



Phylogenetic conservation of protein–lipid motifs in pentameric ligand-gated ion channels☆

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ABSTRACT

Using the crosstalk between the nicotinic acetylcholine receptor (nAChR) and its lipid microenvironment as a paradigm, this short overview analyzes the occurrence of structural motifs which appear not only to be conserved within the nAChR family and contemporary eukaryotic members of the pentameric ligand-gated ion channel (pLGIC) superfamily, but also extend to prokaryotic homologues found in bacteria. The evolutionarily conserved design is manifested in: 1) the concentric three-ring architecture of the transmembrane region, 2) the occurrence in this region of distinct lipid consensus motifs in prokaryotic and eukaryotic pLGIC and 3) the key participation of the outer TM4 ring in conveying the influence of the lipid membrane environment to the middle TM1–TM3 ring and this, in turn, to the inner TM2 channel-lining ring, which determines the ion selectivity of the channel. The preservation of these constant structural–functional features throughout such a long phylogenetic span likely points to the successful gain-of-function conferred by their early acquisition. This article is part of a Special Issue entitled: Lipid–protein interactions.

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“Three Rings for the Elven-Kings under the sky....

...One Ring to rule them all,

One Ring to find them,

One Ring to bring them all....”

The Lord of the Rings, J.R.R. Tolkien

1. 4,000 million year-proof architectural design of pentameric ligand-gated ion channels

Nicotinic acetylcholine receptors (nAChR) are members of the superfamily of ligand-gated ion channels (pLGIC), a collection of neurotransmitter receptors which also includes γ -aminobutyric acid type A or C (GABA_{A/C}) receptors, glycine receptors, the subtype 3 of the serotonin (5-HT₃) receptors and glutamate-gated chloride channels (GluCl) (see reviews in [1–3]). From a functional point of view, members of the superfamily can be divided into two distinct types: the cation-selective channels such as the nAChR and 5-HT₃ receptor, and the anion-selective channels such as the glycine and the GABA_A and GABA_C receptors and invertebrate GluCl. Furthermore, these functionally distinct classes of ion channels employ their unique combination of

ionic selectivity and cellular/tissue/species distribution to mediate and/or modulate either excitatory or inhibitory chemical transmission. The amino-terminal of all their subunits contains extracellular halves of a pair of disulfide-bonded cysteines separated by only 13 residues, the so-called Cys-loop, and this motif has served for almost two decades as a common identifier to encompass the various receptor families present in Metazoa, comprising the superfamily of LGIC coded in eukaryotic genes. But more recently the finding of prokaryotic structural homologues of the eukaryotic pLGIC [4–8] reviewed in [1,3,9–11] has added a new twist, since although these bacterial channels do not possess a Cys-loop and their sequence identity with other members of the LGIC is rather low, the 3-D structural similarity with the eukaryotic LGIC is so remarkable that it appears more convenient to group all these proteins together under the common denomination of pentameric LGIC (pLGIC). Dysfunction of pLGICs plays an important role in several disorders of the central nervous system, including Alzheimer's disease [12], schizophrenia [13], Parkinson's disease [14] and other pathologies. As recently reviewed [1] pLGICs constitute targets for an ever increasing variety of pharmacological and clinically important drugs, including general anesthetics, smoking cessation aids, anxiolytics, anticonvulsants, muscle relaxants, hypnotics, behavior and mood-modifying substances, anti-emetics, and a variety of drugs used in the treatment of epilepsy, insomnia, panic disorders, and other neurological and neuropsychiatric conditions. Some of these drugs activate responses, acting as agonists; others block the ortholog or the allosteric sites, and an ample spectrum of other drugs modulate -potentiating or decreasing- the amplitude of the responses.

Abbreviations: α BTX, α -bungarotoxin; nAChR, nicotinic acetylcholine receptor; pLGIC, pentameric ligand-gated ion channels

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The combination of abundant quantities of nAChR protein in the electric organ of electric fish [15] and the availability of the competitive cholinergic antagonist α -bungarotoxin from the snake *Bungarus multicinctus* [15,16] were the two key elements that led to the isolation, biochemical purification, pharmacological and functional characterization of the nAChR, a saga which started more than forty years ago [17]. Muscle-type AChRs are expressed in the peripheral nervous system and neuronal-type AChRs in both peripheral and central nervous systems as well as in other non-neural cells such as immune cells, lymphocytes, lung epithelium and others. In brain, the nAChR is present in two principal forms: the heteropentameric receptor formed by $\alpha 4$ and $\beta 2$ subunits and the homopentameric receptor formed exclusively by $\alpha 7$ subunits [11,18]. The nAChR rapidly became the reference neurotransmitter receptor, and continues to be the prototype for the pLGIC superfamily. The 30-year-improvement in the electron microscopy resolution of the *Torpedo* nAChR structure, currently at about 4 Å resolution [19–24] and the crystal structure of the water-soluble homologue of the nAChR extracellular domain (the molluscan ACh-binding protein, AChBP) by X-ray diffraction techniques [25,26], were also first hits in the structural characterization of neurotransmitter receptors and structural homologues, and have had an important impact on our knowledge of pLGIC structure. A recent appearance on the scene is the X-ray crystal structure of various complexes between pLGIC and pharmacologically relevant drugs or endogenous ligands such as the competitive antagonist α -bungarotoxin bound to the extracellular domain of the nAChR α subunit at 1.94 Å resolution [27]; the ligand-binding domain of a nAChR chimera in complex with agonist [28]; and acetylcholine (the endogenous neurotransmitter of the nAChR), which acts as a competitive antagonist when bound to an aromatic cage in the extracellular domain of the bacterial protein ELIC, at 2.9 Å resolution [29]. New crystal structures have followed: the mouse 5-HT₃ receptor at 3.5 Å resolution [30], the first eukaryotic anionic-gated channel (from the worm *Caenorhabditis elegans*) in the apo-state and in complex with the anti-parasitic drug ivermectin [31], the bacterial ELIC channel in the presence of memantine, a drug used in the amelioration of cognitive deficits in Alzheimer's patients [32]; the 2.99 Å resolution X-ray structure of GLIC bound with the general anaesthetic ketamine, revealing an inter-subunit cavity that partially overlaps with the homologous antagonist-

