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Structure and function meet at the nicotinic acetylcholine receptor-lipid interface

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ABSTRACT

The nicotinic acetylcholine receptor (nAChR) is a transmembrane protein that mediates fast intercellular communication in response to the endogenous neurotransmitter acetylcholine. It is the best characterized and archetypal molecule in the superfamily of pentameric ligand-gated ion channels (pLGICs). As a typical transmembrane macromolecule, it interacts extensively with its vicinal lipid microenvironment. Experimental evidence provides a wealth of information on receptor-lipid crosstalk: the nAChR exerts influence on its immediate membrane environment and conversely, the lipid moiety modulates ligand binding, affinity state transitions and gating of ion translocation functions of the receptor protein. Recent cryogenic electron microscopy (cryo-EM) studies have unveiled the occurrence of sites for phospholipids and cholesterol on the lipid-exposed regions of neuronal and electroplax nAChRs, confirming early spectroscopic and affinity labeling studies demonstrating the close contact of lipid molecules with the receptor transmembrane segments. This new data provides structural support to the postulated "lipid sensor" ability displayed by the outer ring of M4 transmembrane domains and their modulatory role on nAChR function, as we postulated a decade ago. Borrowing from the best characterized nAChR, the electroplax (muscle-type) receptor, and exploiting new structural information on the neuronal nAChR, it is now possible to achieve an improved depiction of these sites. In combination with site-directed mutagenesis, single-channel electrophysiology, and molecular dynamics studies, the new structural information delivers a more comprehensive portrayal of these lipid-sensitive loci, providing mechanistic explanations for their ability to modulate nAChR properties and raising the possibility of targetting them in disease.

1. Introduction

Cholinergic synapses in the central and peripheral nervous system are complex molecular machineries that despite differing in their detail, share two fundamental components: the natural neurotransmitter acetylcholine (ACh), a small organic molecule (146.2 M.W.) synthesized in and released from the presynaptic nerve ending, and the nicotinic acetylcholine receptor (nAChR), the key component of the cholinergic postsynaptic apparatus. The primary role of this large membrane-bound macromolecule (Mr ~ 290 kDa) present in the muscle endplate [1], the electric fish electromotor synapse [2], and neurons [3], and to a lesser extent in non-neural tissues [4], is to decode the chemical signal encrypted in the simple ACh molecule and transduce it into an ion-mediated electrical signal at the target membrane of excitable tissues. ACh also interacts with the structurally and functionally distinct muscarinic receptors [5,6], i.e., metabotropic receptors that belong to the large G-protein coupled receptor (GPCR) superfamily [7], the most pharmacologically targeted therapeutic class of membrane-bound proteins [8].

There are two families of neuronal nAChRs: homomeric and heteromeric. Homomeric nAChRs are built from five identical α7 or α9 subunits; heteropentameric neuronal nAChRs result from multiple combinations of α and β subunits [9–12]. The most abundant neuronal nAChRs in the mammalian brain are heteropentameric α4β2 receptors and homopentameric α7 nAChRs, the latter composed exclusively of α subunits [13,14]. The α4β2 receptors exhibit two possible stoichiometries (2α:3β and 3α:2β) [15] and carry two orthosteric binding sites at the α/β subunit interfaces, whereas homomeric nAChRs have five orthosteric ligand-recognition sites plus allosteric binding sites [16]. Both types of receptors are primarily localized at pre- and peri-synaptic compartments, and participate in key brain functions like memory, learning and cognition [17–20]. The α4β2 has been implicated in

Abbreviations: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine.

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long-term potentiation, the synaptic mechanism common to these higher brain functions, including working memory, speed of processing, and social cognition [21–23]. α7 nAChRs also play roles in neurodevelopment [24,25], neuroprotection [26–30], and in counteracting inflammation [28,31–33]. Their distinct functional properties -in particular their mode of activation and desensitization- offer a wider range of possibilities to up- or down-regulate them via their allosteric rather than their orthosteric sites, thus augmenting pharmacological selectivity [34]. Therapeutic strategies based on potentiation of the α7 nAChR in neurodegenerative diseases like Alzheimer and Parkinson diseases have recently been examined [35]. For other nAChR subtypes see [3,16]; for the specialized α9/α10 in hair cells see [36]. The muscle-type nAChR, present in the muscle endplate and the electric fish electromotor synapse, is the pharmacological target of the competitive antagonist curare and synthetic derivatives widely used in anesthesiology [37,38]. Autoimmune diseases like myasthenia gravis and myasthenic syndromes preferentially aim at the peripheral nAChR protein, though other postsynaptic proteins can be the focus of the autoimmune atack [39–41].

This review critically analyzes the current status of our knowledge of the nAChR-lipid interface, focusing on the recently available atomistic structural data and the mechanistic insights that the former provide to understand receptor function. The analysis also illustrates from a historical perspective how advances on this topic are intimately linked to the development and successful application of experimental biophysical methodologies. Due to space limitations several relevant aspects of lipid-receptor interactions are not dealt with in this review and for which the reader is referred to related work: membrane organization in general [42] and of the postsynaptic membrane [43], membrane nanodomains [44] and nAChR nanodomains in particular [45], the influence of lipids on membrane-embedded proteins and ion channels [46,47], the effect of the lipid nanoenvironment on neurotransmitter receptors [48], the action of individual lipid classes on the nAChR, e.g., fatty acids [49], involvement of lipids in diseases of the neuronal nAChR such as Alzheimer and Parkinson diseases [50–54], or autism spectrum disorders [55–57]. Another important facet of the role of nAChRs in the central nervous system concerns nicotine dependence and alcohol, morphine, and cocaine addiction, and the potential targetting of these receptors for pharmacological intervention in cessation of drug use [58–67]. The link between nAChRs and lipids in addiction is exemplified by the crosstalk between nicotinic receptors and the G-protein-coupled endogenous cannabinoid receptors in midbrain, hippocampal and amygdala synapses, and the on-demand produced lipid endocannabinoids, N-arachidonoyethanolamine (anandamine) and 2-arachidonoylglycerol that operate on the endocannabinoid receptors, modulated by nicotine [68].

2. Evolutionary pedigree of the nAChR and associated lipid

Digging into the evolutionary history of molecules is a fascinating endeavour. Akin to molecular anthropology and paleontology research, one has to travel back millions of years to establish the origin and kindredness of molecules and to understand how optimal structural designs occurred early in phylogeny and were then adopted. The nAChR protein is one such case; it is a member of the large superfamily of pentameric ligand-gated ion channels (pLGICs) that evolved from a common proto-receptor more than 4000 million years ago. pLGICs comprise several families of evolutionarily related membrane proteins coded by a few dozen genes [69,70]. The actual discovery of the *Erwinia chrysanthemi* and *Gloeobacter violaceus* prokaryotic pentameric ion channels was a seminal contribution to establishing the kindredness of these proteins and their metazoan eukaryotic counterparts [71].

pLGICs are ubiquitously represented in major taxonomic groups with the exception of multicellular plants and fungi and are present sparingly in unicellular taxa, suggesting a non-essential character and a high rate of gene loss [72]. In most metazoan and all vertebrate members of this superfamily, pLGICs are also known as Cys-loop receptors because in their amino-terminal extracellular halves, their subunits contain a pair of disulphide-bonded cysteines separated by only 13 residues. This conserved loop, which has a key role in coupling agonist binding to ion channel gating [73], was independently discovered in 1987: the strychnine-binding subunit of the glycine receptor was found to possess such a loop and exhibited homology with nicotinic receptors [74] and the sequencing analysis of the γ-aminobutyric acid type A ($GABA_A$) receptor showed a similar S-S bond and similarities to other neurotransmitter receptors [75]. “Cys-less” receptors devoid of Cys-loop cysteines are common in prokaryotes and invertebrate metazoans [72]. Hénin and coworkers indicate that “true” Cys-loop receptors form a subgroup within pLGICs that acquired their character and have conserved it ever since [72].

