membrane order. These bulk membrane parameters may not be sufficiently robust to capture the complex physical environment of a reconstituted nAChR membrane. It is also difficult to probe the influence of bulk membrane properties, such as membrane hydrophobic thickness, curvature stress and bilayer ordering, without changing the bilayer lipid chemistry, complicating the interpretation of the experimental findings. A recent study, however, showed that while the nAChR in PC membranes containing di18:1 or shorter acyl chains is locked in a non-activatable "uncoupled" conformation, the nAChR in relatively thick PC membranes with di22:1 acyl chains undergoes agonist-induced conformational transitions, even in the absence of cholesterol or anionic lipids [61]. The latter provides definitive evidence that membrane properties influence agonist-induced state transitions, and highlights the need for further studies to better understand the link between the nAChR and its membrane physical environment.

Finally, it should be noted that the lipid requirements of the nAChR in simple reconstituted membranes may not accurately reflect the lipid requirements of the nAChR in natural membrane environments. Biological membranes are asymmetric with an uneven distribution of lipids in the two leaflets of the lipid bilayer [15]. The possibility that lipids have different effects on nAChR function when included in different leaflets has not yet been rigorously tested. As well, natural membranes exhibit lateral separations of lipids into micro-domains/lipid rafts, with the lipid composition in the microenvironment surrounding the nAChR not matching the bulk membrane lipid composition. Finally, an intriguing study showed that the inclusion of only 1.6 mol% PA in a complex synthetic membrane composed of PC, PS, PI, and cholesterol has a measurable effect on the proportion of agonist-responsive nAChRs, while the inclusion of 1.6% PA in a minimal PC membrane does not [62]. It appears that PA has synergistic effects with other anionic lipids on nAChR function. These studies collectively show that the levels of a particular lipid required to measurably influence nAChR function in a simple homogeneous reconstituted membrane do not necessarily match the levels of the same lipid that are required in a biological membrane to influence function, where lipid distributions are heterogeneous and where synergistic effects on function likely occur. Even trace lipids may have an important role in nAChR function in biological membranes. The possibility that lipids involved in signaling influence nAChR function is obviously of considerable importance, particularly if signaling lipids are enriched in the nAChR's lipid microenvironment.

In summary, although many lipids and membrane properties influence nAChR function, mixtures of both cholesterol and anionic lipids are particularly effective at stabilizing the nAChR in a functional conformation. Bulk membrane physical properties have an important, yet poorly understood role. Additional research is still required to elucidate how a variety of biologically relevant lipids influence nAChR function, especially in the context of the complex lipid compositions and heterogeneous distributions of lipids that are found in biological membranes. An important remaining goal is to extrapolate the findings from studies of lipid–nAChR interactions in reconstituted nAChR membranes to biological membranes. Such studies are essential to understanding the role of lipid–nAChR interactions at the biological synapse.

3.2. Sites of lipid–nAChR interactions

There are likely both annular and non-annular sites of lipid action at the nAChR [63,64]. Non-annular cholesterol binding sites between transmembrane α-helices were originally proposed based on fluorescence quenching studies with brominated lipids [64], and are supported by both molecular dynamics simulations and bioinformatics studies [65,66] (see also [67]). Cholesterol binding to non-annular cavities located between α-helices within the TMD α-helical bundles stabilizes the transmembrane domain structure, facilitating interactions with the ECD [65]. A putative modulatory PC binding site located at the interface between subunits in the homologous glutamate-activated chloride channel, GluCl, has also been identified (Fig. 3A) [28].

Cholesterol and anionic lipids show a strong preference for the annulus of lipids that surrounds the outer surface of the nAChR [68–70,147]. At any given moment in time, both cholesterol and anionic lipids in this annulus are “bound” to the nAChR surface, although each bound lipid exchanges rapidly with others in the bulk membrane environment. Annular lipid binding sites have been observed in crystal structures of the prokaryotic nAChR homolog, GLIC (Fig. 3B) [23]. In fact, the entire lipid-exposed surface of the nAChR may serve as an “allosteric site” that is sensitive to bulk membrane physical properties, such as membrane hydrophobic thickness.