binding site in eukaryotic pLGICs [33], and the human GABA_A receptor [34]. These and other recent structural findings and analyses of pLGIC [3,9,35] have provided a much-needed architectural framework to better understand and in some cases reformulate several important mechanistic aspects of ligand binding, gating and blockage of pLGIC macromolecules from an entirely new perspective.

2. A phylogenetically conserved architecture of the pLGIC: the transmembrane “three-rings” design

Classically, various topographical regions have been distinguished in the nAChR macromolecule: an extracellular domain exposed to the synaptic gap, a transmembrane (TM) region composed of 20 hydrophobic segments having 20–30 amino acids each, and the cytoplasmic domain made up of loops linking the TM segments. Most relevant to the topic of this review is, of course, the 3-ring cylinder of concentrically arranged TM1–TM4 segments [36]. As shown in Fig. 1, the inner ring is made of five TM2 helices, one from each subunit, which constitutes the walls of the ion channel proper [37–39], devoid of contact with the membrane lipid. The inner ring is the site of the selectivity filter, the region of the nAChR determining which ions permeate the pore and which do not. The selectivity filter consists of rings of amino acid residues pointing towards the ion channel central pore, in the lower portion (i.e. the cytoplasmic end of the TM2 segment) [40–42]. The middle ring is formed by ten helices, the TM1 and TM3 segments, which establish a substantial physical contact with membrane lipids. The outermost ring, made up of five TM4s, is totally embedded in the lipid bilayer. I have proposed that the middle and outer rings constitute an important domain defined by the extensive interface between the protein and lipid moieties, comprising both the lipid-exposed TM portions of the nAChR protein and the nAChR-vicinal lipid, respectively [36,43]. The latter corresponds to the lipid-exposed belt (“shell” “annular”, “boundary”, “nAChR vicinal”) region [44–47], that is, the lipid moiety in the immediate perimeter, including crevices, of the nAChR protein, earlier discovered by Marsh and myself using electron spin resonance (ESR) techniques [44] and further characterized in terms of lipid selectivity and stoichiometry [48–52]. As we will see in this review, sites for

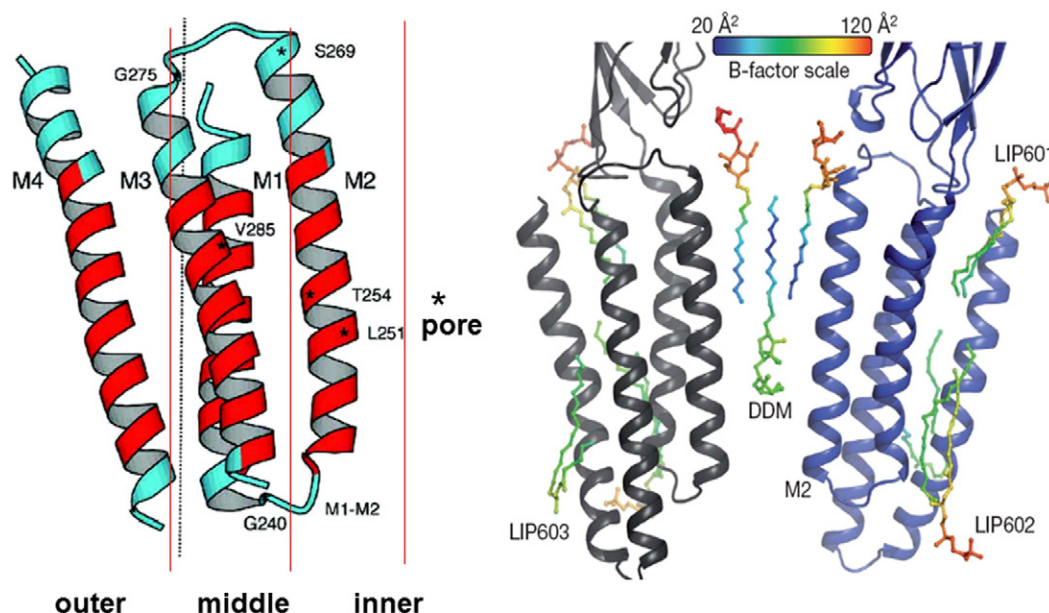


Fig. 1. Location of the three rings in the transmembrane region of the pentameric ligand-gated ion channels. The 20 chains in the eukaryotic nAChR (left) and in the bacterial homologue GLIC (right) are arranged in three concentric rings, probably the structural feature best conserved in pLGIC for about 4000 Ma of channel evolution. Only the helix bundle corresponding to one subunit is shown in the case of the nAChR, and two subunits (gray and blue) in the case of GLIC. Detergent (DDM) and lipid (LIP) molecules co-crystallize with GLIC. The lipids are assumed to be endogenous lipids tightly bound to the protein. The figure on the left has been modified from Unwin and coworkers [179,180] and the right illustration is taken from Ref. [4].

endogenous lipids and pharmacologically important drugs have become a common finding in this interface domain.