Within the pLGIC superfamily, the nAChR and the subtype 3 of the 5-hydroxytryptamine (serotonin, $5\text{-}HT_3$) receptor comprise two families of cation-selective channels, whereas glycine receptors -among the latest to be conferred neurotransmitter receptor status [76]- and the most abundant inhibitory receptor in the central nervous system, the ($GABA_A$) receptors [77] are anion-selective channels. Introduction by site-directed mutagenesis of three amino acids from the M2 segment of the glycine or the $GABA_A$ receptors into the homologous segment of the homopentameric α7 neuronal nAChR suffices to convert the ACh-gated cation-selective channel into an anion-selective channel [78]. Another example of this type of conversion is given by the homopentameric nAChRs from *Lymnaea,* which can adopt cationic or anionic ion selectivity, suggesting that molluscan anionic nAChRs probably evolved from cationic ancestors [79]. For a review of charge-selectivity for Cys-loop receptors in general see ref. [80].

Another manifestation of kindredness among pLGICs is the common basic mechanism of signal transduction. The nAChR can exist in several interconvertible conformational states associated with different functional states. The release of ACh from the presynaptic terminal finds most postsynaptic receptors in the so-called resting state. Upon binding to the receptor protein, ACh triggers a conformational change leading to the transient opening of its intrinsic cation-specific channel across the postsynaptic membrane. This is accomplished by the concerted action of an array of five different but highly homologous combinations of α and β subunits in neuronal-type nAChRs and additional subunits δ and γ (or ε) in the stoichiometry α2βγδ in embryonic or α2βεδ in adult skeletal muscle, respectively [81]. The four muscle-type nAChR subunits share a common muscle-type β-subunit ancestor. β-subunits are the evolutionarily less conserved subunits, and are the only subunits that do not contribute to the two orthosteric agonist binding sites in the receptor monomer. Recently daCosta and coworkers reconstructed this ancestral receptor that forms homopentameric ion channels, opens spontaneously, and displays the single-channel properties of the muscle-type nAChR [82]. These functional properties are agonist-independent, leading these authors to speculate that agonism evolved subsequently as an additional layer of regulation in the pLGIC superfamily.

As we will discuss in the section on nAChR structure, the full structure of three neuronal nAChRs has been resolved in resting, activated and desensitized conformations [83–85]. An interesting intersection of evolutionary aspects and structure of neuronal receptors is provided by a recent work employing functional divergence analysis on nine neuronal nAChR subunits from vertebrate species. Specifically, 44 unique residues were identified for the homomeric α7 nAChR, and 22 residues that were likely candidates for the specific features of other heteromeric nAChR subunits were also identified [86]. By mapping these sites onto the 3D structure of the human α7 nAChR, the functional divergence-related sites were found to cluster in the ligand binding domain, the β2-β3 linker close to the N-term α-helix, the intracellular linkers between transmembrane domains, and the "transition zone". Pan and coworkers conclude that these divergent sites may have undergone altered evolutionary rates. They further speculate that the former two regions may be potential binding sites for α7 subtype-specific allosteric modulators, while the latter region is likely to be a subtype-specific

allosteric modulatory site in heteropentameric descendants such as the α4β2 nAChRs [86].

In relation to the core subject of this review, the molecular architecture of the nAChR membrane-embedded domain is also conserved in its overall arquitectural design (see review in [87] and section below), both within and across species: each chain contains four membrane-spanning hydrophobic segments, 20–30 amino acids in length, referred to as M1-M4. Of these, the innermost M2 segment from each subunit is the key contributor to the walls of the ion channel proper, with additional involvement of M1 and M3, whereas M4, the transmembrane segment most exposed to the bilayer lipid, does not directly participate in the ion permeation pathway, although it is allosterically connected to the M1 segment, modifying energetically the motions of the latter [88]. M1 and M3 effectively incorporate membrane-partitioning photoactivatable probes and are also exposed, at least partially, to the lipid phase, as analyzed below. Thus, despite the wide variability of the amino acid residues at the protein-lipid interface in pLGICs, this region appears to be functionally conserved from prokaryotes to eukaryotes [89,90], pointing to the early stabilization of optimal structural designs that serve their purpose.

3. The lipid-nAChR interface

Electron spin resonance (ESR) spectra of lipids bound to integral membrane proteins or motionally hindered by interactions with their surface can be resolved from lipids in the fluid bilayer regions of the membrane. ESR studies supplied the initial evidence of motionally-restricted lipid in contact with the nAChR in native *Torpedo* membranes [91–93]. The direct contact between receptor and vicinal or boundary lipid was confirmed in subsequent ESR experiments using reconstituted nAChR [94]. A minimal number of 45 lipid molecules per nAChR molecule were estimated to be necessary to support ion flux activity; below that figure, inactivation occurred [95]. Jones and McNamee used brominated sterol to define these "annular" lipid sites and found 5–10 "non-annular lipid" sites per nAChR monomer having ~20-fold higher affinity for cholesterol [96]. Ellena et al. showed that another sterol, androstanol, displayed a higher selectivity for the nAChR (relative to phosphatidylcholine), with a total of 38 sites per nAChR [94]. The distinction between the less mobile receptor-associated fraction of lipids and the bulk membrane lipids was subsequently corroborated using fluorescence spectroscopy [97,98]. ESR studies also produced early evidence of nAChR-immobilized phosphatidic acid [94].

The spectroscopic studies described above did not provide information on the sidedness of the lipid sites. This was dealt with in an early molecular modelling study in which 5 cholesterol molecules were docked on the lipid-exposed surface of the nAChR in crevices between M1, M3 and M4 from adjacent subunits in each leaflet of the membrane, making a total of ten sterol sites per receptor molecule [99]. The functionally important amino phospholipid phosphatidylserine (13% of the *Torpedo* electrocyte lipids [100]) and phosphatidylethanolamine were found predominantly (ca. 80%) at the cytoplasmic-facing membrane hemilayer of rat myotubes in areas where nAChRs occurred [101]. Selective enzymatic hydrolysis with sphingomyelinase showed that about 60% of the sphingomyelins, another minoritary lipid species (~5% of *Torpedo* electrocyte lipids), predominate in the outer leaflet [102]. Recent work on the prokaryotic pLGIC, ELIC, demostrated that the anionic phospholipid phosphatidylglycerol exerts a modulatory action on channel activity at the outer leaflet of the membrane [103]. A single phosphatidylglycerol molecule appears to suffice to stabilize the open-channel (activated) conformer of ELIC. Phospholipid asymmetry may be a more general property, shared by members of the LGIC superfamily, a subject worthy of further investigation.

Ordered lipids - so-called lipid "rafts"- have been described as necessary for the maintenance of α7-type neuronal nAChRs in somatic spines of ganglionic neurons [104]. Other studies have suggested that such ordered lipid domains are required for the clustering of muscle-type receptors in muscle cells [105–107]. Some authors have indicated that it is necessary to trigger the clustering phenomenom in these discrete lateral lipid heterogeneities by a "pulse" of agrin [108,109]. The distinction between membrane fractions that are soluble or insoluble in the detergent used to extract integral membrane proteins from membranes has been operationally employed to define the biochemical counterpart of a "lipid raft" in a live cell or a liquid-ordered (lo) phase in synthetic lipid mixtures. Using lipid mixtures mimicking the composition of a lo phase (1:1:1 cholesterol:palmitoyloleoylphosphatidylcholine:sphingomyelin), no preferential partitioning of the nAChR into this mixture was found [110]. Induction of compositional asymmetry across membrane hemilayers by varying the sphingomyelin composition produced a relative enrichment of the receptor in the lo lipid mixture [111]. It should be stressed that these two studies explored a rather limited set of lipid mixtures, and the detergent solubility criterion is too broad to extrapolate to the conditions present in the cell ([112,113].