Chemical labeling studies in the early 1990s showed that the M4 transmembrane α-helix forms the predominant interface between the TMD of each subunit and the lipid environment [71,72]. The location of M4 at the periphery of the TMD led to the suggestion that M4, in particular, plays a central role in lipid-sensing [73–75]. In support of this assertion, a photoactivatable cholesterol analog labels sites on the

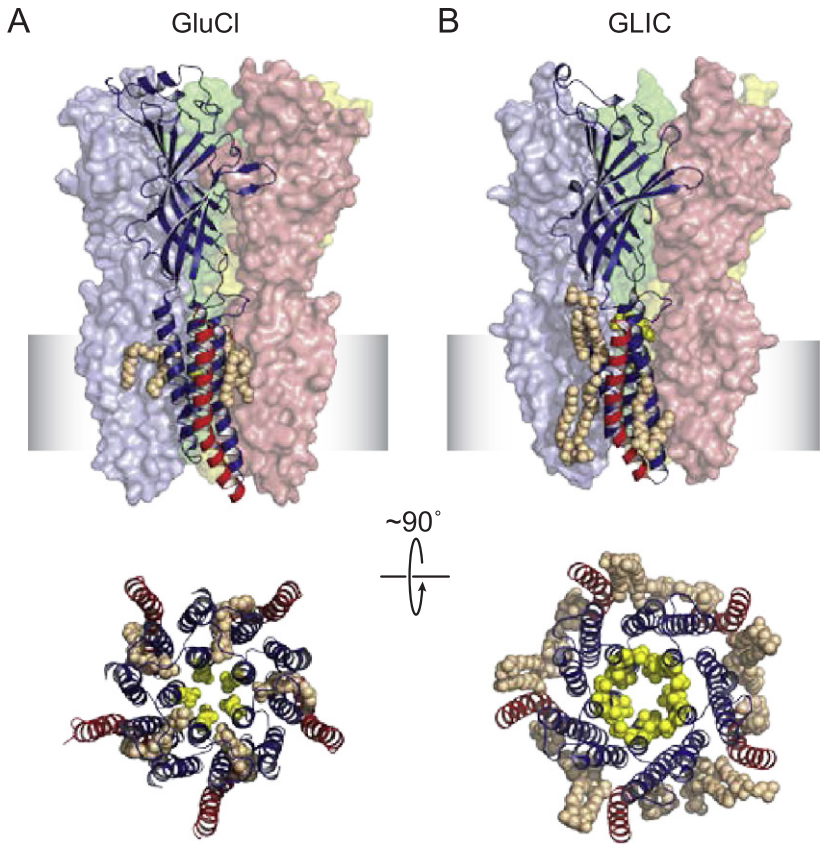

Fig. 3. Phospholipids bound to the surface of A) GluCl and B) GLIC. *Top panel*: GluCl (PDB ID: 4TNW) and GLIC (PDB ID: 3EAM) are shown in a membrane-embedded surface representation, with one subunit as a blue cartoon diagram and the lipid-exposed M4 α-helix in red. Residues contributing to the channel gate are highlighted as yellow spheres. Lipids modeled into the electron density are presented as tan spheres. Three lipids are bound to each subunit in GLIC. In GluCl, the lipid binding site is at the inter-subunit interface. *Bottom panel*: top view of the TMD looking from the extracellular membranous surface.

lipid-exposed surface of the nAChR, including predominant residues on M4 [76,77]. Two of the three lipid binding sites observed in the crystal structure of GLIC are found at the interfaces between M4 and the adjacent TMD α-helices M1 and M3 (see Section 4.1). Numerous mutations of M4 lipid-facing residues on both *Torpedo* and muscle-type nAChRs, which alter M4–lipid interactions, also alter channel function [10,78–82], with the amino acid changes studied to date typically affecting channel opening and closing rates [81,83,84]. Finally, M4-swapped chimeric constructs of the homologous glycine receptor α1 and α3 subunits demonstrate that subunit-specific agonist efficacy is driven in large part by M4, with agonist sensitivity mediated by differences in M4–lipid interactions [85]. M4 thus likely plays a lipid-sensing role in all members of the broader pLGIC family.

3.3. Conformational selection and kinetic mechanisms

To understand the mechanisms underlying lipid–nAChR interactions, research initially focused on elucidating the structural changes induced in the *Torpedo* nAChR in the presence of activating lipids. Although preliminary studies using infrared spectroscopy reported substantial changes in secondary structure in the presence of cholesterol and anionic lipids [86–88], it was subsequently shown that the observed spectral changes were due instead to distinct levels of peptide N-^{1}H/N–^{2}H exchange (note that for technical reasons, infrared spectra are recorded in $^{2}H_2O$) [89,90]. In fact, the non-functional nAChR in PC membranes (PC-nAChR) retains a native-like secondary and quaternary structure [89,91]. PC-nAChR also undergoes thermal denaturation at a temperature comparable to that of resting and desensitized nAChRs [92,93]. The folded PC-nAChR binds agonist, but lacks the capacity to undergo agonist-induced conformational change.