The structural similarities among pLGIC are not restricted to the TM region [1,53]. For instance, Dellisanti et al. [54] have analytically compared the packing of the extracellular domain of some prokaryotic and eukaryotic pLGIC, arguing that loose packing of cavities in eukaryotic pLGIC hydrophobic cores likely developed as an evolutionary strategy aimed at facilitating the allosteric transitions required for rapid ligand-induced switching between closed and open channel states. Although the chemical makeup of the signals that activate or antagonize their action may differ, it is not totally unforeseen that members of the *eukaryotic* superfamily of pLGIC implement common structural designs in their extracellular moiety and in their TM region. The 3-ring architecture, conserved in eukaryotic nAChRs, GABA_A, GABA_C, GluCl and 5-HT₃ receptors, is a vivid example; however, when we observed an identical design in the bacterial homologues GLIC and ELIC [55], the finding came as a total surprise. The stability of the design over an evolutionary period of more than 4000 Ma is also quite remarkable, and probably a manifestation of the success of its early adoption and conserved maintenance.

3. The key contribution of the outer TM4 ring: lipid sensing function

In 2005 we undertook molecular dynamic studies of the entire transmembrane region (180,000 atoms) of the nAChR in a lipid bilayer, with the aim of understanding the mechanics of gating kinetics [56]. However, what was probably the most relevant (and unexpected) outcome of the work was the finding that the TM4 outer ring underwent substantial motions in the time window explored, changing extensively its contacts, in an alternating fashion, with the lipid surrounding its outside boundaries or with the middle ring (TM1–TM3) helix bundle inside its perimeter, respectively. From this modelling exercise we explicitly suggested that “the outer ring of TM4 acts as the vehicle to transfer the influences of the lipid surroundings to the conformational changes of the whole channel” [56]. We further elaborated that this ability of the TM4 ring provided a rationale for the effect of mutations in α TM4, β TM4, and γ TM4 on channel function, and for the influence of the lipid environment on the stability of nAChR functional states, as well as on how several pharmacologically relevant ligands that partition in the lipid bilayer affect nAChR channel function [56,57]. Strong support for the lipid sensing role of TM4 stemming from our molecular modeling and fluorescence studies was provided by the electrophysiological studies of Auerbach and coworkers (e.g. [58]) indicating that TM4 undergoes substantial motion in the course of nAChR gating.

Mutations of amino acid residues at the lipid–protein interface of *Torpedo* and muscle-type nAChRs provided additional experimental evidence for the functional coupling of the outer TM4 ring with the relatively distant ion permeation pathway lined by the inner ring of TM2s, secluded from contact with the lipids by the middle TM1–TM3 ring. Most mutations modify the kinetics of channel gating by altering the rate of the transitions between channel states, some of them to an extent that mimics pathological conditions akin to congenital myasthenic syndromes [59–61].

Baenziger and colleagues have significantly contributed to the development and extension of the concept of TM4 acting as a lipid sensor [53, 62–67], especially by disclosing the functional implications of the terminal region of TM4 located outside the lipid bilayer and therefore termed “post-M4”. This group highlights the role of Gln435, located in the post-M4 extracellular region, and purported to interact with Phe137, located in the extracellular Cys-loop [62]. In this hypothesis, Gln435 and Phe137 would couple the extracellular domain with the transmembrane domain, and thus functionally couple agonist binding with channel gating. Baenziger's idea is that post-M4 interaction with the extracellular Cys-loop would also be subject to lipid modulation, and that ineffective coupling leads to a non-functional, “uncoupled” state of the channel (see below). Early mutagenesis studies which deleted residues or truncated part of TM4, thus effectively shortening its length, were

initially invoked to claim lack of influence of TM4 on nAChR channel function [68], an assertion which is currently invalidated by the wealth of information on mutagenesis of TM4 residues and their effect on ion permeation function. The functional implications of the TM4 truncation/deletion studies of nAChR can thus be reinterpreted in the light/in support of the Baenziger hypothesis of agonist binding with channel activity via coupling of post-M4 with the extracellular domain [53,62, 64,65,67].