A systematic coarse-grained in silico study of the influence of lipid composition on the local composition around the nAChR tested more than 70 different combinations of lipids and concluded that the receptor partitions into a liquid-disordered phase (lo) containing polyunsaturated fatty acids (PUFAs) whenever such phase was present in the simulations [114]. These authors also found that the receptor-vicinal lipid was enriched in the PUFA docosahexahenoic acid. The molecular dynamics study, however, was conducted using a single receptor molecule, thus providing interesting clues as to the type of lipids that may play a role in receptor assembly at low expression levels, such as during embryonic development. In contrast, at early stages of postnatal life and definitely at the mature synapse, nAChRs are within nanometer distances from each other in the plane of the membrane and crosstalk extensively with other receptors and vicinal lipids. This raises the question of the extent of the lateral "area of influence" of the nAChR on adjacent lipids in the more complex arena of a synapse. How many layers of lipid are under the influence of the macromolecule? Between embryonic day 13 and E14 nAChRs form nanoclusters [115], a process that constitutes the beginning of their "socialization" [45], driven by lateral translational motion. At these stages, amenable to study in mammalian heterologous expression systems, nAChRs displace laterally following complex cholesterol-dependent motional regimes, ranging from subdiffusive to superdiffusive motions [116–118]. In postnatal life, muscle-type nAChRs form much larger platforms that eventually lead to the tightly-packed 2D lattice observed in the adult myotube and the mature neuromuscular junction [119], where receptors occur at densities of 10,000–20,000 μm^2 and are essentially immobile in global terms [120]. As pointed out in a previous review, lipids occupy the relatively small interstices between receptor macromolecules [87]; the postsynaptic membrane is not a sea of lipids with isolated receptor molecules floating around, but rather a densely packed protein assembly with lipids filling in the spaces left in between proteins. Since even the highly clustered synaptic nAChRs constitute a disordered 2D array, the degree of lipid immobilization imposed by the protein may vary as a function of the distances between receptor monomers (and dimers) in the lattice. This is so because the first-shell lipid layer surrounding a given receptor monomer is separated by only a few layers from an homologous layer surrounding a different neighbouring nAChR protein [87]. Thus, the notion that "sorting" over the 5–20 nm range is primarily driven by intrinsic differences in membrane organization that would be observed without the receptor [114] may be tenable for nAChR in developing but not in adult synapse. Likewise, the asumption that single receptor-lipid interactions can be extrapolated to multiple receptors in a liquid-disordered domain will require experimental validation. Super-resolution optical microscopy has shown that receptors occur at the plasmalemma both as isolated particles and nanoclusters [117,121,122], a characteristic and dominant feature of membrane-embedded proteins [123] and many neurotransmitter receptors in the central nervous system [124–126].

While limited information on lipid selectivity is available for the *Torpedo* nAChR (see e.g., [127] and review in [87]), the selectivity of the neuronal nAChR is even less known. Explored in silico with molecular dynamics simulations, in all synthetic lipid systems in which domain formation occurred, the nAChR partitions preferentially into cholesterol-poor ld domains, and receptor vicinal lipids are dominated by those lipids in the ld domain, particularly PUFAs [114,128]. More recent coarse-grained molecular dynamics simulations from the group of Brannigan in a neuronal-mimicking membrane containing more than 30 lipid species [129] led to the determination of affinities for two types of sites on the neuronal nAChR: i) for the deeper inter-subunit site formed by M1 and M2 of one subunit and M2 and M3 of the adjacent subunit [130], cholesterol is preferred over phospholipids at both membrane hemilayers, with stronger affinity for the exoplasmic-facing hemilayer. Phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholines (PC) have similar affinities and significantly stronger affinities for these sites than the phosphoinositides PIP1, PIP2, PIP3, which have, in turn, a significantly stronger affinity than phosphatidic acid. From strongest to weakest, they follow the order: PE > PI ~ PS ~ PC ≫ PIP1 ~ PIP2 ~ PIP3 ≫ PA. In contrast, at the M4 site, PI has significantly stronger affinity than PS, and PS has a significantly stronger affinity than PC. ii) In the case of the M4 site, the order of affinities, from strongest to weakest, is: PE > PI > PS > PC ≫ PIP1 ~ PIP2 ~ PIP3 ~ PA [129]. In addition, these authors found that cholesterol selects for concave-shaped inter-subunit sites, whereas n-3 PUFAs have the strongest affinity for convex M4 sites (n-3 > n-6 > monounsaturated > saturated fatty acids). Interestingly, although cholesterol apparently prefers inter-subunit rather than M4 sites, it has the second strongest affinity after the top ranking n-3 PUFA at the M4 sites.

4. Structural studies of the nAChR and beyond: architectural prototyping of pLGICs

A rather limited number of high-resolution structures of membrane proteins were available until recently, mainly because of the inherent difficulties in obtaining three-dimensional crystals of sufficient size and order for X-ray diffraction studies. During the 20th century, only glimpses at the molecular structure of the nAChR were available, obtained primarily through electron microscopy (EM) of electric fish electroplaque membranes, the gold standard of the pioneer structural investigations. The nAChR was not only the first neurotransmitter receptor to be biochemically isolated and characterized in the early 1970´s [131–133] but also the first to be studied with biophysical methods that provided information on the common structural features of the pLGIC superfamily.

In one of the earliest studies on *Torpedo marmorata* native membranes and detergent-solubilized membranes from *Electrophorus electricus*, Changeux and coworkers showed negatively stained regions containing small ordered 2D arrays in the *Torpedo* membrane fragments and isolated 80–90 Å doughnut-shaped particles (“rosettes”) with a stain-filled core [134] that were consistent with the available hydrodynamic data at the time, e.g., Stokes radius of the isolated receptor in ultracentrifugation studies [135,136] and low-angle neutron scattering [136]; a prolate ellipsoid of ~240 kDa. X-ray diffraction data at 20 Å resolution crystals showed that this body traversed the postsynaptic membrane, extending 15 ± 5 Å on one side of the bilayer and ~ 55 ± 5 Å on the other, with an overall length normal to the membrane of 110 Å [137]. Native and reconstituted membranes from *Torpedo californica* also showed the characteristic rosettes [138]. Negatively-stained tubular structures subsequently established the orientation of the nAChR in the plane perpendicular to the membrane normal, indicating that the larger protruding mass corresponded to the extracellular moiety [139]. Freeze-etching electron microscopy showed that arrays of ordered particles of variable size were also present in the intact ventral, innervated *Torpedo* electroplax postsynaptic membrane [140,141].

Alain Brissońs Ph.D. work at Grenoble showed that in vitro ageing of *Torpedo* nAChR-rich membrane fragments led to the spontaneous aggregation of receptor ribbons ending within 3–4 weeks in the formation of a surface lattice enclosed in tubules, that within another couple of weeks, exhibited enhanced order relative to the native membrane vesicles [142]. These long, thin (up to 1.2 μm) 2D surface arrays of receptor particles were far better ordered than native membranes and tubules obtained in previous studies [139], showed a p2 plane group symmetry, and were amenable to negative-staining EM down to 30 Å resolution that revealed the appearance of a pentameric countour in the individual receptor particles [143]. However, the resolution that could be achieved at the time was limited by the small lateral dimensions of the arrays, by the inability to make them perfectly flat, and by the restricted range of tilt views that could be obtained [144].