Early chemical labeling and infrared difference measurements suggested that PC-nAChR does not flux cations because it adopts a desensitized conformation, whereas increasing levels of both cholesterol and anionic lipids in PC membranes stabilizes an increasing proportion of activatable resting state nAChRs [51,52,62,94]. These observations led to the hypothesis that membranes influence nAChR function by modulating the natural equilibrium between resting and desensitized conformations, which in native *Torpedo* membranes strongly favors the resting state [95,96]. Subsequent studies, however, showed that the non-functional PC-nAChR does not exhibit the high agonist-binding affinity characteristic of the desensitized conformation [93,97]. The conformational selection mechanism was thus expanded to include a non-activatable conformation, referred to as the uncoupled state (Fig. 4) (see Section 3.4). Some membrane environments favor activatable resting (PC/PA/cholesterol), while others favor uncoupled (PC alone) and/or desensitized (PC/PS) nAChRs [98]. Lipids also influence the transitions between conformations. Increasing membrane hydrophobic thickness lowers the activation energy barrier between uncoupled and coupled (resting, open, and desensitized) conformations [61]. Mutations to the lipid facing surface of M4 influence both channel opening and closing kinetics. In effect, lipids/membranes influence nAChR function by both conformational selection and kinetic mechanisms. They modify the magnitude of the agonist-induced response by altering the relative proportions of resting, desensitized, open, and uncoupled conformations, as well as the agonist-induced transitions between each of these states.

