

Dialogue between membrane lipids and acetylcholine receptor

F. J. Barrantes

UNESCO Chair of Biophysics & Molecular Neurobiology and Instituto de Investigaciones Bioquímicas, de Bahía Blanca, Universidad Nacional del Sur-CONICET, C.C. 857, B8000FWB Bahía Blanca, Argentina

The nicotinic acetylcholine receptor (AChR) is the archetypal molecule in the superfamily of Cys-looped ligand-gated ion channels. Members of this superfamily mediate fast intercellular communication in response to endogenous neurotransmitters. This review focuses on structural and functional crosstalk between the AChR and the lipids in its membrane microenvironment. Influence on receptor properties is mainly exerted by the AChR-vicinal ('shell', 'boundary' or 'annular') lipids, which occur in the liquid-ordered phase as opposed to the more disordered and 'fluid' bulk membrane lipids. Changes in Förster's energy transfer (FRET) efficiency induced by fatty acids, phospholipids and cholesterol have led to the identification of discrete sites for these lipids on the AChR protein. Electron-spin resonance spectroscopy has established the stoichiometry and selectivity of the shell lipid for the AChR and disclosed lipid sites in the AChR transmembrane region. Combined single-channel and site-directed mutagenesis data fostered the recognition of lipid-sensitive residues in the transmembrane region, dissecting their contribution to ligand binding and channel gating, opening and closing. Experimental evidence supports the notion that the interface between the protein moiety and the adjacent lipid shell is the locus of a variety of pharmacologically relevant processes, including the action of steroids and other lipids.

Keywords: Acetylcholine receptor, membrane lipids, protein moiety, protein-vicinal lipid.

Introduction

In the central nervous system, neurotransmitter receptors and their associated signalling components in the synapse are crucial elements in the supracellular assemblies known as neural circuits. In the peripheral nervous system, the equivalent synaptic apparatus is made up of similar macromolecular building blocks in a complex identified as the neuromuscular junction (NMJ). Both synapses are key to the transmission of chemical signals involved in the communication between neurons and nerve and vertebrate skeletal muscle respectively. A fundamental component of this synaptic machinery is the neurotransmitter

receptor, acting as transducer of the chemical signal. The ligand-gated ion channel (LGIC) superfamily comprises several families of evolutionarily related neurotransmitter receptor proteins coded by a few hundred genes so far identified. Of these, the nicotinic acetylcholine receptor (AChR) is one of the best-characterized. LGICs mediate excitatory and inhibitory chemical transmission.

Several genes coding for AChR subunits have been characterized in central and peripheral nervous systems. They exhibit amino acid sequence homology and presumably higher-order structural motifs¹⁻³. Within the LGIC superfamily, the AChR and subtype 3 of the 5-hydroxytryptamine (serotonin, 5-HT₃) receptor comprise two families of cation-selective channels, whereas glycine and gamma aminobutyric acid type A (GABA_A) receptors are anion-selective channels. Members of this superfamily are also known as Cys-loop receptors because in their amino-terminal all their subunits contain extracellular halves of a pair of disulphide-bonded cysteines separated by only 13 residues. Glutamate and histidine receptors are also Cys-looped receptors, but their structure does not conform to the canonical LGIC superfamily. Until recently, the Cys-loop family was thought to comprise only ion channels produced by eukaryotic genes, but a prokaryotic proton-gated ion channel from the AChR family has recently been discovered⁴.

The basic mechanism of signal transduction is common to all members of the LGIC and results from relatively fast and similarly simple steps: binding of the neurotransmitter followed by conformational transitions in the receptor proteins that lead to changes in the ionic permeability of the postsynaptic membrane. In the specific case of the AChR, upon binding, acetylcholine initiates a conformational change in this protein that triggers the transient opening of its intrinsic cation-specific channel across the postsynaptic membrane. At the molecular level, this is accomplished by the concerted action of four different but highly homologous AChR subunits in the stoichiometry $\alpha_2\beta\epsilon\delta$ in adult skeletal muscle^{5,6}.

Each AChR subunit contains four hydrophobic segments, 20–30 amino acids in length, the M1–M4 membrane-spanning segments. Of these, the M2 segment from each subunit contributes structurally to the formation of the ion channel proper. M4 is considered the most likely segment to be exposed to the bilayer lipid. M1 and M3

e-mail: rtfjb1@criba.edu.ar

effectively incorporate membrane-partitioning photoactivatable probes, and are also likely to be exposed, at least partially, to the lipid phase. It is usually accepted that all four hydrophobic segments M1–M4, referred to as transmembrane (TM) domains, correspond to regions of the protein fully embedded in the membrane. We have proposed that in addition to these two main regions of the AChR, a third important domain is defined at the extensive interface between the protein and lipid moieties, comprising both the lipid-exposed TM portions of the AChR protein and the AChR-vicinal lipid^{7,8}. The latter corresponds to the lipid belt ('shell', 'annulus', 'boundary', 'AChR-vicinal') region, that is the lipid moiety in the immediate perimeter of the AChR protein, earlier discovered by Derek Marsh and myself using electron spin resonance (ESR) techniques⁹ and further characterized in terms of lipid selectivity and stoichiometry^{10–15}. In this review I discuss various experimental approaches that have led to a quite detailed description of the AChR lipid microenvironment and of the cross-talk between the receptor protein and its surrounding lipids. The reader is referred to other reviews covering wider aspects of AChR-lipid interactions⁸ or more specific facets of this topic, e.g. the effects of cholesterol (Chol) on receptor supramolecular structure, stability and dynamics at the cell surface¹⁶.

The AChR is surrounded by lipids in the liquid-ordered phase

Model membranes are relatively well characterized in terms of their physical properties. At high Chol concentrations, phospholipid–Chol mixtures mimic many aspects of the phase state displayed by biological membranes rich in Chol. These mixtures lack a defined lipid phase transition and instead are characterized by a single phase state, the liquid-ordered phase (l_o)¹⁷, with properties between the gel and the fluid lipid phases. For low Chol 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 Chol concentrations, there is phase coexistence of s_o and l_o (below), or of l_d and l_o (above), depending on the temperature relative to T_m . In the particular case of the AChR, early studies from the group of McNamee^{18,19} showed that the phase state of the membrane was important: 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'. Early ESR studies^{9,15,20} made apparent the occurrence of two distinct signals in ESR experiments with native and reconstituted membranes containing AChR at relatively high or low concentrations: one signal corresponded to the bulk membrane lipid and the other was interpreted as stem-

ming 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. Rousselet *et al.*²⁰ found immobilization with fatty acids but not with phospholipids. Ellena *et al.*¹³ confirmed our findings using reconstituted AChR. This series of studies from different laboratories demonstrated that shell or annular protein-vicinal 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.