The phylogenetically conserved structure of the TM4 lipid-sensing region in prokaryotic and eukaryotic ion channels points to the inherent functional correlates; mutations in the TM region of the eukaryotic proteins which provoke changes in the ion permeation properties [69–79] ought to be observed when tinkering with the bacterial TM regions. This appears to be the case. Deletion of aromatic residues at the interface region between TM4 and the TM1–TM3 bundle in GLIC reduces the interaction between the two rings [80], affecting the normal assembly of the pentamer. The alteration in the assembly of the mature oligomer following tinkering of the aromatic residues in the TM segments is also observed in other members of the pLGIC, such as the glycine receptor, leading to the more general conclusion that the geometry of the TM1–TM4 tetrahelical packing is important for the nascent pLGIC subunits to adopt a closed five-fold symmetry [81]. Furthermore, mutations in the TM4 region of ELIC and GLIC which alter the interactions of the TM4 lipid sensing domain with the TM1–TM3 helix bundle indicate that aromatic residues strengthen the interactions between the two rings and hence reduce the crosstalk of TM4 with the lipid microenvironment [66]. These data provide full support to the molecular dynamics data and interpretation of these data in terms of alternating crosstalks of TM4 with its surrounding lipids outside or the TM1–TM3 bundle inside, which gave rise to the lipid sensing hypothesis [56].

4. The physical state of the nAChR lipid microenvironment

In absolute terms, lipid molecules in a biomembrane outnumber those of protein molecules, and the physical properties of the membrane derive predominantly from the physical state of the lipid milieu. The pioneering study of Epstein and Racker [82] on the reconstitution of purified nAChR set the basis for exploring the chemical composition and physical state of the host lipids with a view to understanding the influence of the latter on receptor function upon purification and reconstitution and, by extrapolation, in the native membrane environment. Contemporary with these attempts, the phase state of lipid bilayers in general was being characterized using a variety of physicochemical and biophysical approaches, and it is thus natural that the bulk physical properties of the lipid became an area of research in the receptor field. Model membrane studies showed that cholesterol–phospholipid mixtures at high cholesterol concentrations mimicked many aspects of the phase state displayed by biological membranes rich in cholesterol. These mixtures lack a defined lipid phase transition and instead are characterized by a single phase state, the liquid-ordered (L_o) phase [83], with properties between the gel and the fluid lipid phases. For low cholesterol concentrations, solid-ordered (S_o) or liquid-disordered (L_d) phases are observed, depending on whether the system is above or below its gel–fluid transition temperature (T_m), respectively. When the binary lipid system is at intermediate cholesterol concentrations, there is phase coexistence of S_o and L_o (below) or L_d and L_o (above), depending on the temperature relative to T_m .

Early studies exploring lipid effects on nAChR focused on the influence of lipid composition on the ion permeation properties and the conformational equilibria of the receptor protein reconstituted in defined lipid mixtures [84–86]. The capacity of reconstituted AChR to translocate ions *in vitro* was found to be sensitive to the bulk physical properties of the host membrane, such as its “fluidity” [86–89], and this in turn was found to be sensitive to the chemical composition and cholesterol content of the host membrane [84,86,90–92]. The influence of phosphatidylethanolamines [93], phosphatidylserine [94] and phosphatidic acid

was also studied in detail [63,89,94–97] and, conversely, the influence of the nAChR protein on the physical state of phosphatidic acid-containing membranes [96]. The effect of phospholipid/sterol composition and length and saturation of the phosphoglyceride acyl chains on the conformational states of the nAChR were also the subject of study [62,65,93,98–105]. Acyl chain length determines lipid bilayer thickness, and this physical property influences the orientation of the hydrophobic TM segments of the nAChR [57]. A recent outcome of this series of studies bears on the identification of the functionally “uncoupled” receptor conformation (daCosta et al., 2009 and see below). Phosphatidylcholines lack specificity for the nAChR [44,52,106] and nAChR reconstituted in phosphatidylcholine membranes lacking “activating” lipids is stabilized in the uncoupled conformation, from which it can slowly emerge upon ligand binding and transit to the desensitized conformation if the host membrane is thick enough, e.g. with di22:1 acyl chains [64].

Electron-spin resonance (ESR) studies [50–52,107–110] made apparent the occurrence of two distinct signals in ESR experiments with native and reconstituted membranes containing nAChR at relatively high or low concentrations: one signal corresponded to the bulk membrane lipid and the other was interpreted as stemming from the protein-immobilized lipid. These direct interactions between protein and lipid moieties were observed with fatty acids, phospholipids, and sterols in the native membrane environment. Ellena et al. [109] confirmed our findings using reconstituted nAChR. Rousselet et al. [110,111] found immobilization with fatty acids but not with phospholipids. The L_o nature of the nAChR immediate perimeter was further confirmed in native membranes using fluorescence [112] or a combination of fluorescence and single-channel patch-clamp recordings [113]. This series of studies from different laboratories demonstrated that shell nAChR protein-adjacent lipids are relatively immobile with respect to the rest of the membrane lipids and pointed to the existence of phase lateral heterogeneity in membrane lipids much earlier than the concept of “rafts” came into use.

The lipid “raft” hypothesis proposes that a subset of specific lipid species self-associates to form microdomains or platforms that can intervene in protein partition, signaling and other functional properties [114–118]. Early studies on the nAChR proposed the existence of two cholesterol pools in nAChR-rich membranes from *Torpedo*: an easily extractable fraction that influences the bulk fluidity of the membrane and a tightly bound receptor-associated fraction [119]. Evidence has become available indicating that nAChRs interact with cholesterol-rich lipid domains in vitro and in vivo [120–125]. In our laboratory it has also been demonstrated that cholesterol plays a key role in the trafficking of the nAChR along the early secretory [126] and endocytic [127] pathways and also affects nAChR distribution in the plasma membrane [127,128].