Together with Peter Zingheim and Joachim Frank, our approach relied on imaging of negatively-stained, freshly prepared nAChR-rich membrane fragments and application of the approach that Joachim Frank had developed to average single molecules in native membranes (see review in his Nobel Lecture [145]). This approach was absolutely novel in the field of membrane proteins and was received with scepticism. Based on available biochemical evidence, we reasoned that the receptor molecules were pentamers formed by subunits of varying size that may not necessarily form symmetric oligomers, and that native membranes were unlikely to exhibit ordered structure, making the case for single-molecule averaging techniques. A highly positive aspect of the native membranes that we could produce with a gentle isolation procedure was that the receptor protein formed densely-packed 2D arrays of many individual molecules that could be simultaneously subjected to low-radiation dose EM, thus enabling us to collect multiple projections of the particles and analyze them using the novel single-molecule averaging procedures. This approach was employed to image the nAChR at 20 Å resolution [146], characterize the nAChR dimers [147], and localize the two non-symmetric α-bungarotoxin sites at 18 Å resolution [148].

When Jacques Dubochet and coworkers perfected the technique of rapid-freezing specimens in a thin aqueous film, in which water could be retained without dehydration in the high-vacuum column of the electron microscope in a solid amorphous phase instead of ice crystals [149], biological cryo-EM came of age. Applying this technique to *Torpedo* nAChR specimens, some tubules with circular cross-section exhibited vitrified regular helical arrays of the protein, allowing Nigel Unwin to apply the Fourier image reconstruction methods that Aaron Klug had developed for studying helical virus [150] and thus precluding the need to tilt the specimens and facilitating the averaging of well-ordered tubules [151]. The five membrane-spanning subunits could be resolved and shown to lie at pentagonally pseudo-symmetrical positions around the central pore of the molecule over a large fraction of their length and the position of rapsyn, the 43 kDa receptor-anchoring scaffolding protein [152], was established relative to that of the receptor proper. The supramolecular organization of nAChR-rapsyn complexes into a 2D meshwork was subsequently refined using cryoelectron tomography and Fourier transformation [153]. It took several decades to improve the resolution down to 9 Å [154], 4.6 Å [155], and 4.0 Å [156], a structure (PDB 2BG9) that remained as a landmark for several years.

In 2001 Sixma and coworkers produced via X-ray diffraction the crystal structure of the molluscan water-soluble ACh-binding protein (AChBP), a structural and functional homologue of the amino-terminal ligand-binding domain of an nAChR α-subunit [157]. The protomers in the AChBP homopentamer were described as having an immunoglobulin-like topology, with the ligand-binding sites located at each of five subunit interfaces, a structural feature that was later also found in the actual neuronal α7-type homomeric neuronal nAChR molecule [157]. The AChBP is released into the synaptic region in a molluscan cholinergic synapse in response to the natural agonist ACh [158]. AChBP acts as a decoy signal, and is released by glial cells into the synaptic cleft, providing a mechanism through which the glia modulates the efficacy of cholinergic neurotransmission. The availability of the

AChBP crystal structure provided the opportunity to formulate inferences on the agonist recognition site of the nAChR [159–161].

The first truly atomic (1.94 Å resolution) crystal structure of the nAChR, albeit of the extracellular domain only, was reported for the α1 subunit in complex with α-bungarotoxin obtained by the X-ray diffraction technique [162,163]. This was followed by the X-ray crystal structures of the human neuronal α9 extracellular domain and of its complexes with the antagonists methyllycaconitine and α-bungarotoxin at resolutions of 1.8 Å, 1.7 Å and 2.7 Å, respectively [164]. The structures of complexes of nAChR with elapid toxins have been of great interest to understand the action of competitive antagonists. A recent cryo-EM structure of the muscle-type *Torpedo* receptor in complex with ScNtx, a recombinant short-chain α-neurotoxin, bungarotoxin, has thrown light on the dissimilar antagonist action of the former on muscle and neuronal (α7 type) nAChRs [165]. Analogous structural studies undertaken in the presence of *d*-tubocurarine, the active component of the poison arrow toxin curare, have shown that this competitive antagonist stabilizes the electromotor (*Torpedo*) muscle-type receptor in a closed, desensitized-like state independently of the presence or absence of agonist [166]. At variance with α-bungarotoxin, *d*-tubocurarine binds to the two non-equivalent orthosteric sites *and* two allosteric sites.

While most of the recent information on nAChR structure acquired through biophysical studies has relied on X-ray diffraction of 3D crystal samples or cryo-EM of single molecules embedded in lipid nanodiscs, a large proportion of the functionally important intracellular moiety of the nAChR has remained elusive. This due to its inherent structural flexibility, with disordered regions that account for a large section of the domain. The nAChR and many other membrane-embedded signaling proteins require such structural flexibility to accommodate the pleiotropic roles associated with this region (e.g., receptor assembly, trafficking, subcellular localization) and the great variety of downstream interacting partners in the intracellular milieu. For instance, in addition to its ionotropic character, the neuronal α7-subtype nAChR can signal metabotropic intracellular cascades mediated by heterotrimeric G-protein coupled receptors via its intracellular loop [167–170]. Recent work has resorted to nuclear magnetic resonance (NMR) to characterize this large loop of the α7 nAChR, which accounts for ~60% of the intracellular structure, primarily in highly flexible regions as far as 50 Å from the ion pore axis [171].

5. Structural studies of the nAChR transmembrane region

5.1. Early structural studies of lipid sites

The X-ray diffraction study at 2.9 Å resolution of GLIC, the prokaryotic proton-activated ion channel from *Gloeobacter violaceus*, delivered the first pieces of information about sites attributable to lipid molecules in the transmembrane region of a pLGIC [172]. Well-defined electron densities were observed in grooves between M4 and both M1 and M3 close to homologous amino acid residues labeled with hydrophobic probes in the *Torpedo* nAChR [173,174]. To date, a sizeable number of lipid sites have been identified with structural methods in the superfamily of pLGICs [175]. The transmembrane topography of the archetype pLGIC, the nAChR, was first elucidated using frozen nAChR tubular specimens of the *Torpedo* receptor arranged in a regular surface lattice, thus amenable to imaging, Fourier transformation, and 3D maping. High-density regions that corresponded to the presumptive M2 helices known from molecular biology and biochemical studies to line the ion-permeation pore could be identified, and low-density areas of the images were attributed to the M1, M3, and M4 transmembrane segments, proposed to have a β-sheet secondary structure based on their lack of symmetry [154]. The correct helical secondary structure was subsequently gained with the advent of methodological advances such as direct-electron detectors and improvements in software technology that made feasible the extraction of information from low-contrast regions. The extracellular, outer leaflet of the bilayer was found to exhibit a non-uniform distribution of densities at the level corresponding to the phospholipid headgroups, which were interpreted as cholesterol-rich areas [176], and suggested to (i) stabilize the nAChR transmembrane α-helices, and (ii) participate in the ion-channel gating by restricting the mobility of the δ subunit.

5.2. Lipid nanodisc technology and initial crystallization approaches of neuronal nAChRs

The group of Lasalde-Dominici embarked on a systematic search for purification of the nAChR in lipid-mimicking detergents and with the addition of the cholesteryl ester, cholesteryl hemissuccinate [177], optimization of subunit stoichiometry of the expressed receptor [178], and other conditions that paved the way for current studies on the neuronal nAChR exploiting cubic lipid phase / nanodisc approaches [179,180], reviewed in [181]. Advances in nanodisc technology enabled Hibbs and coworkers to apply X-ray diffraction and single-molecule cryo-EM techniques to disclose the crystal structure of the α4β2 nAChR isomers at 3.94 Å resolution in a presumably non-conducting, nicotine-induced desensitized conformation [182]. In the case of the homomeric α7 nAChR, the receptor protein fused to soluble cytochrome b562 was crystallized in various forms, one of them with bound epibatidine, a natural chlorinated alkaloid secreted by the Ecuatorian frog *Epipedobates anthonyi* and poisoned dart frog of the *Ameerega* genus (PDB: 7KOQ) [83]. Only a few months before, the group of Tzartos had solved the structure of the epibatidine-liganded extracellular domain of the human neuronal nAChR α2 subunit [183]. Remarkably, the agonist induces α2 subunits alone to adopt the form of a pentamer.