Functional studies contributed to understanding the role of lipids in AChR ion permeability. The need to include sterols and certain phospholipids to preserve this property of the AChR in reconstituted systems was subsequently demonstrated²¹. The relative contributions of phospholipid and sterol could be established in various studies *in vitro*^{22–24} and the minimal number of lipid molecules (~45) per AChR was ascertained in early ESR experiments^{13,25}. AChR-vicinal lipids appeared to be an inherently relevant environmental feature of the AChR native membrane, but the nature of the interaction between protein and lipid moieties was still obscure, as were the possible functional implications proposed in early work⁹.

In order to define the physical state of the AChR membrane lipids, we subsequently resorted to fluorescence methods using the so-called Generalized Polarization (GP)^{26,27} of the fluorescent probe Laurdan (6-dodecanoyl-2-dimethylamino naphthalene). This approach was introduced to learn about the dynamics of the AChR and some physical properties of the protein-vicinal lipid^{28–30}. Towards this end we fostered two hitherto unexploited properties of Laurdan: (i) its ability to act as a Förster-type resonance energy transfer (FRET) acceptor of tryptophan emission³⁰ and (ii) the resulting differences in FRET efficiency upon displacement of Laurdan by exogenous lipids^{28,29}. Laurdan is a particularly advantageous fluorescent probe; it localizes itself at the hydrophilic–hydrophobic interface of the lipid bilayer, with its lauric acid moiety at the phospholipid acyl chain region and its naphthalene moiety at the level of the phospholipid glycerol backbone. Its spectral properties are extremely sensitive to the polarity and molecular dynamics of dipoles in its environment. This is due to dipolar relaxation processes that are reflected as relatively large spectral shifts. Excitation of Laurdan under FRET conditions using the Trp residues of the AChR-rich membrane as donors constituted a new tool to learn about the properties of the lipids in the immediate vicinity of the AChR and to compare them with those of the average, bulk lipid in the rest of the bilayer. The first application of this approach was the determination of distances between the AChR protein and adjacent lipid. From the spectral overlap integral for the AChR membrane–Laurdan pair we calculated R_0 , the Förster critical distance, to be 29 Å³⁰. We modelled the AChR

as a cylinder of about 80 Å in diameter with donor Trp residues lying in a ring within the perimeter of its TM portion. From the electron microscope data available at that time³¹ this region of the protein was assumed to have a radius of 32.5 Å. The height of the plane of AChR tryptophan residues was set with respect to the plane of acceptors by using the parameter H , the distance between donor-acceptor planes normal to the membrane surface, which was allowed to vary between 0 and 50 Å in view of the long-axis dimensions of the AChR molecule and the width of the lipid bilayer. Using the above model we found differences between the AChR-*vicinal* lipids within a 14 Å radius of the AChR surface on the one hand, and the bulk lipids on the other. When no relaxation occurs, GP values are high, indicating low water content in the hydrophilic/hydrophobic interface region of the membrane. GP observed under FRET conditions from the intrinsic protein fluorescence exhibited higher absolute values than those obtained by direct excitation of the probe, indicating the lower polarity of the lipid in the protein-*vicinal* lipid microenvironment of the AChR. The main dipoles sensed by Laurdan in the membrane are water molecules. Thus, this series of studies showed that AChR-*vicinal* lipids are more rigid and exhibit a lesser degree of water penetration than bulk lipids, and that a single thermotropic phase with the characteristics of the so-called liquid-ordered phase defines the entire AChR-rich postsynaptic membrane, at least in fish electrocytes³⁰.

AChR-lipid interactions as viewed from the protein moiety

Electron microscopy experiments over twenty years ago already indicated that about half the mass of the AChR protein protrudes into the extracellular space, about 30% corresponds to TM domains, and the remainder is in the cytoplasmic compartment^{32–35}. The agonist-recognition domains of the AChR were also located in the extracellular portion of the macromolecule³³ (and see review in ref. 36) at a distance of about 25 Å from the apex of the AChR³⁷ and about 30 Å from the membrane surface^{31,38,39}.

Site-directed mutagenesis of the AChR combined with patch-clamp electrophysiological and photoaffinity labelling experiments with noncompetitive channel blockers support the notion that one of the TM AChR regions, the M2 domain, lines the walls of the pore. The data are also indicative of α -helical periodicity in the residues exposed to the lumen of the AChR channel⁴⁰. NMR studies of the M2 segment of the δ subunit⁴¹ indicate that this domain is inserted in the bilayer at an angle of 12° relative to the membrane normal, in a totally α -helical configuration. Analogously, a synthetic peptide corresponding to the *Torpedo* α M2 segment in organic solvents also adopts a totally α -helical configuration⁴². Cryoelectron microscopy data confirm that M2 forms the innermost ring of mem-

brane-spanning segments, isolated from membrane lipids^{43,44}.

During the nineties, cryoelectron microscopy revealed the relatively featureless appearance of the other putative TM domains (M1, M3 and M4). A large portion of this AChR region was postulated to be arranged in the form of a β -barrel outside the central rim of M2 channel-forming rods⁴⁵. This interpretation contrasted with photoaffinity labelling studies, in which the observed periodicity of the lipid-exposed residues in M4 and M3 was consistent with an α -helical pattern^{46–48}, and with deuterium-exchange Fourier transform infrared spectroscopy studies indicating a predominantly α -helical structure in the AChR TM region⁴⁹. In addition, secondary structure analysis (CD and Fourier transform infrared spectroscopy) of isolated and lipid-reconstituted TM AChR peptides indicated an α -helical structure for M2, M3, and M4 segments⁵⁰. Furthermore, a synthetic peptide corresponding to the α M3 segment of *Torpedo* AChR exhibited a totally α -helical structure by 2-dimensional ¹H-NMR spectroscopy⁵¹; NMR studies of a synthetic γ M4 peptide are also compatible with an α -helical secondary structure⁵².

Considerable advance in defining the structure of the AChR at atomic resolution resulted from crystallographic studies of a water-soluble ACh-binding protein from a snail⁵³. The structure of this protein, highly homologous to the water-soluble extracellular domain of the AChR protein proper⁵³, provided the first truly high-resolution data of the region of the AChR putatively involved in agonist recognition, the first step in the cascade leading to channel opening. More recent work has resulted in the crystallization of the actual water-soluble extracellular domain of the mouse AChR α -subunit bound to α -bungarotoxin. The crystal structure was solved at 1.94 Å resolution⁵⁴.