The structural requirements for steroid stabilization or disruption of lipid domains containing nAChR were systematically investigated using fluorescence techniques [129] and more recently the purified and reconstituted nAChR was found to partition into roughly similar proportions between L_o and L_d domains [130]. In living cells, the distribution of nAChR diffraction-limited (nano)-clusters at the cell surface appears to follow the same trend, and changes in the cholesterol content modify the proportion of nAChR sub-resolution clusters associated with either L_o or L_d domains [131].

5. Functional effects of cholesterol and steroid on nAChR

The term pLGIC alludes to the two functional properties of this superfamily of receptors/ion channels: 1) ligand binding and 2) ion permeation. Cholesterol and other sterols, and various types of steroids affect both the ligand recognition ability and the ion channel functions of these proteins. Cholesterol and water-soluble analogues modify the equilibrium between the agonist-dependent functional states of the nAChR [86,132–136]. Chol depletion increased the probability of channel opening and the duration of the channel by ~30% already

after 15 min. By contrast, Chol enrichment resulted in an ~33% diminution in the channel mean open time [127]. Since Chol depletion accelerates endocytosis and thus reduces the amount of nAChR at the cell surface [127,137], the changes in channel kinetics (increased open dwell time) may represent a transient compensatory mechanism to maintain AChR function until new receptors are targeted to the cell surface. Whether these interactions are direct or indirect has been a matter of debate (see reviews in [47,138]). Whereas indirect interactions depend on changes in the free energy difference between diverse protein conformational states, apply to membrane proteins in general and are allosteric in nature, direct interactions imply the binding of cholesterol to the protein, sometimes in a stereospecific mode [139]. Where this binding occurs, the exact stoichiometry and the precise nature of the interactions of cholesterol with the nAChR are still not fully elucidated, nor are the mechanisms by which these interactions are finally transduced into the observed epiphenomenological changes in the receptor's ligand binding, i.e. ion permeation.

The effects of steroids have been characterized in detail on two “cousin” members of the pLGIC superfamily: central nervous system GABA_A receptors [140,141] and both muscle-type and neuronal-type nAChRs [50,142–154]. In the central nervous system neurosteroids modulate GABA_A receptors producing sedation, anesthesia, anti-anxiety and anticonvulsive effects. In the case of the neuronal nAChR, steroids, and neurosteroids in particular, also affect nAChRs. The neurosteroid 17 β -estradiol potentiates $\alpha 4\beta 2$ nAChRs, but not rat $\alpha 4\beta 2$ nAChRs [150,151]. Glucocorticoids are also modulators of nAChR channel function, and an allosteric mechanism was proposed to account for the mechanism of action of these ligands [144]. Using an alanine-substituted quadruple mutant of four putative lipid-exposed residues in TM4 (Leu411, Met415, Cys418 and Thr422) Garbus et al. [153] dissected the effect of hydrocortisone into four Ala-substituted receptors, and found that the Thr422, a residue located close to the extracellular-facing membrane hemilayer in the α -subunit TM4, had direct bearing on the inhibition exerted by hydrocortisone on the receptor. It is interesting to note that the interface between the α and β subunits in the TM domain of the GABA_AR, in the same region as the Ivermectin site in the nematode GluCl protein, has been identified through mutagenesis as the probable site of action of allopregnanolone and THDOC, two neurosteroids that display positive allosteric modulatory effects [155].

6. The “uncoupled” nAChR conformation

Baenziger and coworkers have extensively studied the conformational equilibria of the nAChR and the influence of lipids on nAChR state transitions [104]. This group has discovered that *Torpedo* nAChR reconstituted into phosphatidylcholine membranes lacking cholesterol and anionic lipids adopts a conformation in which agonist binding is uncoupled from channel gating [62], reviewed in [64]. These authors have also systematically explored the contributions of anionic lipids like phosphatidic acid to this phenomenon [63,65,94,95] and in particular the mechanism for re-activating uncoupled nAChRs back to their functional state, with interesting biomedical implications [65]. Hénault et al. [67] and Mowrey and coworkers [35] have recently reviewed the structural studies on the bacterial homologue ELIC, the crystal structure of which exhibits many of the properties purported to correspond to the uncoupled conformation, as supported by electrophysiological data [156]. Further insight into the “uncoupled conformation” of the nAChR and its functional implications can be found in another review of this series (Ref. Baenziger et al., 2015).

7. The search for cholesterol sites on the nAChR and pLGIC

The three-ring TM region of the pLGIC is the site of action of a variety of chemical substances, including the endogenous lipids present at the host membrane –essentially phosphoglycerides and cholesterol, and exogenous compounds of pharmacological interest, ranging from anti-