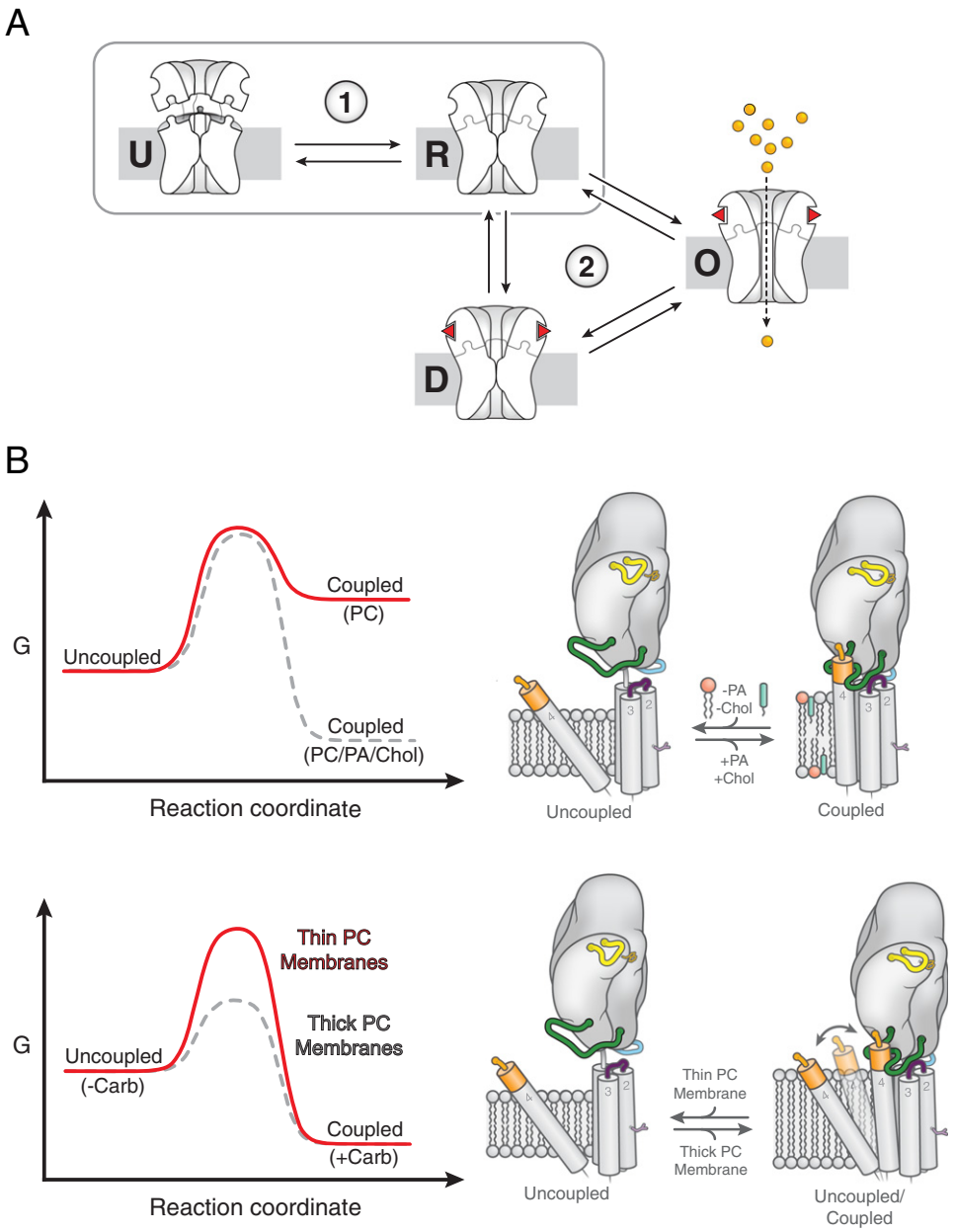

Fig. 4. Conformational selection and kinetic mechanisms of lipid–nAChR interactions. A) nAChR function is usually interpreted in terms of a conformational scheme involving resting (R), open (O), and desensitized (D) conformations (scheme 2). The membrane-reconstituted *Torpedo* nAChR can also adopt a non-responsive uncoupled (U) conformation (scheme 1). B) *Left panel*: Reaction coordinate diagrams illustrating qualitatively the effects of both lipid composition and membrane bulk properties affect the structure and function of the nAChR. Anionic lipids and cholesterol influence the relative proportion of uncoupled versus coupled conformations by interacting preferentially with "coupled" conformations (i.e., conformations where agonist binding and gating are allosterically coupled), thus lowering their energy relative to uncoupled conformations (*a conformational selection mechanism*). Membrane thickness influences the transitions between uncoupled and coupled states by lowering the activation energy between these conformations (*a kinetic mechanism*). Note that the reaction coordinate diagrams are qualitative representations. Individual lipids or lipid mixtures may influence function by a combination of both conformational selection and kinetic mechanisms. *Right panel*: Proposed models of uncoupling in the context of conformational selection versus kinetic mechanisms of lipid–nAChR interactions.

Note that to fully understand the mechanisms underlying lipid–nAChR interactions, one must have intimate knowledge of the structures of each conformation/transition state, and then elucidate how each lipid/membrane environment interacts preferentially with these states to influence their relative populations, as well as the transitions between them. While this alone is a complex undertaking, a detailed mechanistic understanding is further confounded by the aforementioned possibility that lipids influence function via both direct and indirect mechanisms. The latter raises the possibility that a particular lipid could have one effect on channel function, by preferential binding to one conformation (for example, the resting state), while at the same time have an opposite effect on channel function by changing bulk membrane physical properties to favor another conformation (for example, a non-activatable desensitized or uncoupled conformation). Deciphering the mechanisms underlying nAChR–lipid interactions remains a daunting task.

3.4. The uncoupled nAChR

The proposed conformational selection and kinetic mechanisms that underlie lipid–nAChR interactions likely apply to all membrane proteins, with subtle changes in lipid composition/bilayer physical properties having subtle effects on channel function. As described above, a unique feature of lipid–nAChR interactions is that a functionally silent,

uncoupled conformation is stabilized when the *Torpedo* nAChR is reconstituted in PC membranes lacking cholesterol and anionic lipids. The uncoupled conformation exhibits resting-state-like agonist binding, but does not flux cations or undergo agonist-induced transitions to the high affinity agonist-binding desensitized state [98]. The fact that the uncoupled nAChR still binds agonist, but does not desensitize indicates that the activation energy barrier between uncoupled and coupled conformations is insurmountably high over the minute time scale of most biophysical measurements. This implies that the structural differences between uncoupled and coupled conformations are energetically more substantial than those between resting, open, and desensitized nAChRs [61].

The identification of a lipid-dependent uncoupled conformation of the *Torpedo* nAChR may be relevant to human cholinergic biology, as anecdotal evidence suggests that neuronal nAChRs also adopt a functionally equivalent conformation. A large proportion of α4β2 nAChRs expressed on the surface of human embryonic kidney cells bind agonist, but do not undergo agonist-induced channel gating, consistent with the existence of a large pool of uncoupled receptors [99,100]. Some nAChR subtypes, including those with the α6 subunits, exist in oligomeric structures on cell surfaces but do not flux cations in response to acetylcholine binding [101,102]. In fact, the β3 subunit is essential for the biogenesis of α6α4β2β3 nAChRs, yet β3 suppresses acetylcholine-evoked currents. It is possible that the β3 subunit favors formation of an uncoupled state. The normalized current (current per acetylcholine binding site on the surface of the cell) also increases with time after injection of muscle-type cRNA into oocytes, suggesting a time-dependent maturation in "folding" to activatable conformations [103]. Chronic nicotine exposure upregulates nAChR trafficking to the cell surface, but there appears to be a role for inactive nAChRs in this process [104], with chronic nicotine exposure potentially leading to the activation of previously assembled but inactive nAChRs [105].