The cryo-electron microscopy data of Unwin and co-workers^{43–45} at 4 Å resolution provided inspiring insights into the structure of the AChR, and particularly relevant to the subject of this review, the electron microscopy data revealed interesting features of the membrane-embedded domains of the AChR protein. I described the occurrence of three concentric rings in the AChR region⁷: (a) an inner ring exclusively formed by five M2 segments, corresponding to the walls of the AChR ion pore, which have no contact with the membrane lipid; (b) a middle ring, formed by ten helices corresponding to the M1 and M3 TM segments. This middle ring is separated from the inner five-member ring of M2s, and its outer face is exposed to lipids and also to (c) the outermost ring, consisting of five M4 segments (Figures 1–3). The proton-gated ion channel protein recently found in the cyanobacterium *Gloeobacter violaceus*⁴, Glvi, shares only 20% amino acid identity with one of its homologues in *Homo sapiens*, the α 7 neuronal AChR. However, some key regions contributing to the gating properties of AChRs are conserved, such as the Cys-loop, the proline in M1, the equatorial ring of hydro-

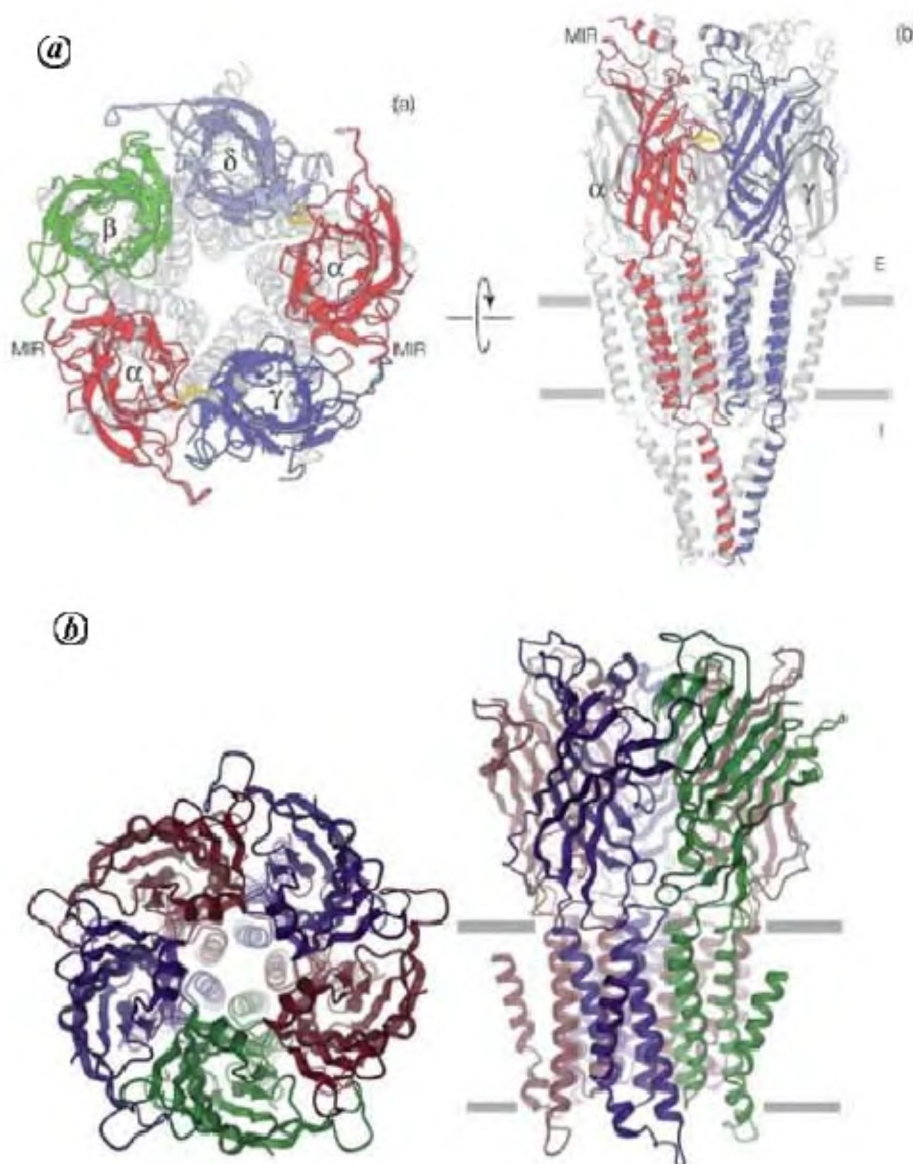


Figure 1. *a*, Structure of *Torpedo* AChR derived from cryoelectron microscopy data at 4 Å resolution (from ref. 44). *b*, ELIC, a pentameric proton-gated channel from the bacterium *Erwinia chrysanthemi* at 3.3 Å resolution (from ref. 55).

phobic residues in M2, and the four TM segments. The latter feature is reinforced by the recently available X-ray structure of an ortholog of the *Gloeobacter* channel, the pentameric protein 'ELIC' from *Erwinia chrysanthemi* at 3.3 Å resolution, which exhibits only 16% sequence identity with the AChR and lacks the cytoplasmic region⁵⁵. The crystal structure of ELIC clearly shows the three concentric rings in the TM region which I described for the AChR⁷, and suggests that its equivalent segments M1, M2 and M3 in the two inner rings of AChRs are involved in interactions at subunit interfaces, whereas M4, located at the peripheral, outer ring, only loosely interacts with M1 and M3 of the same bundle and is not involved in subunit–subunit interactions⁵⁵ (Figure 1).

Influence of the lipid environment on AChR secondary structure

The lipid environment exerts a modulatory effect on AChR secondary structure. Early studies reported that Chol increased the α -helix content of the AChR. The sterol was postulated to stabilize AChR structure by packing its rigid planar ring into grooves of TM helices⁵⁶. CD spectroscopy⁵⁷, Raman spectroscopy⁵⁸ and ¹H/³H exchange studies⁵⁹ detected no large differences in structure or solvent accessibility between resting and desensitized AChR. One study reported no changes in secondary structure in the presence of Chol⁶⁰. In contrast, evidence from cryoelectron microscopy³⁹ indicated differences between resting and

desensitized and resting and activated AChR respectively. Furthermore, the accessibility of AChR fluorophores to membrane probes between resting and desensitized forms of the AChR is different⁶¹, as is the accessibility of residues near the ligand-recognition site⁶² and the TM regions⁶³. Castresana *et al.*⁶⁴ reported that the helical content of the AChR was not affected by the addition of agonist, whereas the proportion of β -structure decreased to 24% concomitantly with an increase in disordered structure. Prolonged exposure to the agonist, leading to desensitization, resulted in significant rearrangement of the AChR structure. Lack of Chol in an asolectin reconstitution system produced an increase in disordered structure in *T. marmorata* AChR⁶⁵. Addition of exogenous Chol resulted in restoration of the proportion of AChR ordered structure in asolectin liposomes but not in liposomes prepared from egg PCs, leading these authors to suggest that a component other than PC is needed for the restoration of AChR structure in the presence of Chol. Fernandez-Ballester *et al.*⁶⁵ also suggested that the desensitization phenomenon does not depend on the presence of Chol or other lipids. Methot *et al.*⁶⁶ found 39% α -helix, 35% β -sheet, 20% random coil and 6% β -turn in *T. californica* AChR reconstituted in DOPC : DOPA : Chol (3 : 1 : 1) by FTIR, in other words sufficient α -helical content to form four-helical TM segments and a substantial portion of the extracellular region. It is interesting to note that much earlier, Finer-Moore and Stroud⁶⁷ had predicted a high helical content

(44%) and 27% β -sheet in a theoretical analysis using a Fourier transformation of the AChR primary structure. Methot *et al.*^{66,68} found no significant agonist-induced changes in the secondary structure of the AChR upon exposure to the agonist Carb or to the local anesthetic tetracaine, regardless of the presence or absence of Chol in the reconstitution system.