emetics to anti-epileptics or cognitive enhancing drugs. The latter fall within the category of positive allosteric modulators and in the case of the nAChR, and the neuronal $\alpha 7$ subtype in particular, these modulators comprise PNU-120596, TQS, and various other chemically diverse compounds purported to bind to sites at the TM domain and slow down the rate of desensitization at the molecular level, while having cognitive enhancing effects on brain function [12]. The search for site(s) and mechanism(s) of action of cholesterol on the nAChR and other members of the pLGIC has made the researchers interested in the field for quite some time [45,86,92,136,145,146,149,157–166]. Electron–spin resonance, fluorescence quenching and reconstitution of purified nAChR have provided useful information on this topic. Photoaffinity labelling techniques have been the experimental approach of choice in the search for cholesterol binding sites on the nAChR protein. Early experiments were targeted at the characterization of labelling to the intact subunits [145,157] and/or employed photoactivatable sterols that were purported to be functional cholesterol analogues [149,166]. More recent photoaffinity labelling studies confirmed earlier results and led to the identification of putative cholesterol–nAChR interaction sites at the TM4, TM3, and TM1 segments of each subunit, fully overlapping the lipid–protein interface of the nAChR [159]. The TM4 segment showed the most extensive interaction with the cholesterol analogues. For α TM4, the labelling pattern was consistent with the incorporation of an azido derivative of cholesterol into α Glu-398, α Asp-407, and α Cys-412, i.e. amino acid residues that lie in a rather shallow region in the NH-term of the α TM4 transmembrane segment. Using fluorescence quenching techniques, sterol sites were also identified in all TM regions of native nAChRs, and their depth in the membrane bilayer could also be established [167].

The search for cholesterol sites on the nAChR has also relied on in-silico approaches. Molecular dynamics simulations of the nAChR in the presence or absence of cholesterol led Brannigan et al. [168] to conclude that the nAChR possesses multiple cholesterol binding sites, some of which correspond to non-annular cavities located between the nAChR transmembrane α -helices, deeply buried in the protein, and that the nAChR collapses in the absence of the sterol. Their argument is based on the observation of “holes” in the electron density maps of the nAChR cryoelectron microscopy images of Unwin [21], which could accommodate up to 15 cholesterol molecules. From ESR experiments [106] we calculated that the outer perimeter of the nAChR could accommodate up to 15 cholesterol molecules which readily exchanged with bulk lipids in these experiments; this would not occur in the case of the deeply buried cholesterol postulated from in-silico calculations. More recent works from our laboratory resulted in the identification of cholesterol recognition motifs in the TM region not only of the nAChR and other members of the pLGIC, but also conspicuously present in other transmembrane proteins such as the GPCR superfamily [163, 169] (Fig. 2).

Cholesterol sites have been recently identified at the TM surface of the human GABA_AR based on the structure of the glutamate-gated GluCl of *C. elegans* [162] using a combination of several molecular modelling approaches, one of them based on the analogy with the Ivermectin molecule docked on GluCl crystals [170].

8. Presence of lipid and general anaesthetic sites in the bacterial homologues of pLGIC

The cyanobacterium *Gloeobacter violaceus* does not possess cholesterol. However, the X-ray studies of Nury et al. [8,171] on the nAChR prokaryotic homologue GLIC have revealed that binding of general anaesthetics occurs at a site located at the protein–lipid interface where nearby ordered lipids were previously identified [4,5,172] (see Fig. 1). One of the lipid molecules lies in a crevice between TM1 and TM4; this lipid is observed in two of the structures (apo and des-flurane) but is displaced in the structure when the general anaesthetic propofol is bound to GLIC. General anaesthetics such as propofol and desflurane

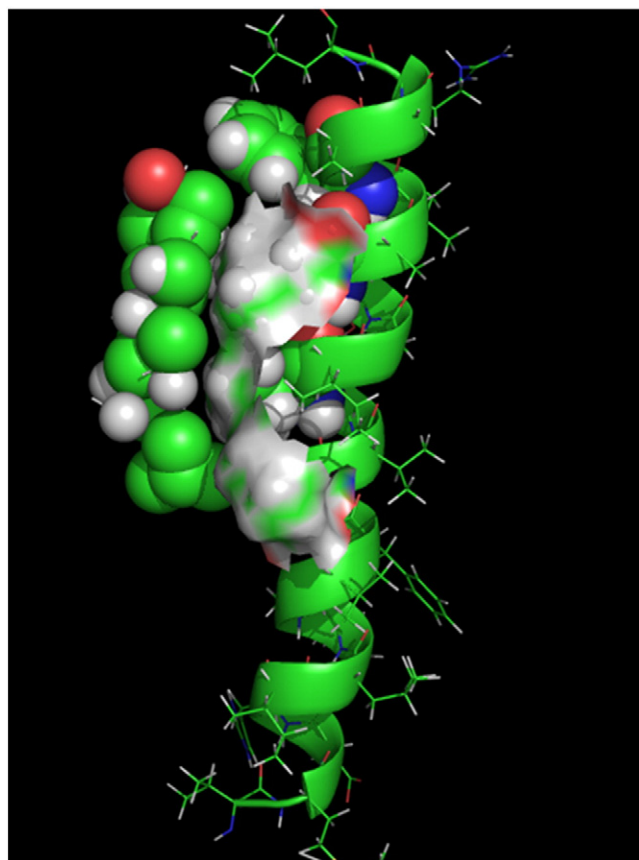


Fig. 2. A cholesterol molecule docked on the surface of the CARC cholesterol recognition domain [163,169] in the TM4 of the *Torpedo* nAChR α -subunit. The polypeptide chain is shown in line/ribbon representation except for the area in the proximity of the bound cholesterol, which is displayed in surface-rendering mode. The lipid is shown in CPK rendering.