Subsequent work from Hibb́s group used cryo-EM and subtype-specific Fab fragments as fiducial markers to dissect the structures of the two possible α4β2 nAChR isoforms [15] with stoichiometries 2α:3β and 3α:2β respectively in the same sample, in the presence of the full agonist nicotine [84]. Cecilia Gotti and her group discovered that nicotine, when present at high concentration in the cell culture during, but not after, pentamer assembly, elicits a ~5-fold increase in cell-surface 2α:3β subtype [184]. This is due to the increased stability (i.e., less prone to proteasomal degradation) and trafficking of receptors assembled in the presence of the drug. The opposite condition is found in clinical practice in a form of epilepsy termed autosomal-dominant nocturnal frontal lobe epilepsy [185], where the 3α:2β isoform predominates [186].

5.3. Cholesteryl ester sites identified on neuronal nAChR transmembrane domain

Heteromeric α3β4 nAChRs are highly expressed in certain brain regions, such as the olfactory bulb, hypothalamus, medial habenula and the nucleus interpeduncularis pathway [187,188], where they modulate reward-related behaviours and as such constitute potential drug targets for addiction and, conversely, for aversion [67]. The structure of the 2α:3β isoform of the neuronal (ganglionic) α3β4 nAChR (PDB: 6PV7) followed [85]. Reconstitution of the α3β4 nAChR into nanodiscs employed a mixture of lipids that supported ion channel activity and cholesteryl hemisuccinate, a water-soluble cholesteryl ester that we introduced in nAChR reconstitution studies [189] and was subsequently used by Keith Miller and colleagues [190]. The reconstitution mixture also included phosphatidic acid, a metabolically very active phospholipid in the electromotor synapse [191] that is important for stabilizing the nAChR in its resting conformation [192] and is laterally segregated by the nAChR in reconstituted systems [193]. *Torpedo* nAChR retains ion permeation when reconstituted in the presence of cholesteryl hemisuccinate, as judged by Na^{22} ion flux experiments [189], but partly inhibits ACh-evoked currents in *Xenopus oocytes* [177]. Patch-clamp experiments of HEK293 cells exposed to the cholesteryl ester exhibit briefer mean open times [194]. In their cryo-EM maps Hibbs and

coworkers identified densities that were interpreted as corresponding to the cholesteryl ester molecules at the periphery of the α3β4 nAChR transmembrane domain, at both the M4-M1 and the M4-M3 interfaces of each subunit [85]. Two such putative cholesterol sites had previously been found per receptor subunit along the intracellular half (i.e., cytoplasmic facing) of the transmembrane domain in the case of the α4β2-subtype receptor [84]. The site on the principal subunit was described as "bowl-shaped", with contributions from the M3, M4, and the MX helices: a single non-isosteric amino acid located at the penultimate turn of the M3 helix (Cys292 in β2 and Phe300 in α4 subunits) in the two types of neuronal nAChR subunits. Interestingly, each cholesterol molecule interacted almost exclusively with a given subunit and with another adjacent cholesterol molecule in a pairwise fashion, a motif recently corroborated by Nigel Unwin in the *Torpedo* electromotor nAChR [195] (see below). The position of the cholesteryl ester sites in the α4β2 structures suggests that the orientations of the two molecules at an interface are dictated by the identity of the subunit on the so-called "principal side (+)" of the subunit interface (in reference to cholesterol binding). Walsh et al. state: "*When the principal is β2, the cholesterols are oriented parallel to the pore axis; when the principal subunit is α4, the apical ends of both molecules are tilted relative to the principal face*" [84].

The cryo-EM structure of the homomeric α7-subtype of neuronal nAChR was next solved by the Hibbs and Lindahl's groups in resting (in the presence of the competitive antagonist α-bungarotoxin), active (bound to the agonist epibatidine), or desensitized (bound to only epibatidine) conformations in the presence of soybean lipids:cholesterol (75:25% by w.) nanodiscs [83]. The pose of a positive allosteric modulator (PNU) on the α7 nAChR was in fact calculated through in silico molecular dynamics [83]. The localization of lipids in this receptor subtype was not attempted in this study, but a subsequent work from Erik Lindahl's group in collaboration with the Hibbs'group reported the α7 nAChR structure in the presence of a PNU and the role of cholesterol in receptor desensitization [196] (see below). The actual structure of the α7 nAChR in complex with the PNU-120596 was solved at 3.02 Å resolution a few months later by the group of Changlin Tian [197].

These structural studies on neuronal nAChRs confirmed the overall architecture of the transmembrane helices that was available at low resolution from the *Torpedo* nAChR: the M2 inner ring bundle lining proximally the ion channel pore, the outer ring of M4 conforming the concentric 3-ring motif described for this region of the receptor [198]. The new cryo-EM data further provides strong architectural support to the initial explicit postulation of the "lipid sensor" ability of M4 and the modulatory effect of the lipid milieu, and the outer ring of M4 transmembrane domains in particular, on nAChR function (see below).

5.4. Cryo-EM studies of the muscle-type Torpedo nAChR in lipid nanodiscs and tubules

Given the wealth of information accrued on the muscle-type nAChR, especially on the lipid-dependence of the *Torpedo* electromotor synapse, its high-resolution structure was long-awaited. The crystal structure of the embryonic muscle-type nAChR was initially solved at 2.7 Å resolution (PDB: 6UWZ) by single-particle cryo-EM of the detergent-solubilized nAChR protein from *Torpedo (Tetronarce) californica* in complex with α-bungarotoxin [199]. The nAChR was reconstituted as predominantly dimers in the complex soybean lipid mixture, mainly containing phosphatidylcholine, and finally examined in soybean lipid mixture-saposin nanodiscs in monomeric form. The cryo-EM structure provided new details of the toxin sites, the ion permeation pathway, the amphipatic MX helix, which forms a submembrane rim parallel to the plane of the membrane at the intracellular-facing hemilayer, and mapped the location of mutations that result in some of the congenital myasthenic syndromes. The initial data on the lipid-contacting transmembrane region of the electromotor nAChR were more scanty, except for the observation of densities "consistent with a bound lipid" at the base of each α subunit M4 segment that extended to the adjacent αCys418 residue, and an additional density consistent with a palmitic acid covalently bound to Cys451 of the γ subunit at the α-γ interface. McNamee and coworkers were the first to conduct site-directed mutagenesis on *Torpedo* αCys418, providing the first strong indication that M4 was involved in channel gating of the nAChR [200,201]. Substitution of αCys418 for Trp increased ion permeability per cell surface area by about 40-fold [202]. Systematic mutational studies corroborated the influence of the M4 lipid-exposed residues on muscle-subtype [203–206] and neuronal [207,208] nAChR ion channel gating kinetics.

The new information stemming from Hibbs et al.'s study [199] facilitated interpretation of electron densities in a subsequent study in which Unwin identified the phosholipid head groups of both membrane hemilayers in the tubule specimens, which were, remarkably, separated by only 30 Å [195]. The location of cholesterol molecules in the bilayer was also tentatively assigned in this study (Fig. 1). Regions of the bilayer attributed

to cholesterol microdomains were tentatively identified based on the observation that they did not contribute density at the level of the phospholipid headgroups. In contrast to the exclusively intracellular hemilayer location described by Hibbs' group for the cholesteryl hemisuccinate sites [84], the endogenous cholesterol sites described by Unwin were present in *both* hemilayers occupying equivalent sites on all subunits, involving M4, M1 and M3 helices in the outer hemilayer and M4, M3 and MX in the inner hemilayer (the latter consistent with Hibbs' data), close to only α and δ receptor subunits and forming networks of bridging microdomains or patches. Larger patches were described at the interface of δ-δ and α_γ-α_γ nAChR monomer contacts. Unwin subsequently suggested that cholesterol is almost certainly needed to stabilize the splayed-apart arrangement of adjacent M4-M1-M3 helices in the exoplasmic hemilayer by wedging between helices at their interfaces [195], a feature consistent with thermodynamic and molecular dynamics studies [130,209].