The canonical 4-helix TM models^{69,70}, increasingly validated by cryoelectron microscopy data^{43,44} and more recently by X-ray diffraction studies⁵⁵, placed between 17% and 20% of the AChR mass inside the membrane bilayer. Earlier studies^{71,72} had suggested that each of the membrane-spanning segments was independently stable in the lipid bilayer. Corbin *et al.*⁵⁰ reported that in a membrane environment the AChR TM segments have the intrinsic propensity to adopt an α -helical secondary structure. The discussion about the secondary structure of the AChR TM region has further implications for current models of the other regions of the AChR molecule. Thus, compatibilizing the most recent experimental work with the theoretical models that assume four TM helices implies placing an important proportion of β -sheet structure

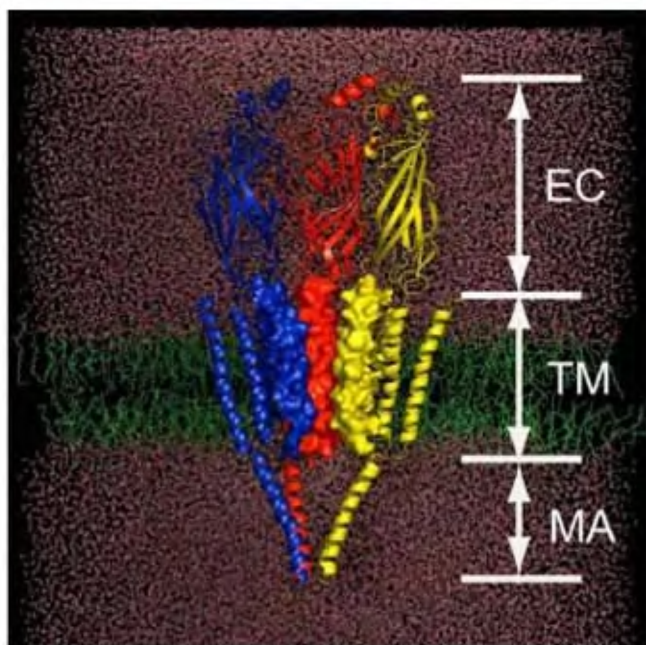


Figure 2. Schematic representation of the molecular dynamics system constructed on the basis of the cryoelectron microscopy structure of the AChR at 4 Å resolution⁴⁴. The receptor protein is embedded in a DPPC lipid bilayer (green) and water molecules (red). Only three subunits (colour-coded in red, blue and yellow) are displayed for clarity. The innermost ring of M2 helices is rendered with electrostatic potential surface to highlight the pore shape (from ref. 137).

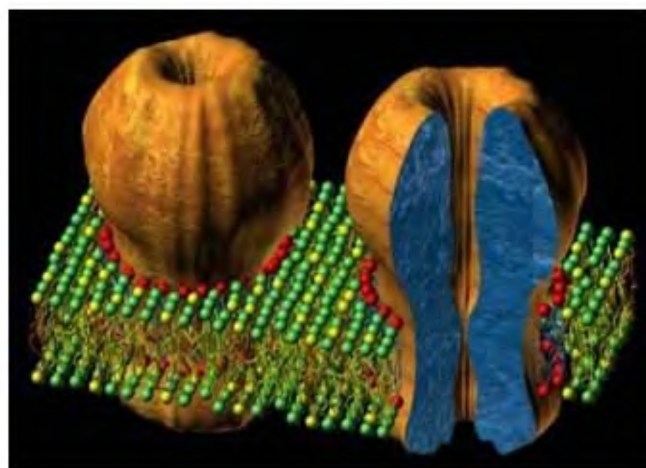


Figure 3. The AChR and its lipid milieu. The bulk lipid region (depicted as green and yellow phospholipid molecules) constitutes the membrane framework. Red spheres immediately adjacent to the AChR molecules represent the polar head regions of the phospholipids in the first-shell ('belt layer', 'annular') region. Unlike the vast majority of individual proteins in cell-surface membranes ('iceberg-like embedded proteins in a sea of lipid'), the AChR molecules form tightly packed 2-dimensional arrays in the postsynaptic membrane, in which lipid molecules fill in the interstices. Only three to four phospholipid shells of bulk lipid separate the protein-vicinal first shell from a homologous, adjacent one surrounding a neighbour AChR protein. Both bulk and shell lipids in the membrane are in the liquid-ordered (l_o) phase, with decreasing polarity towards the protein molecule. That is, the protein-vicinal lipid-belt region is more ordered than the bulk, bilayer lipid, and yet the two lipid regions form a continuum from the physicochemical point of view. The protein-vicinal first-shell lipid is relatively immobilized with respect to motions both around and perpendicular to the long molecular axes of the lipid molecules, respectively, with rotational correlation times about 50–100 times longer than is typically found with fluid bilayer lipid. Notice that even the first-shell lipid is relatively distant from the ion pore in the receptor protein, as made apparent in the sectioned AChR molecule on the right.

in the extramembranous regions (extracellular and cytoplasmic-facing) of the AChR, a feature that appears to find validation at least in the case of the AChBP, the structural homologue of the AChR extracellular moiety⁵³. The AChBP has extensive β -sheet structure. Görne-Tschelnokow *et al.*⁷³ concluded from their FTIR data that the TM region of the AChR contains 40% β -sheet plus turn structure. Using the same technique, Baezinger and Methot⁴⁹ produced experimental evidence supporting an all-helical model of the AChR TM region. Additional FTIR studies^{49,68} indicate that $^1\text{H}/^2\text{H}$ exchange kinetics is slower in the presence of DOPA or Chol, suggesting that the lipid environment modulates the conformational dynamics of the membrane-embedded peptide hydrogens that exchange with deuterium.