As noted, thicker membranes, such as those found in lipid rafts [57,106], promote slow conformational transitions from uncoupled to coupled conformations [61]. Neuronal nAChRs require lipid rafts for trafficking to the cell surface [107,108]. The above observations raise the intriguing speculation that a lipid raft-dependent transition from uncoupled to coupled conformations represents a final step in the folding and trafficking of nAChRs to the cell surface, as discussed in more detail in Section 5.

3.5. Structural insight into lipid–nAChR interactions

Both crystallographic and functional studies have increasingly provided insight into the nature of nAChR structure and conformational change, opening the door for a more detailed understanding of lipid–nAChR interactions. Kinetic studies show that the structure of the lipid-exposed α-helix, M4, is dynamic when the nAChR shifts between resting and open conformations. When gating, the two αM4 α-helices in the $(\alpha1)_2\beta1\varepsilon\delta$ pentamer move synchronously, each moving as a unit roughly halfway along the reaction coordinate between agonist binding and the open transition state [109]. The motions of the εM4 and βM4 follow that of αM4, while δM4 has no apparent motion during gating. The temporal differences in various M4 positions should permit the pentamer to present a unique binding surface to the lipid bilayer in each distinct conformation and/or while transitioning between them. Movement of M4 has also been suggested in the desensitized state [110]. Such structural changes in M4 could expose or mask lipid binding sites, allowing for stronger or weaker interactions with the lipid bilayer that preferentially stabilize different conformations or promote/inhibit transitions between these states. In addition, biophysical measurements and molecular dynamics simulations suggest that the orientation of M4 relative to the membrane normal depends on the presence of cholesterol and bilayer thickness [73,74]. Lipids may preferentially stabilize different orientations of M4 relative to the remainder of the TMD, altering gating kinetics as M4 moves along the reaction coordinate leading from agonist binding to the open state.

A role has also been proposed for M4 in formation of the lipid-dependent uncoupled conformation. The uncoupled nAChR undergoes more extensive peptide N-^{1}H/N–^{2}H exchange than either resting or desensitized nAChRs. Curve-fitting the peptide hydrogen/deuterium exchange curves shows that uncoupling is accompanied by the solvent exposure of previously buried peptide hydrogens [93]. Although the regions of the nAChR that become solvent-exposed have not been defined, the critical importance of the ECD/TMD interface in channel function [40,111] led to the hypothesis that uncoupling results from structural changes at this interface. One model proposes that weakened interactions leading to an increased physical separation between the ECD and TMD accounts for both the loss of function and the increased solvent accessibility in the uncoupled state [93]. The proposed structural independence of the ECD is supported by a number of crystallographic studies [30,31,112].

The nAChR structure also shows that the C-terminus of M4 (post-M4, specifically Gln435) extends beyond the lipid bilayer to interact with a conserved residue (Phe137) in the Cys-loop (Fig. 2). Given that the Cys-loop plays a central role at the interface between the ECD and TMD, it was postulated that interactions between post-M4 and the Cys-loop are important for coupling agonist-binding to channel gating [93]. Considerable functional data support a role for post-M4 in channel function [113–115]. The M4 lipid-sensor model proposes that lipids influence M4 binding to M1/M3 to modulate post-M4/Cys-loop interactions and thus channel function. Ineffective post-M4/Cys-loop interactions could lead to partial dissociation of the ECD from the TMD to form the uncoupled state [93]. Modulatable binding has been discussed in terms of both the conformational selection and kinetic effects of lipids on nAChR function (Fig. 4) [61].

4. Prokaryotic pLGICs as models of lipid–nAChR interactions

4.1. Insight from crystal structures of prokaryotic pLGICs

The homologous homopentameric prokaryotic pLGICs, GLIC and ELIC, are expressed in *Escherichia coli* at levels sufficient for both biochemical and structural studies, and each has yielded several high resolution crystal structures (Fig. 1) [21–23,116–119]. These structures reveal two key features that impact on our understanding of lipid–nAChR interactions.

First, the crystal structures of GLIC exhibit electron density located at the periphery of the TMD, suggesting the presence of partially-ordered lipid molecules (Fig. 3) [23]. Two of the three lipid binding sites on each subunit are found at the interfaces between M4 and the adjacent TMD α-helices M1 and M3. One of these bridges interactions between post-M4 (F315) and the β6–β7 loop (F121), the loop analogous to the Cys-loop in eukaryotic pLGICs. Intriguingly, the lipid bridging post-M4 to the β6–β7 loop in the GLIC structure is displaced slightly by the inhibitory anesthetic propofol, suggesting that the lipid occupies an allosteric site [26]. The finding that lipids bind to the M4–M1/M3 interface in the GLIC structure supports the hypothesis that lipids influence M4–M1/M3 interactions.