are known to be positive allosteric modulators of GABA_A receptors in eukaryotic pLGIC [10] and exert the opposite action, i.e. negative allosteric modulation, on prokaryotic GLIC [173]. The lipids identified in the crystal structure were not exogenous lipids added in the course of purification/crystallization, and were therefore interpreted as endogenous lipids present in the native membrane where GLIC is inserted, indicating a tight binding between the protein and lipid moieties [4]. It has also been suggested that the alteration of GLIC–lipid interactions caused by binding of the general anaesthetic molecule might contribute to functional inhibition [171]. In the case of the eukaryotic GluCl from *C. elegans*, phospholipid molecules have recently been identified in the crystal structure, as shown in Figs. 3 and 4. The phospholipids compete with the drug Ivermectin and potentiate glutamate binding to the GluCl channel. The binding site is located between TM1 and TM3 helices of adjacent subunits, with the phosphocholine head group pointing towards the center of the pore and the two alkyl tails at the periphery of the transmembrane domain [31]. In the prokaryotic organism ELIC general anaesthetics bind more promiscuously to three different types of sites. The binding site located at the ion channel proper (the pore site, “PS” in Fig. 5) is presumably responsible for the non-competitive type of blockage that general anaesthetics exert on ELIC and other ion channels [174]. A second site, (“TS”) is present at the TM region (Fig. 5), in a crevice formed at the interface between two subunits. The walls of the crevice are formed by the middle (TM1) and outer (TM4) rings of one subunit and the middle ring (TM3) of another subunit. General anaesthetics may thus compete with endogenous lipids in the case of GLIC or ELIC, but also possibly with cholesterol [43] and/or neurosteroids [155,175] in the case of eukaryotic pLGICs such as the nAChR or

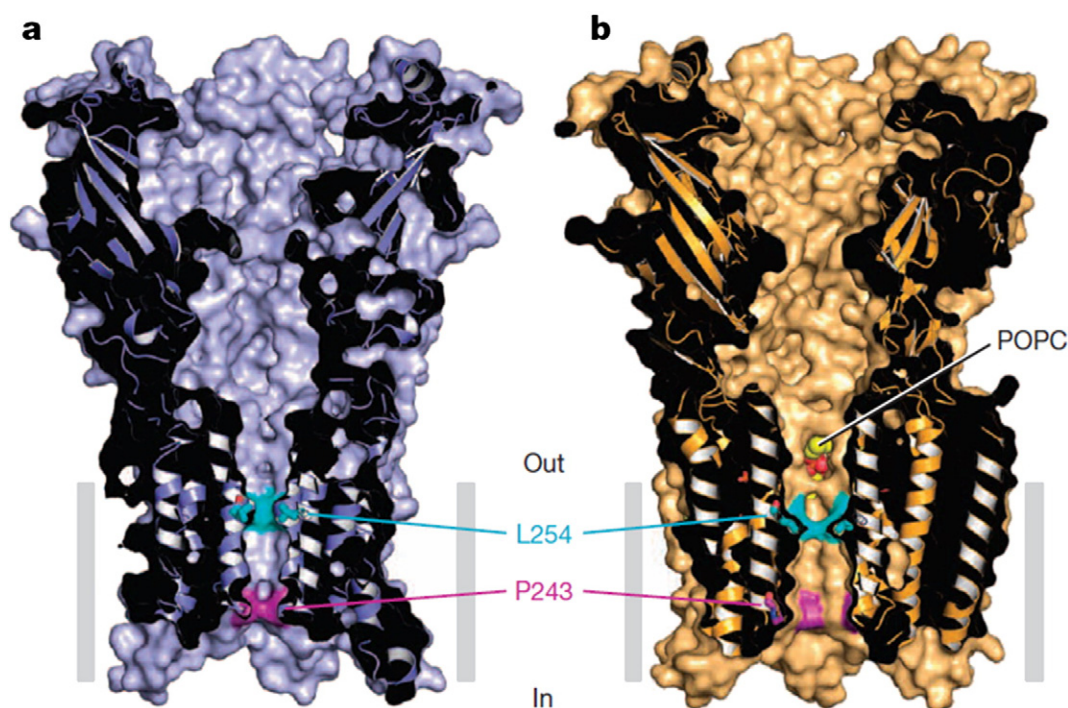


Fig. 3. Recent crystallographic studies of Gouaux and his group [31] have established the atomic position of phosphatidylcholine (POPC) in the pentameric GluCl channel of the nematode *C. elegans*: The head group of one of the POPC molecules (yellow ball) is shown wedged between the TM1 and TM3 transmembrane helices, that is in the middle ring motif [36] common to all pLGIC (from [31]).

GABA_A receptors (see previous sections). It is interesting to note that general anaesthetics exert the same type of pharmacological effect on GLIC and nAChRs: both are inhibitory (see review by [10]. By contrast, glycine and GABA_A receptors are mostly potentiated by general anaesthetics. Using MD simulations, Nury et al. [171] also showed changes in the lipid cavities caused by motions of TM2 and TM3, suggesting that the shape and volume of the cavity are coupled to channel gating. The observation of changes in the shape of the lipid cavities in GLIC upon binding of the general anaesthetic propofol was also reported recently by Gosh et al. [176], who challenge the recent crystallographic data of Nury et al. [171], maintaining that in the resting state propofol does not bind in the TM domain intrasubunit cavity as observed in the crystal structure of GLIC with bound propofol.