Physical contact between lipids and the AChR surface: fluorescence studies

The vicinity of AChR protein to lipids in the membrane bilayer was apparent in early fluorescence quenching studies (AChR intrinsic fluorescence) performed on native membranes⁶¹ and in reconstituted systems¹⁹. The fluorescence emission of the AChR is typical of that of other integral membrane proteins. Fifty-one Trp and 80 Tyr residues are present in *T. californica* AChR⁷⁰, but the spectrum appears to be dominated by the Trp emission⁶¹. Fluorescence studies⁷⁴ and sequence topology⁷⁵ indicate that only Trp⁴⁵³ in the γ -subunit is present in the membrane-embedded region in *Torpedo californica*, with the addition of one more Trp residue in the α -subunit in the case of *Torpedo marmorata*. These intrinsic fluorophores of the AChR are accessible to quenching by lipid analogues from the bilayer region; the heterogeneous nature of the fluorophore population is reflected in the occurrence of various quenching constants. From the study of other systems such as the sarcoplasmic reticulum Ca^{2+} -ATPase⁷⁶ paramagnetic quenching of membrane-bound fluorophores is assumed to be predominantly static in nature. This is due to the fact that the measured phospholipid lateral diffusion in membranes, D , is about $10^{-8} \text{ cm}^2 \text{ s}^{-1}$, i.e. relatively slow with respect to the time window of these measurements and thus the distance between fluorophore and quencher does not change appreciably during the lifetime of the former. In the case of the quenching of AChR intrinsic fluorescence, the mechanism is more complex, probably mixed in nature, because of the dynamic components arising from rotational motions and peptide chain wobbling. Paramagnetic quenching requires a minimum distance between quencher and fluorophore of about 5 Å. The occurrence of effective paramagnetic quenching by spin-labelled lipids thus constitutes strong evidence that quenching occurs in the TM regions of the AChR, and that the latter contain discrete sets of intrinsic fluorophores accessible to the nitroxide probe. The K_Q 's and the

apparent fraction of available fluorophores (f_a) can be obtained from the modified Stern–Volmer equation:

$$F_0/(F_0 - F) = 1/(f_a K_Q [Q]) + 1/f_a, \quad (1)$$

where F_0 is the initial AChR intrinsic fluorescence intensity, and F is the fluorescence intensity of AChR in the presence of a given concentration, $[Q]$, of nitroxide-labelled lipids. The value of $1/K_Q$, the spin-labelled lipid concentration at which 50% of the initial intensity is quenched assuming that all fluorophores are fully accessible to quencher, provides a quantitative estimate of the efficiency of the spin-labelled probes as quenchers of the fluorescence of membrane-embedded Trp residues. In the case of *T. marmorata* AChR in its native membrane this follows the sequence¹⁴:

$$\text{CSL} > \text{ASL} > 16\text{-SASL} > 12\text{-SASL} > 5\text{-SASL}.$$

The higher efficiency of the steroid analogue spin-labelled cholestane (CSL) than spin-labelled androstane (ASL) in quenching AChR intrinsic fluorescence can be explained in terms of the different location, of the paramagnetic nitroxide group relative to the membrane surface. CSL is located close to the lipid/water interface, which most probably enables this spin label to quench Trp residues either in the extramembranous domain of the AChR, or in shallow regions of the TM region.

Stoichiometry and selectivity of the protein-vicinal lipid

ESR is an excellent biophysical method for studying the mobility, stoichiometry and selectivity of lipids at the intramembranous surface of integral membrane proteins, because its dynamic sensitivity is optimally matched to the time-scale of lipid rotational motions in biological membranes, in the order of nanoseconds. ESR spectra of spin-labelled lipids sense molecular motions corresponding to rotational frequencies of about 10^8 – 10^5 s^{-1} (ref. 77). Thus, spectra of lipids motionally restricted by interactions with the surface of, or bound to, integral membrane proteins are suitably resolved from those corresponding to the fluid bilayer regions of the membrane. We have reported that lipid mobility at the lipid shell surrounding the AChR protein (the AChR-vicinal lipid, see Figure 3) is reduced relative to that of the bulk membrane lipid, giving rise to a two-component ESR spectrum from which the number and selectivity of the lipids at the lipid–protein interface may be quantitated (see e.g. ref. 15). In this way we were first able to demonstrate protein-induced restrictions on the mobility of spin-labelled fatty acids and ASL⁹ and of spin-labelled phospholipids¹⁵ in AChR-rich membranes from *T. marmorata*. Subsequent work demonstrated the preferential association of AChR with spin-labelled ste-

roids, phosphatidic acid (PA), and fatty acids, rather than with other kinds of lipid^{13,20}. The possible functional implications of this topographical relationship between lipid and AChR protein became apparent in studies showing that Chol and negatively charged phospholipids were required to support the ion-gating activity of the AChR^{22,23,78} whereas fatty acids blocked the ion-flux response⁷⁹. The latter was interpreted as the perturbation of the functionally significant interaction between AChR and Chol or negatively charged phospholipids.

The FRET studies using the SM fluorescent derivative also yielded information on the relative affinity of the SM derivative for the AChR protein. Thus, Py-SM exhibited a moderate-to-low selectivity for the protein-vicinal lipid domain, with a calculated relative affinity $K_r \cong 0.55$ (ref. 80). This figure should be added to the list of known selectivities of other lipids for the AChR calculated from early ESR experiments: PS (0.7), PC (1.0), PE (1.1), PA (2.7) and stearic acid (4.1) by Ellena *et al.*¹³, and to those determined by us more recently, also using ESR techniques⁸¹, allowing a classification of lipids according to their selectivity for the AChR protein. Mantipragada *et al.*⁸¹ provided the first detailed description of the dynamic composition of 'first-shell' lipids in the belt region surrounding a receptor protein. AChR-vicinal lipids fall into three categories: (a) a high specificity group, constituted by fatty acids like stearic acid, cardiolipin and phosphatidylinositols^{81,82}, androstanol^{15,82}, and phosphatidic acid¹³; (b) an intermediate group, made up of SM⁸⁰, PS and PG⁸¹, and (c) a moderate-to-low specificity group, where we find PC, PE, and the gangliosides GD1b, GM1, GM2 and GM3⁸¹.

It is interesting to note that owing to the high-packing density of AChR molecules in the postsynaptic membrane, only three to four phospholipid layers of 'bulk' lipid separate the protein-vicinal first layer from the nearest-neighbour first-layer lipid surrounding an adjacent AChR protein⁸. In *Torpedo* receptor-rich native membranes prepared from electrocytes, *all* lipids in the membrane are in the liquid-ordered (l_o) phase, with decreasing polarity towards the AChR protein molecules³⁰. This is because the protein-vicinal lipid-belt region is more rigid and ordered than the bulk bilayer lipid, as determined by Laurdan GP measurements using FRET^{29,30}. However, the receptor-vicinal and the bulk lipid regions form a single, liquid-ordered phase from the physico-chemical point of view.