Second, crystal structures of ELIC solved in the presence of agonist exhibit electron density in the agonist site, but show no structural rearrangements in the TMD pore relative to crystal structures solved in the absence of agonist [118,120,121]. In fact, no movement of the pore-lining M2 α-helices was detected with mutants that prolong channel opening and exhibit no propensity to desensitize. It has been suggested that the ELIC structures reflect a conformation that is refractory to agonist binding [121] — i.e., a conformation that is functionally equivalent to the lipid-dependent uncoupled state of the *Torpedo* nAChR [122]. Significantly, the ELIC structures exhibit multiple proposed features of the uncoupled nAChR (Figs. 1 and 2): 1) The M4 α-helix is partially unwound and tilted away from the remaining TMD, with several

C-terminal M4 residues unresolved. 2) In contrast to the nAChR structure, ELIC shows no direct contact between post-M4 and the β6–β7 loop, and 3) the tilting of M4 away from the remainder of the TMD is accompanied by reduced contact between both the β1–β2 and β6–β7 loops of the ECD and the M2–M3 linker of the TMD. In the nAChR structure, the extended side chains of the β1–β2 and β6–β7 loops engage the M2–M3 linker in a fashion reminiscent of vice grips attached to a metal pipe. In ELIC, the β1–β2 and β6–β7 loops no longer surround the M2–M3 linker, which itself tilts toward the membrane surface so that it approaches the β8–β9 loop on the complementary face of the adjacent subunit. In effect, reduced interactions between M4 and M1/M3 are accompanied by weakened interactions between the ECD and TMD — as predicted by the M4 lipid-sensor model.

4.2. Testing the role of M4 in pLGIC lipid-sensing

The noted difference in the position of M4 in the crystal structures of ELIC and GLIC is intriguing given the demonstrated importance of aromatic residues in the binding of M4 to M1/M3 during folding of the homologous glycine receptor [123]. GLIC has nine aromatic residues at the interfaces between M4 and M1/M3 that are involved in extensive π–π interactions (Fig. 5A). These strong interactions likely contribute to the apparent tight interactions between M4 and M1/M3 in the GLIC crystal structure. Four of these aromatic residues at the M4–M1/M3 interface are conserved in ELIC. The remaining five aromatics are replaced in ELIC by aliphatic residues (Fig. 5B). In particular, ELIC lacks a cluster of three aromatic residues located near post-M4 that may be essential in GLIC for effective binding of post-M4 to the remainder of the TMD and/or the β6–β7 loop. The absence of this post-M4 cluster in ELIC may lead to the aforementioned tilting of the C-terminus of M4 away from the main body of the TMD in the crystal structure of ELIC. The lower number of aromatic interactions at the M4–M1/M3 interface may render M4 binding to M1/M3 weaker in ELIC and thus make ELIC more susceptible to the perturbing effects of detergent solubilization during crystallization than GLIC [122].

In light of the above structural differences, aromatic substitutions were used to test the effects of altered M4–M1/M3 interactions on pLGIC activity (Carswell et al., revised manuscript under review). Ala substitutions of aromatic residues were introduced into the M4–M1/M3 interface of GLIC to weaken M4 binding [123], with every aromatic substitution leading to reduced channel function. Conversely, aliphatic to aromatic substitutions were introduced at the same interface in ELIC to promote M4 binding, with every aromatic addition leading to enhanced channel function. In fact, the introduction of a single pair of interacting aromatic residues on the C-terminus of M4 and the N-terminus of M3 decreased the EC_{50} for ELIC channel gating almost 10-fold, implying close to a 10-fold potentiation of channel function. The data support a key tenet of the M4 lipid-sensor model, that enhanced interactions between M4 and M1/M3 promote coupling between the agonist binding site and channel gate.

Additional studies suggest that modulatable M4 binding to M1/M3 plays a role in pLGIC lipid sensing. Protocols for reconstituting GLIC and ELIC into lipid bilayers have been developed [124–128]. As noted, the extensive aromatic network at the M4–M1/M3 interface in GLIC contributes to relatively strong M4–M1/M3 interactions along the entire length of M4. In contrast to the nAChR, which exhibits few aromatic interactions at the M4–M1/M3 interface, GLIC retains robust agonist-induced gating in the minimal PC membranes that stabilize the uncoupled nAChR [127]. In fact, electrophysiological measurements suggest that GLIC function is relatively insensitive to its lipid environment [124,126]. The intrinsic strength of M4–M1/M3 interactions in GLIC may be sufficient to maintain effective M4–M1/M3 interactions and retain channel function, even in PC membranes lacking cholesterol and anionic lipids. In contrast,