A recent study from Hibbs' group reported the cryo-EM structures at 2.51 Å resolution of the *Torpedo* nAChR in the absence of ligands (PDB: 7SMM, "apo", closed ion channel), in complex with the agonist carbamoylcholine or the competitive antagonist *d*-tubocurarine, or a mixture

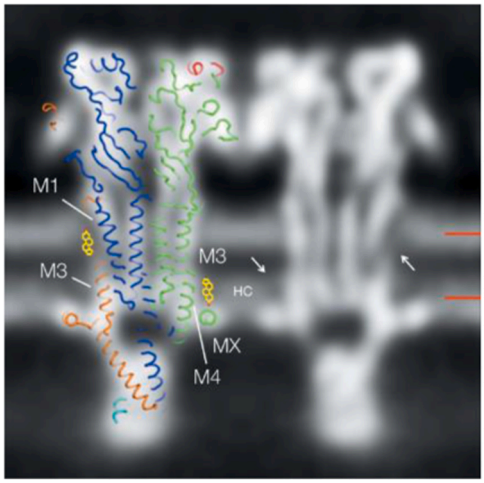

Fig. 1. A section through the lipid bilayer showing densities attributed to tight packing of the phospholipid hydrocarbon chains in the inner-leaflet hydrophobic core. The densities (HC) extend into the hydrophobic core by about the same distance as would the sterol group of cholesterol. A matching slice through the model (PDB: 6uwz) and two manually inserted sterol groups are shown next to one of the nAChRs to indicate the locations of two of the identified cholesterol sites. Arrows point to the equivalent sites on the other (two-fold related) receptor. Bars on the right indicate the levels of the transmembrane domain. The figure is shown in inverted contrast. Reproduced from ref. [195], an open access article distributed under the terms of the Creative Commons CC BY license.

of both, to define the resting, active, and desensitized forms of the receptor [166]. Additionally, the study contrasts the apo-nAChR in a cholesterol-poor, soybean lipid mixture (PDB: 7SMM) with the cholesterol-enriched soybean lipid mixture (PDB: 7SMQ). As shown in Fig. 2, Hibbs and coworkers could diffferentiate between cholesterol and phospholipid-associated densities, and defined high- and low-affinity cholesterol binding sites on the transmembrane domain.

In contrast to their previous study [199], the new data showed lipid densities not only in the vicinity of the cytoplasmic-facing hemilayer but on both hemilayers. In the "low cholesterol condition" (i.e., the apo-nAChR without added cholesterol, which retains ~4–5 (presumably endogenous) cholesterol molecules after eluting from the affinity column), the densities interpreted as cholesterol sites in the inner leaflet (i.e., equivalent to the cytoplasmic-facing hemilayer in a native membrane) of the soybean lipid nanodisc, close to the MX helix, were interpreted as *high-affinity* sites (3 per nAChR monomer, Fig. 2). These high affinity sites are hydrophobic pockets formed by M4, M3 and MX on the principal face of the two α subunits and the single β subunit, occupying the same positions in three interfaces: α/γ, α/δ, and β/α but not in γ/α_δ or δ/β. The cholesterol molecules contact a Val and an Arg residue in M3 and a Val/Ile and Phe residues on MX. When samples were supplemented with cholesterol, about ~25 bound cholesterol molecules were calculated to be present in the nanodisc, and the corresponding densities were more clearly observed in the transmembrane segments located in the outer leaflet of the nanodisc bilayer in the vicinity of M4 (i.e., equivalent to the extracellular-facing hemilayer in a native membrane), and interpreted as corresponding to *low-affinity* cholesterol binding sites. Early biochemical work showed that *Torpedo californica* nAChR-rich native membranes are rich in cholesterol [211], as are those of other Torpedinidae like *Torpedo marmorata* and *Discopyge tschudi* [100]. The neutral lipid represents 40–46% of the total lipids [100,211]. In the recent Rahman et al. cryo-EM study, 27–28 cholesterol molecules were estimated per nAChR monomer in native membranes. In purified nAChR samples *supplemented* with cholesterol the total number of cholesterol molecules per *Torpedo* nAChR monomer estimated by cholesterol biochemical assays was 25–26, of which 4–5 molecules remained bound through the purification procedure in the soybean lipid mixture [166]. Electron spin resonance experiments [127] and thermodynamic and molecular dynamics simulations of *Torpedo* nAChR [209] yielded stoichiometries of ~15 cholesterol molecules per nAChR molecule. It should be noted, however, that the number of lipid sites so far identified in cryo-EM studies varies significantly with the biochemical procedures used in the purification of the nAChR and its reconstitution into nanodiscs, particularly regarding the scafolding protein (MSP2N2 (Nury's group [212]) or saposin (Hibb's group [166,199]) required for nanodisc formation [213]. Saposin A can adopt different stoichiometries in the liposome, leading to nanodiscs of different diameters. The size of reconstituted nAChR-containing liposomes also varies considerably as a function of cholesteryl hemisuccinate concentration, as assessed by transmission electron microscopy [214]. Recently the size of the nanodisc has been reported to affect the cryo-EM structure of a prokaryotic pLGIC [215]. Remarkably, and despite the variable preparative procedures, all sterol and phospholipid binding sites identified thus far on the nAChR by cryo-EM lie on its surface, with no evidence of lipid sites buried deep inside the molecule [114,130]. Brannigan and coworkers define three types of sites in order of increasing depth within the nAChR: site A (in the groove behind M4 and in direct contact with the phospholipid); site B (at the interface between subunits, bordered by M1 and M2 of one subunit and M2 and M3 of the adjacent subunit); and site C (in the subunit center, bordered by M1, M2, M3, and site A [130].

5.5. *The occurrence of tandem cholesterol molecules on the same transmembrane domain*

A further correspondence between the cryo-EM studies of Hibbs and coworkers and our previous studies is the observation of two cholesterol recognition domains sitting in tandem on the *same* transmembrane segment, separated by one or 2 amino acids residues. This is clearly observed in the linear sequences of *Homo sapiens* α4 and β1 M4, close to the MX helix and is repeated at two other interfaces, but not all (Figs. 3 and 4). The linear consensus cholesterol-binding motifs CARC and CRAC are characterized by a triad of basic (Lys, Arg), aromatic (Tyr, Phe), and aliphatic amino acid residues [209]. The presence of two