The protein-vicinal lipid is relatively immobilized with respect to motions both around and perpendicular to the long molecular axes of the lipid molecules, i.e. with rotational correlation times ~ 50 – 100 times longer than is typically found with fluid bilayer lipid⁹. The protein-vicinal lipid also exhibits a lower degree of penetration of water molecules, thus rendering it less polar than the bulk bilayer lipid^{29,30}. Another dynamic aspect that characterizes the two lipid regions is the relatively high exchange between the two moieties: although the AChR-vicinal

lipid is expected to have a lateral diffusion coefficient 50–100 times slower than that of the fluid bilayer lipid, i.e. $\sim 10^5 \text{ s}^{-1}$ (ref. 9), the lipid exchange process between the AChR-vicinal lipid and the bulk lipid exhibits rates in the order of 1 – $5 \times 10^8 \text{ s}^{-1}$. Lipid species displaying selectivity for the AChR protein spend on average a longer period in the immediate vicinity of the protein; they are concentrated relative to those lipids exhibiting little or no selectivity. In systems where the selectivity of the lipid for the protein is changed by varying the pH or the ionic strength, it has been shown that the on-rate remains constant, whereas the off-rates reflect the specificity of a given lipid, which is independent of the lipid/protein ratio⁸³.

We have also found that some local anaesthetics, non-competitive antagonists of the AChR, exert their action at the AChR-lipid interface^{11,14,81}. The effect of local anaesthetics contrasts with those of general anaesthetics: both reduce the motionally restricted boundary or shell-lipid, but the latter also fluidize the bulk lipid, an effect that we have not observed with local anaesthetics at the concentrations used⁸¹. It is also interesting to note that local anaesthetics compete more effectively with the phospholipid PI than with the fatty acid analogue, although both lipids display similar relative association constants for the AChR. Unwin and colleagues suggested the possibility that alcohols and local anaesthetics bind to the AChR in water-filled cavities in between the M2 channel-lining ring and the middle M1–M3 TM ring⁴³.

Changes in the physical state of the native AChR membrane induced by exogenous lipids

When Laurdan GP was measured in *Torpedo* native AChR membrane (either by direct excitation or under FRET conditions) in the presence of exogenous lipids, GP and by inference the 'fluidity' and order of the membrane were found to diminish upon addition of oleic acid and DOPC, and not to vary significantly upon addition of Chol hemisuccinate, indicating an increase in the polarity of the single, ordered-liquid lipid phase in the two former cases²⁸.

Complementary information about the bulk lipid order was obtained from measurements of fluorescence anisotropy of DPH and two of its derivatives. The membrane order diminished in the presence of oleic acid and DOPC. The location of Laurdan was determined using the parallax method of Chattopadhyay and London⁸⁴. Their method is based on the relative position of a fluorescence probe embedded in the membrane and its quenching by probes having nitroxide spin labels at different positions along their acyl chains. The parallax determination is accomplished by pairwise comparison of quenching parameters with different pairwise combinations of the PC analogues with spin labels in carbons 7, 10 and 12.

When the temperature dependence of Laurdan fluorescence was studied in the native AChR-rich membrane,

dipolar relaxation was found to gradually increase with increasing temperature³⁰, probably reflecting an increment in water content and disorder in the shallow membrane region sensed by the probe, both in AChR-vicinal and bulk lipid regions. Similar conclusions were reached from a study of fluorescence anisotropy of DPH and derivatives, which sense the thermally-induced disorder in other regions of the membrane²⁸.

Energetics of channel gating in living cells measured by Laurdan fluorescence

The characterization of Laurdan thermotropic behaviour was subsequently extended to living mammalian cells expressing endogenous or heterologous AChR. Interestingly, the differences in physical properties of cell membranes measured by Laurdan GP in a variety of cells, reflecting the molecular dynamics of water molecules, could be correlated with the energetic changes in the AChR ion channel occurring as a function of temperature, as measured in single-channel recordings⁸⁵. Laurdan expends energy in solvent (water) reorientation, as evidenced in the red shift of its emission spectrum. The decrease in Laurdan GP upon increasing the temperature reflects an increase in water diffusion into the membrane. Water penetration into the membrane is facilitated by the increased thermal-induced disorder in the bilayer lipid. The higher AChR channel conductance upon increasing the temperature is a manifestation of the augmented ion and water permeability in the AChR channel or 'pore' region as we observed in single-channel recordings with the patch-clamp technique. Our study⁸⁵ further indicated that AChR channel kinetics depends not only on intrinsic properties of the AChR protein but also on the physical state of the membrane in which the receptor is embedded.

Topology and lipid selectivity of individual AChR membrane-embedded domains

The topography of the pyrene-labelled Cys residues in TM regions of the AChR with respect to the membrane was experimentally determined by differential fluorescence quenching with spin-labelled derivatives of fatty acids, PC, and the steroids cholestane and androstane⁸². TM peptides were obtained by controlled enzymatic digestion from purified *Torpedo californica* AChR, derivatized with *N*-(1-pyrenyl)maleimide (PM), purified, and reconstituted into asolectin liposomes. For the intact AChR, PM fluorescence mapped to proteolytic fragments consistent with labelling of cysteine residues in α M1, α M4, γ M1 and γ M4. Stern-Volmer plots of whole AChR quenching by spin-labelled lipid analogues showed no deviation from linearity. Stearic acid and androstane spin label derivatives were the most effective quenchers of the pyrene fluorescence of whole AChR and derived TM

peptides. In the case of spin-labelled stearic acid derivatives, the 5-SASL isomer quenched more effectively than the 7-SASL and the 12-SASL analogues, indicating a shallow location of the pyrene-labelled Cys residues⁸⁶. In fact, the quenching studies indicated that all labelled Cys residues are located in shallow positions with respect to the membrane bilayer in the AChR and derived TM peptides. In the case of γ M4, and by inference in other M4 segments of the AChR, this is compatible with a linear α -helical structure (α Cys⁴¹² and γ Cys⁴⁵¹ are located near the N-terminus of the TM segment). In the case of α M1, 'classical' models locate α Cys²²² and Cys²³⁰ at the center of the hydrophobic segment in an extended α -helical structure and therefore alternative structures/topologies for the M1 domain must be considered. One possibility is that the M1 segment contains non-helical structure, and/or kinks. In a mixed α -helix/ β -sheet model of the AChR⁸⁷, α M1 was constructed as a three-strand β -sheet interrupted by short loops generated by searching in the database of known structures for an appropriate backbone conformation. The proline residues themselves could not lie within a β -strand, so they were positioned in the loops. The same model can be extended to γ M1, having a proline residue (Pro²²⁹) immediately adjacent to Cys²³⁰ and two other (Pro²²² and Pro²⁴⁴) at the end of the TM region. Thus, the conserved proline residues in the M1 segments of the AChR might introduce 'curls' or kinks in a manner analogous to that reported for one of the TM segments of a K⁺ channel^{88,90}. The occurrence of proline residues – potential helix-disrupting residues – is a striking feature of M1 in the AChR and all members of the rapid LGIC superfamily⁹¹. Another possibility is that a portion of the N-terminus of the M1 TM α -helix extends beyond the lipid bilayer, therefore placing Cys²²²/Cys²³⁰ near the lipid/water interface. This interpretation appears now more likely in view of electron microscopy data^{43,44}.