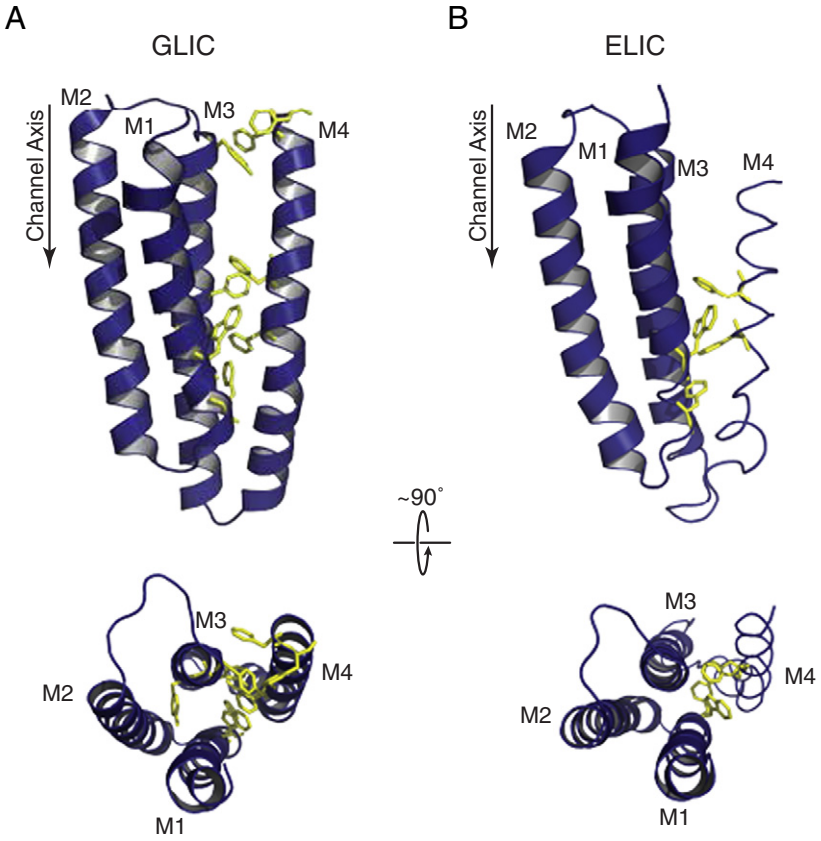

Fig. 5. Aromatic residues at the M4–M1/M3 interface of A) GLIC and B) ELIC. *Top panel*: Side view of a single TMD from both GLIC and ELIC highlighting aromatic residues at the M4–M1/M3 interface as yellow sticks. *Bottom panel*: Top view from the extracellular surface of a single TMD.

although the conserved aromatic residues at the cytoplasmic end of the M4–M1/M3 interface in ELIC appear to be sufficient to stabilize M4–M1/M3 interactions in this region, ELIC does not gate open in response to agonist in these minimal PC membranes. This lack of function may be due to the absence of effective post-M4 aromatic interactions. Significantly, engineering the post-M4 aromatic cluster into ELIC restores the ability of ELIC to gate open in PC bilayers [128]. Modulatable post-M4–M1/M3 interactions thus play a role in ELIC lipid-sensing.

5. Lipids and the folding and trafficking of nAChRs

The proposed role of M4 binding to M1/M3 in lipid sensing may also be relevant to the folding, assembly, and trafficking of nAChRs to the cell surface. Although many factors are known to influence mammalian nAChR trafficking [129–131] (see also [132]), a role for M4 binding to M1/M3 has been clearly demonstrated in the homologous glycine receptor. When expressed in oocytes, the glycine receptor is cleaved within the intracellular loop, but still undergoes agonist-induced channel gating. A truncated glycine receptor containing both the ectodomain and the first three TMD α-helices fails to traffic to the cell surface [123]. Co-expression with the M4 α-helix, however, rescued both folding and cell surface expression, with the cell-surface expressed channels indistinguishable from wild type channels in terms of their gating behavior. Aromatic interactions at the interface between M4 and M1/M3 were found to be critical for M4 binding and thus in the folding and trafficking of these pLGICs.

Post-M4 appears to be critical for the surface expression of *Torpedo* nAChRs in frog oocytes [113]. Deletion of C-terminal residues from M4 in the homologous GABAρ1 [133] and 5-HT3A [134] receptors also leads to non-functional channels. In the latter case, elimination of 2 or more residues prevents trafficking to the cell surface, suggesting that post-M4 plays an important role in cell surface trafficking of many pLGICs. M4 binding to M1/M3 in a homomeric α7/5HT3A chimera locks the chimera in a functional conformation via direct post-M4/Cys-loop interactions [114].