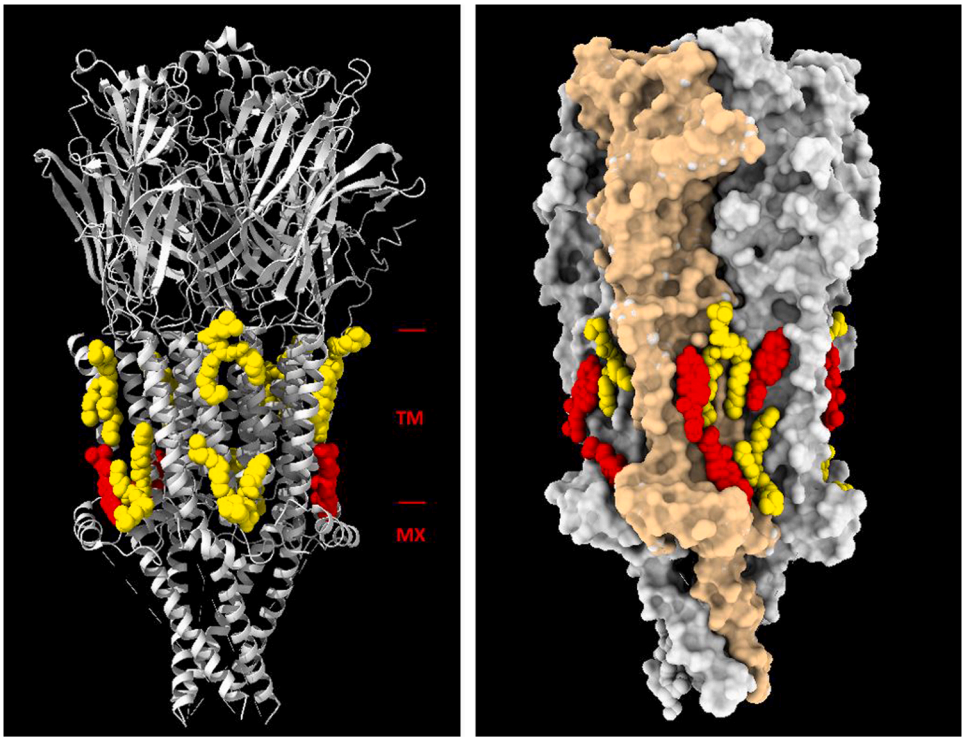

Fig. 2. "Apo"-nAChR (PDB: 7SMM, left) with 8 phospholipid molecules (POPC, yellow, three at the back of the model) on both hemilayers of the transmembrane region and the postulated three high-affinity cholesterol sites occupied by cholesterol molecules (red, one at the back) on the cytoplasmic-facing hemilayer only, in contact with the MX domain. The cholesterol-supplemented cryo-EM sample (PDB: 7SMQ) on the right shows the presumptive low-affinity cholesterol sites in the exoplasmic-facing hemilayer and the high-affinity sites on the inner membrane leaflet. The α subunit is highlighted in orange. From ref. [166]. Molecular graphics performed with UCSF ChimeraX [210].

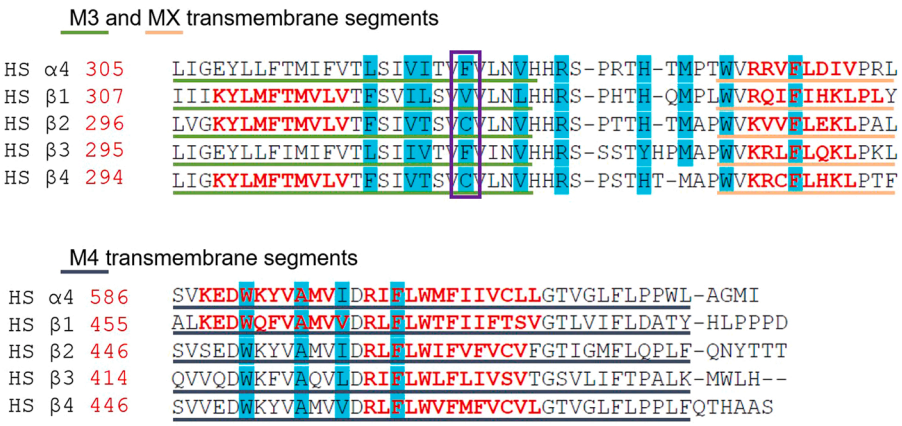

Fig. 3. Co-alignment of transmembrane segments encompassing putative cholesterol binding sites disclosed by cryo-EM [84,85] in the *Homo sapiens* α4β2 and α3β4 neuronal nAChR and cholesterol-recognition domains (amino acid residues in red) (Baier et al., 2011). The M3, MX and M4 segments are underlined in green, orange and magenta, respectively. Residues in cholesterol binding pockets revealed by cryo-EM are highlighted in light blue. The amino acid position implicated in differential cholesterol binding is enclosed in a blue box, and falls outside the consensus domains in M3. Arg301 (M3-MX loop) and Phe316 (MX) are conserved residues across subunits and species and are postulated to form part of the high-affinity cholesterol sites [166]. Note that the conserved Phe residues in MX, and Phe in M4 are within the cholesterol-consensus motifs in both segments. CARC cholesterol-recognition domains could constitute the low-affinity cholesterol sites occupied under desensitizing conditions (see discussion below).

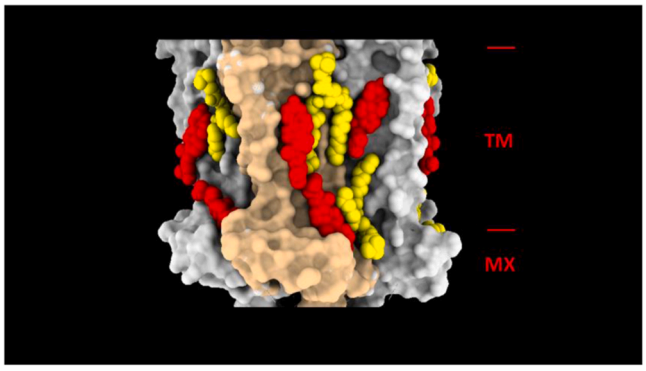

Fig. 4. Presence of cholesterol "doublets" in the model of the cholesterol-supplemented cryo-EM sample of *Torpedo* nAChR resolved at 2.7 Å (PDB: 7SMQ). The model shows the presumptive low-affinity cholesterol sites in the external, exoplasmic-facing hemilayer and the high-affinity cholesterol sites in the inner, cytoplasmic-facing hemilayer of the nAChR α subunit (highlighted in orange) and on the β subunit at its left. The high-affinity cholesterol binding sites sit on the MX helices. Among the five subunit interfaces, the high-affinity cholesterol molecules occupy the same positions in three interfaces: α/γ, α/δ, and β/α, but not γ/α_δ and δ/β. From ref. [166]. Molecular graphics performed with UCSF ChimeraX [210].

cholesterol-recognition domains within the same membrane-spanning domain [216,217] may allow the simultaneous binding of two cholesterol molecules, one in each membrane leaflet, as now revealed for various subunit interfaces in the *Torpedo* nAChR [166,212]. The corresponding molecular model (Fig. 4) shows the transmembrane region of the Torpedo nAChR with the cholesterol "doublets" on one of the α subunits and on the β subunit [166]. These doublets are reminiscent of the "mirror code" disposition of tail-to-tail or head-to-head cholesterol molecules in GCPRs [216].

A recent work by Unwin departs from previous studies in which the density maps were obtained by helical reconstruction upon combining 3D data from multiple tubule subsets; instead, he employed a single tubule subtype with the same curvature and lattice dimensions [218]. Unwin reported that the inner leaflet, which has the highest cholesterol content, is thicker, and the densities interpreted as lipids form close-packed ordered linear arrays, with periodicities (6.2 Å) resembling those of pure lipid monolayers interrogated with electron diffraction (Fig. 5).