We studied the γ M4 AChR domain incorporated in liposomes made of 60% POPC-40% Chol by means of fluorescence spectroscopy⁹². In a pure phospholipid (POPC) system in the I_d phase, the lipid-peptide interactions predominated over interactions between helices, and γ M4 occurred as an isolated peptide, matching the hydrophobic region of the bilayer. In contrast, in a mixed 60% POPC-40% Chol system in the I_o phase, peptide-peptide interactions prevailed, and peptide aggregation occurred. These Chol-dependent properties of a representative membrane-embedded segment of the AChR may bear relevance to the organization of the γ -subunit α -helical bundle motif and the AChR membrane-spanning region at large. The tendency of the hydrophobic γ M4 peptide to maximize peptide-peptide interactions in the presence but not in the absence of Chol may be related to the ability of this sterol to stabilize the α -helix content of the native AChR^{78,93}. Diffusion coefficients of $D = 14 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ (for 7% peptide) and $D = 12 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ (for 3% peptide) were obtained for the γ M4 peptide. These values

have the same order of magnitude or are slightly higher than those typically found for diffusion in an l_d phase ($D = 1.1 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$)⁹⁴, $D = 1-3 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ for trans-membrane proteins and $D = 9-14 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ for a fluorescent lipid derivative⁹⁵ and the fluorescent lipid analogue NBD-phosphatidylethanolamine ($D = 8 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$)⁹⁶. The lateral diffusion of the AChR reconstituted in pure DMPC bilayers was studied with fluorescence recovery after photobleaching techniques. D values in the range of $10^{-8} \text{ cm}^2 \text{ s}^{-1}$ were observed for both the AChR monomer and dimer in the l_o phase⁹⁶. Additional multiple-component recoveries with D values of less than $5 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$ were found below the lipid phase transition. Thus the translational diffusion of the γ M4 peptide is not significantly different from that of the 9S AChR monomer or the 13S dimer in the low concentration limit; neither the peptide nor the whole AChR encounter hindrance to lateral diffusion on the part of the lipid bilayer itself, whereas in native membranes, the densely packed AChR molecules are almost totally immobile (see ref. 91 for review).

Functional effects of the lipid environment on the AChR

Several channels and receptors, the AChR protein included, are embedded in the postsynaptic membrane. Given the extensive contacts between membrane lipids and the AChR, the physicochemical properties of the constituent lipids are likely to influence the physicochemical and functional properties of the protein moiety, and likewise, the latter is bound to modify the corresponding properties of the membrane lipids. This prediction finds extensive experimental validation, since various types of lipid have been found to affect the function of channels and receptors, as analysed in this section. One functionally relevant example is the phospholipid regulation exerted on the ATP-sensitive potassium channel (K_{ATP} channel), the channel that (i) controls the movement of K ions in and out of the pancreatic β cells, thus linking blood glucose concentration with insulin secretion, (ii) modulates the tone of smooth muscle in blood vessels and (iii) regulates the length of the action potential in cardiac muscle. K_{ATP} channel has been shown to be modulated by phosphatidylinositol-4,5-bisphosphate (PIP_2)^{97,98}.

Exogenous, synthetic steroidal substances of therapeutic use, as well as endogenous steroids have been shown to bind to⁹ and affect muscle and neuronal-type AChR⁹⁹⁻¹¹⁰.

Lipids and agonist-induced state transitions: Chol and negatively charged phospholipids

When AChRs were reconstituted into lipid vesicles made up of only PC they were found not to be functional^{22-24,111}. The presence of Chol and anionic lipids such as PA or PS

restored the capacity to undergo agonist-induced state transitions¹¹². However, contradictory interpretations still remain. Thus Rankin *et al.*¹¹³ concluded that AChR reconstituted in PA/PC mixtures were unable to undergo agonist-induced state transitions, whereas Baezinger *et al.*¹¹² used AChR preparations reconstituted in Chol and found that they are able to undergo agonist-induced structural transitions, though anionic lipids were needed for full activity. Recent studies stress the importance of acidic phospholipids like PA (e.g. POPC liposomes containing POPA) in the reconstitution mixture to preserve nearly complete functionality of the AChR. Addition of Chol (POPC/POPA/Chol, 3:1:1) had a positive synergistic effect¹¹⁴. These results further suggest that this lipid composition stabilizes the receptor in the resting state and allows agonist-induced state transitions, as previously observed with FTIR spectroscopy and radioactive ion-flux experiments¹¹⁵.

Lipid effects on AChR agonist-induced conformational states

The requisite lipid composition for maintenance of agonist-induced affinity transitions between conformational states is not necessarily optimal for adequate receptor-controlled ion translocation^{22,23}. The latter property has been reported to depend on the presence of neutral and anionic phospholipids²⁴. In one of our studies, unsaturated PE in combination with approximately 30% of a cholesteryl ester proved the best lipid mixture for the reconstitution of the AChR-mediated ion-permeation in artificial membranes²³. The less saturated the acyl chains of PE, the higher the observed response.

Other lipids and hydrophobic molecules are also known to modulate AChR activity^{8,116,117}. Fatty acids and sterols are of particular significance. Their selectivity for the AChR microenvironment was demonstrated in early experiments^{9,116}. The major lipid components of biological membranes in general are phosphoglycerides, Chol, and sphingolipids. In the case of the AChR-rich membranes prepared from the electric organ of Torpedinidae species, the major phospholipid constituents are choline-(40%), ethanolamine-(35%), and serine-(13%) glycerophospholipids. The effect of phosphoglyceride composition on AChR function has been the focus of several studies²²⁻²⁴. Chol and steroids have also been shown to play a major role in AChR function^{82,116,118}. Sphingomyelins (N-acyl-sphingosine-1-phosphorylcholine or ceramide-1-phosphorylcholine, 'SM'), the simplest class of the sphingolipids, amount to about 10% of cellular lipids in mammalian cell membranes. They are more abundant in the plasmalemma than in intracellular membranes and like the other choline-containing phospholipid, PC, are enriched in the outer, exoplasmic leaflet of the bilayer^{119,120}. Sphingolipids are important lipid constituents, essential for cell growth¹²¹.

Aminophospholipids such as PS or PE, on the other hand, are enriched in the inner, cytoplasmic leaflet of the membrane¹²². SM has also been implied to play a key role in the so-called 'SM cycle', in which SM-derived ceramide acts as a lipid second messenger¹²³. In the case of the AChR-rich membrane from *Torpedinidae*, SM accounts for about 5% of the total phospholipid content^{124,125}. We have established its topography in the *Torpedo* AChR-rich membrane and its moderate-to-low affinity for the receptor protein⁸⁰. More recently, we found that inhibition of sphingolipid biosynthesis resulted in the accumulation of unassembled AChR oligomers in the endoplasmic reticulum, leading us to suggest a 'chaperone-like' effect of sphingolipids on the AChR biosynthetic pathway, affecting both the efficiency of the assembly process and subsequent receptor trafficking to the cell surface¹²⁶.