As noted above, thick membranes support slow agonist-induced conformational transitions from uncoupled to coupled conformations. Lipid rafts/microdomains are typically enriched in cholesterol, which orders and increases the hydrophobic thickness of the bilayer [57,135]. 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 [107,108,136–138]. Disruption of these rafts leads to both altered cell surface exposure and altered nAChR function [138,139]. Integral membrane proteins with shorter transmembrane α-helices tend to remain in intracellular membrane compartments, possibly because they hydrophobically match the thinner intracellular membranes more favorably [57,140]. Partitioning of the nAChR into thicker lipid rafts could favor transitions from uncoupled to coupled conformations, as we observe in the thicker di22:1PC model membranes. If this transition leads to a preferential alignment of M4 perpendicular to the membrane surface, the increased hydrophobic length of M4 should promote trafficking to the cell surface. Such a mechanism could explain why nicotine acts as a chaperone to promote cell-surface expression of the high affinity $(\alpha4)_2(\beta2)_3$ nAChR versus the lower affinity $(\alpha4)_3(\beta2)_2$ nAChR [141]. Preferential nicotine binding could lead to maturation of the high affinity $(\alpha4)_2(\beta2)_3$ nAChRs toward coupled (resting or desensitized) conformations where M4 is aligned closer to the bilayer normal to promote cell-surface expression. In this scenario, limited binding would thus have minimal effect on the trafficking of the low affinity $(\alpha4)_3(\beta2)_2$ nAChR.

6. Role of lipids in the clustering of nAChRs in the plasma membrane

Both the clustering of nAChRs on the plasma membrane and the internalization of cytoplasmic membrane-located nAChRs are lipid raft-dependent [142]. Lowering cholesterol levels to disrupt lipid rafts leads to altered nAChR clustering and mobility on cell surfaces, rapid internalization, and ultimately enhances function of the surface-retained nAChRs [139,143,144].

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 [55,145]. The nAChR concentrates cations, including protons, at the bilayer surface, facilitating the ordering of nearby anionic lipids [56,146]. There appear 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 [54]. The hydrolysis product of PA, diacylglycerol, is a potent nAChR activator [54]. A nAChR-associated phospholipase activity could 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 head groups 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 could create less favorable interactions between the nAChR and its surrounding lipids, which may ultimately lead to nAChR trafficking from a less favorable to more favorable lipid micro-environments. 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 complexities underlying nAChR–lipid interactions and the potential importance of these interactions *in vivo*.

7. Conclusions

Three decades of research have revealed both an exquisite sensitivity of the *Torpedo* nAChR to its surrounding lipid environment and a tremendous complexity to lipid–nAChR interactions. While many lipids likely influence function, cholesterol and anionic lipids play a vital role in stabilizing the nAChR in a functional conformation. Membrane physical properties also play a poorly understood role. Recent research has shown that even trace lipids found in complex membrane environments can impact on nAChR function. Considerable research is still required to fully understand how different lipids influence nAChR function in a biological context.

Lipids/membranes influence the nAChR by both conformational selection and kinetic mechanisms. Different lipids/membranes stabilize varying proportions of pre-existing resting, open, desensitized, and uncoupled conformations, as well as influence the transitions between these conformational states. Although the mechanisms by which lipids/membranes interact preferentially with these different conformations/transition states to influence function remain obscure, increasing evidence points to a role for the outermost transmembrane α-helix in lipid sensing. Both lipid binding and bulk membrane effects likely influence M4 interactions with M1/M3 to alter function. One hypothesis is that lipid-sensitive interactions between M4 with M1/M3 ultimately influence interactions between post-M4 and the Cys-loop to regulate nAChR activity; although this still hypothesis requires rigorous testing.

A lipid-dependent uncoupled conformation has been identified that may form as a result of an M4-dependent loss of physical contact between the ECD and the TMD. While this conformation has only been conclusively demonstrated for *Torpedo* nAChRs in reconstituted membranes, it may also exist for other pLGICs. Functionally equivalent conformations have been detected with neuronal nAChRs, and with the detergent-solubilized prokaryotic pLGIC, ELIC. An important goal will be to test for existence of uncoupled nAChRs in biological membranes,

and whether lipids or other allosteric modulators enhance the transition from uncoupled to coupled conformations to enhance synaptic communication.

There is considerable interest in the role of nAChRs in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, as well as in other neurological disorders, such as epilepsy and schizophrenia. The role of lipid–nAChR interactions in disease progression remains to be studied. Altered lipid profiles leading to altered lipid–nAChR interactions could lead to altered cholinergic activity, with small functional changes resulting in severe physiological effects. Fundamental knowledge of how different neuronal nAChRs respond to changes in their lipid environments may prove important for understanding the mechanisms of altered cholinergic activity during the course of human disease.

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