The cryo-EM structural data have brought new insights into the functional effects of cholesterol on the neuronal nAChR [196]. Based on the α7 nAChR cryo-EM data [83], Hibbs and coworkers performed atomistic coarse-grained in silico simulations under conditions that mimic electrophysiological experiments, leading to the proposal that agonist-triggered opening of the α7 receptor Ca^{2+} permeation pore (activation) is accompanied by compression of the receptor-vicinal lipid

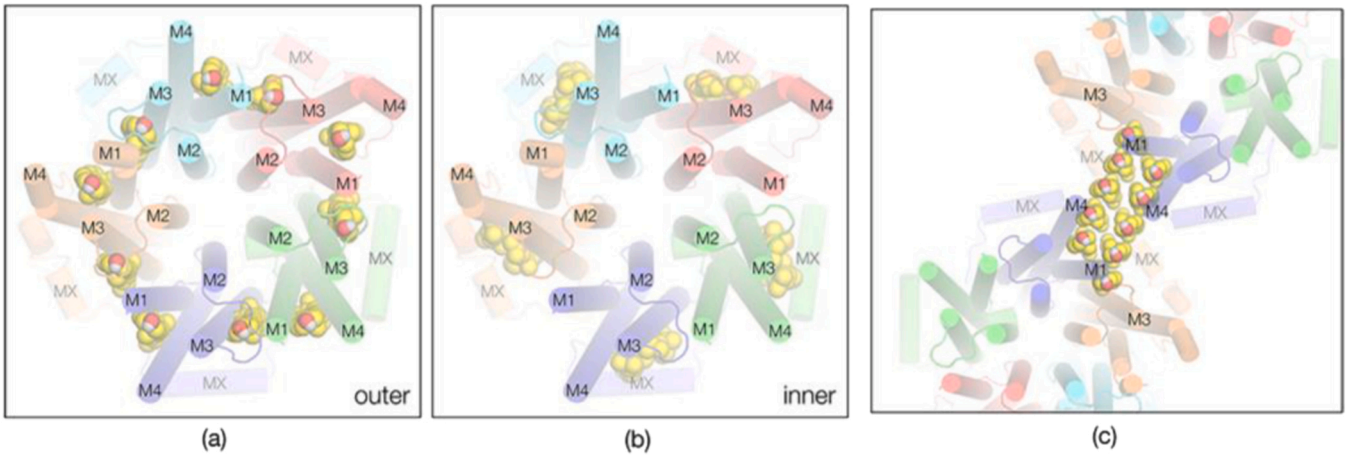

Fig. 5. Hypothetical arrangements of cholesterol molecules based on the cryo-EM images from Torpedo tubules: (a) wedging between helices M4–M1 and M1–M3 in outer leaflet; (b) supporting helices M3 and M4 in inner leaflet; (c) bridging the δ–δ dimer in the outer leaflet. Reproduced from ref. [219], an open access article distributed under the terms of the Creative Commons CC BY license.

bilayer (from 42 Å in the resting or desensitized states to 37–39 Å in the activated state) and increased contacts of the MX helices with the cytoplasmic-facing membrane hemilayer. As is well known, sustained agonist exposure leads to the non-conducting desensitized state, with a characteristically very rapid onset for α7 receptors. Desensitization would partially contract the pore, and this conformational transition would be accompanied by a shift in the preferential binding of cholesterol from the lower hemilayer to a site at the center of the bilayer with increased interaction with amino acid residue Met253 in M2. Hibbs and coworkers further postulate that binding of the PNU competes for this central bilayer sterol site and stabilizes the activated state, thus counteracting the desensitization phenomenon [196]. But probably more interesting from the perspective of cholesterol-nAChR interactions is the observation that cholesterol molecules are no longer present at the inner hemilayer sites of the neuronal α7 nAChR but appear only in the outer hemilayer upon agonist-mediated desensitization (Fig. 4) [196]. One possible explanation is that in the desensitized conformation the high-affinity cholesterol sites in the inner membrane leaflet are no longer accessible for binding by the neutral lipid, leaving available only low-affinity sites in the outer hemilayer. An alternative possibility is that cholesterol molecules actually move -translocate- from the high- to the low-affinity sites. The trans-bilayer motion or "flip-flop" of cholesterol is a well-known phenomenon in membrane dynamics occurring in the sub-millisecond time scale [220], and hence probably accessible to the time window in silico simulations. Whichever of these two non-exclusive mechanisms is operative, the CARC cholesterol-recognition domains shown in Fig. 3 beginning with KYL at M3 α4, β1, β2 and β3 subunits, and the conserved (RIFLW or RLFLW) CARC domains in all α and β M4 segments of the human nAChR would be the recipients of the cholesterol molecules previously bound to the conserved high-affinity sites in M3 (starting with LSI or FSI) or M4 (WKY or WQF), thus identifying these CARC domains as the low-affinity cholesterol sites (see Fig. 3 above).

5.6. Phospholipid sites

In addition to the densities that can be modeled as sterol molecules, the *Torpedo* structures show sites attributable to phosphoglyceride molecules [166,199,212]. In the latter study (PDB: 7QL5) six to eleven bound phospholipids could be modeled on the nAChR depending on the presence/absence of ligand and the agonist/antagonist nature of the ligand. The study describes with particular detail the location of the phosphatidylcholine sites: i) a conserved site per subunit at the inner, cytoplasmic facing hemilayer, sandwiched between a conserved Arg (or His) residue immediately after the M3 helix of the principal subunit, and another charged residue (Lys or Arg) in the complementary M4 and ii) an exoplasmic, outer leaflet site, consisting of a shallow cavity with no conserved amino acid motif, flanked by a positively charged residue in M3, the M2-M3 loop and the Cys loop from the principal subunit together with the M1 from the complementary ligand subunit [212]. Molecular dynamics calculations performed by these and other [129] authors show that phosphatidic acid can also bind to the outer leaflet site, lending support to the reported ability of the *Torpedo* nAChR to recruit this acidic phospholipid species to its vicinal entourage [193]. A single anionic phospholipid site has also been found in the outer leaflet of reconstituted ELIC (*Erwinia chrysantemi* pLGIC). The anionic phospholipid phosphatidylglycerol occupies this site and was found to play a crucial role in positively stabilizing the activated, open-channel conformation of ELIC [221].

6. Concluding remarks

Cryo-EM studies of neuronal- and electromotor- (muscle-type) nAChRs are affording valuable structural information on the location of phospholipid and sterol sites. Further studies are needed to determine lipid selectivity and sidedness in a reproducible manner, as well as the absolute number of lipid sites and their stoichiometry using the available armamentarium of structural biophysical techniques. While cryo-EM will certainly continue to deliver much valuable information, differences due to sample preparation conditions need to be resolved in order to establish why for example some of the cholesterol sites reported for *Torpedo* nAChR [166] are not apparent in other studies [212]. Nuclear magnetic resonance will probably complement these efforts [222–224] and play a role in deciphering the structure of the less structured and more plastic regions of the receptor [171], including those lodging highly mobile lipids and loosely bound small ligands.

From a historical perspective it is rewarding to witness how cryo-EM has in various instances identified sites previously uncovered via photolabelling of the incumbent amino acid residues [173,174,225–228] or through spectroscopic studies employing lipid spin labels and fluorescence quenching [127,227,229]. The considerable orientational flexibility of M4 while maintaining a linear α-helical structure, and its ability to vary its tilt depending on bilayer width and cholesterol content, especially in the inner membrane bilayer [230,231], also finds verification in the new structural studies. A functionally relevant mechanistic insight is the correlation between the new high resolution cryo-EM data with studies showing the crosstalk between *Torpedo* M4 with both the surrounding vicinal lipid at certain times, and its change of tilt to interact with M1 and M3 domains at other times [231], an observation that provided experimental support to the notion that M4 acts as a "lipid sensor" [87,198], a concept subsequently developed in depth by the group of Baenzinger [232–234] and extended to other pLGICs [235–237]. The cryo-EM studies of Nury and coworkers [212] and Hibbs and coworkers [166] show that M4 tilts away from the other transmembrane segments upon agonist (carbamoycholine) binding, as observed in our previous molecular dynamics study [231]. A most interesting recent finding, also from in silico modelling studies, is that upon agonist-induced desensitization cholesterol molecules do not bind to the presumptive high-affinity sites at the inner, cytoplasmic-facing hemilayer of the neuronal α7 nAChR. Under cholesterol supplementation mimicking the cholesterol levels in *Torpedo* membranes, additional, presumptively low-affinity sterol molecules populate the outer, exoplasmic-facing hemilayer of the bilayer [196], putting cholesterol center-stage in the receptor gating cycle.

CRediT authorship contribution statement

Francisco J. Barrantes conceived, wrote, created molecular models and revised the manuscript.

Declaration of interests

None. The author has no competing interests to declare.

Data availability

Data will be made available on request.

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