9. Perspectives and future developments in the field

During the last few decades experimental evidence has provided a solid foundation to the assertion that the most important consequence of the cross-talk between the membrane lipid environment and the nAChR is the functional modulation exerted by lipids on the protein. nAChR functional properties affected by the membrane surroundings encompass the processes of channel gating, the kinetics of opening and closing of the channel proper, the kinetics of desensitization, the supramolecular distribution and translational dynamics of the protein at the cell surface (reviewed here and in [43, 177]). The availability of high resolution X-ray diffraction structures of various representative members of the pLGIC in which the

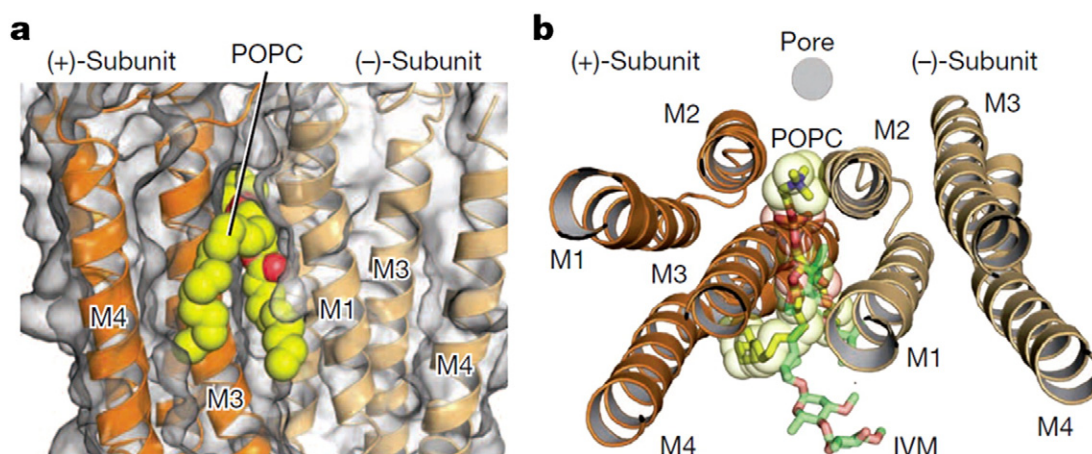


Fig. 4. Location of the phospholipid POPC at one of the inter-subunit sites in GluCl. Phospholipids compete with the antiparasitic drug Ivermectin and potentiate glutamate binding to the GluCl channel of the nematode *C. elegans*. a and b, POPC binding site between TM1 and TM3 helices of adjacent subunits viewed parallel (a) and perpendicular (b) to the membrane. The location of the pore in (b) is shown by a gray circle and the overlap between the phospholipid and Ivermectin is displayed in 'stick' representation. The phosphocholine head group points towards the center of the pore and the two alkyl tails are located at the periphery of the transmembrane domain. From Ref. [31].

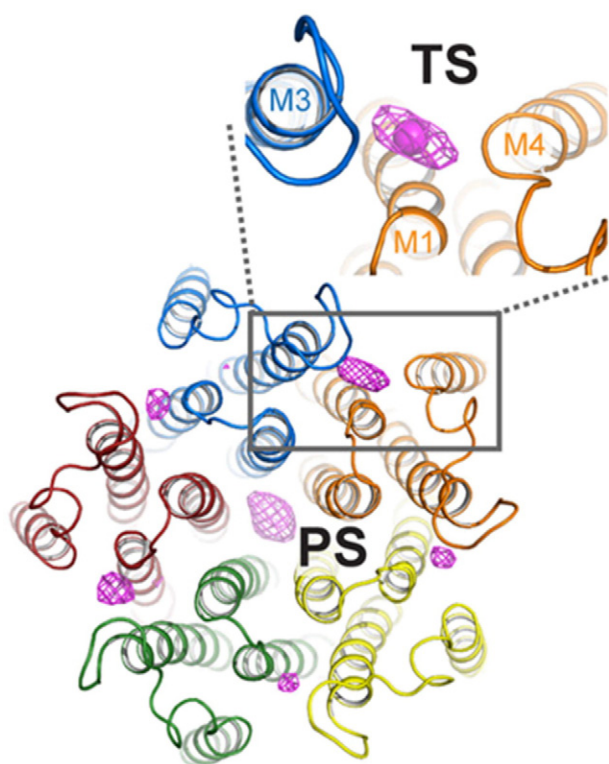


Fig. 5. Transmembrane middle (TM1–TM3) and outer (TM4) rings of the bacterial homologue ELIC provide binding sites not only for lipids but also for a variety of pharmacologically relevant compounds, like general anaesthetics. Here the TM binding site (“TS”) of a chloroform derivative (magenta) is formed at the interface between two subunits. The walls of the crevice are formed by the middle (TM1) and outer (TM4) rings of one subunit (orange) and the middle ring TM3 polypeptide chain of another subunit (blue) in the pentameric bacterial homologue ELIC. An additional binding site (pore site, “PS”) for the general anaesthetic lies within the ion channel, and is presumably responsible for the non-competitive type of blockage. From Spurny et al. ([174]).

presence of lipids, anaesthetics or pharmacologically important compounds is apparent, as reviewed here, will likely continue to expand our comprehension of the detailed structure–function correlations needed to understand the physiology and dysfunction of the nAChR and other members of the pLGIC.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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