A correlation between the structural characteristics of some lipids and AChR conformation could be established in fluorescence studies with the open channel blocker crystal violet. This probe exhibits a higher affinity for the desensitized state than for the resting state of the receptor. Fatty acids decreased, albeit to different extents, the K_D of CrV in the absence of agonist. However, only *cis*-fatty acids, which increase membrane fluidity, caused an increase in K_D in the presence of agonist. Steroids produced a concentration-dependent diminution of the K_D of the resting AChR state, which approached that of the K_D of the desensitized state. However, the presence of steroids did not alter the desensitized state of the AChR, a result that concurs with the fact that the steroids tested did not change the polarity of the membrane sensed by Laurdan GP¹²⁷.

Lipid sites on the AChR molecule

The presence of binding sites for hydrophobic molecules distinct from the annular ones, on the surface of the $(Ca^{2+}-Mg^{2+})$ -ATPase, has been deduced from both FRET between Trp residues and dansyl-undecanoic acid, and quenching of the intrinsic protein fluorescence by brominated lipids^{128,129}. The simple addition of Chol had no effect on the intrinsic fluorescence of the ATPase reconstituted in dioleoylphosphatidylcholine (DOPC) liposomes containing brominated PC. However, reconstitution of the ATPase with mixtures of DOPC and dibromo-Chol resulted in fluorescence quenching. From these data a model was proposed in which Chol is excluded from the lipid-protein interface, i.e. from the annular binding sites, but is still able to bind at a second set of sites from which phospholipids are excluded, namely non-annular binding sites. The same explanation was furnished in the case of the AChR by McNamee's group²⁵ and non-annular binding sites for fatty acids, Chol and its analogue cholesteryl hemisuccinate were postulated to occur on the surface of the receptor, but distant from the protein-lipid interface

or 'annular' lipids. In an early modelling study, we 'docked' Chol molecules on the lipid-facing surface of the AChR TM region in crevices between M1, M3 and M4 from adjacent subunits¹³⁰. Such crevices were apparent even in low-resolution electron microscope images of the AChR protein³⁹. Five such sites were located in each leaflet of the membrane, making a total of ten steroid sites, in general agreement with the discrete number of sites postulated by McNamee's group^{19,25}. A second outcome of this early model was that only a few lipid molecules could gain simultaneous access to more than two TM segments. This concept was further elaborated in a subsequent model based on Unwin's suggestion³⁸ of similarities between the tertiary structure of the B5 pentamer of *E. coli* enterotoxin and the AChR. In their description of the Chol sites on the AChR protein, Jones and McNamee¹⁹ postulated that these sites were at interstitial regions of the AChR. Miller and coworkers¹³¹ further elaborated on the topology of the Chol sites, which they located at a distance of about 0.6 nm from the first shell or annular lipids.

Early work on AChR-rich membranes demonstrated the natural abundance of Chol^{124,132} and the protein-induced restriction in mobility of spin-labelled androstanol⁹. The latter biophysical work suggested the occurrence of sites for steroid-like molecules in the AChR microenvironment. The search for such sites involved the use of other spin-labelled sterols¹³ and photoaffinity labelling techniques¹³³ which demonstrated the close proximity of a Chol analogue and the AChR. The radioactive photoaffinity label [³H]cholesteryl diazoacetate, a carbene-generating probe, was incorporated into all AChR subunits. Spin-labelled cholestane was also shown to be incorporated into AChR-rich regions of the membrane¹¹. Gonzalez-Ros and coworkers¹³⁴ showed that a photoactivatable analogue of Chol can be displaced from the AChR by unlabelled Chol and that the labelling is sensitive to the desensitization phenomenon.

Jones and McNamee¹⁹ used brominated sterol to define annular (about 45 per AChR monomer) and non-annular (about 5–10 per AChR) sites, the latter with ~20-fold higher affinity for Chol. Ellena *et al.*¹³ showed that another sterol, androstanol, exhibited a higher selectivity relative to PC, occupying about 38 sites on the AChR. Subsequent ESR work showed preference of the AChR for a PS analogue (~60% perturbation by the membrane protein) over other lipids (~30%)¹². After proteolytic removal of the extramembrane portions of the membrane-bound receptor, binding sites for the spin-labelled androstanol and stearic acid disappeared, but not for phospholipids and sphingomyelin analogues. The occurrence of androstanol sites in extracellular AChR domains has been challenged by subsequent work¹³¹. Photoaffinity labelling studies with azido-Chol also do not support the occurrence of extracellular sites for the sterol¹³⁵ as found with the GABA_A receptor.

Fernández-Ballester *et al.*⁶⁵ studied the effect of Chol on the secondary structure and cation permeability of reconstituted *Torpedo* AChR. They found that the presence of phospholipids was necessary in the reconstitution mixture to support the modulatory effect exerted by Chol on AChR ion permeability *in vitro*. Blanton *et al.*¹³⁶ used the steroid promegestone to photolabel *T. californica* AChR. The probe was incorporated into each of the AChR subunits in approximately equal amounts both in the presence and in the absence of Carb. While no evidence of [³H]promegestone incorporation was detected in the inner ring M2 segment, residues reacting with the steroid in the outer ring M4 domains were identified and found to be identical to those previously shown with other ligands reported to be in contact with the lipid bilayer. The steroid promegestone was further found to be a noncompetitive antagonist of the AChR, allosterically affecting the receptor by interacting with residues situated at the lipid-protein interface¹³⁶. Corbin *et al.*¹³⁵ used the photoactivatable Chol analogue 3 α -(4-azido-3-[¹²⁵I]iodosalicylic)-cholest-5-ene (azido-Chol) to label the TM regions of *T. californica* AChR. In α M4, residues Cys⁴¹², Val⁴¹³, Phe⁴¹⁴ and Met⁴¹⁵ were labelled. This stretch corresponds to outer ring residues embedded in the cytoplasmic-facing hemilayer of the membrane.

Another strategy for determining the occurrence of lipid sites in the AChR exploited the decrease in FRET efficiency (*E*) between the intrinsic fluorescence of AChR-rich membranes and Laurdan, induced by different lipids. *E* between AChR (donor) and Laurdan (acceptor) was calculated using the equations below. *E* is given by

$$E = R_o^6 / (R_o^6 + r^6),$$

where *r* is the intermolecular distance and *R_o* is a constant parameter for each donor-acceptor pair, defined as the distance at which *E* is 50%. *E* can also be calculated as:

$$E = 1 - (\phi / \phi_D) \approx 1 - (I / I_D),$$

where ϕ and ϕ_D are the fluorescence quantum yields of donor in the presence and absence of the acceptor, respectively, and *I* and *I_D* are the corresponding emission intensities. FRET efficiency was found to decrease upon addition of exogenous lipids, which displace Laurdan molecules from the AChR-microenvironment. The maximal decrease in *E* resulting from the addition of a fatty acid (18:1) amounted to about 60%, whereas Chol or phospholipid reduced *E* by 35% and 25% respectively. The sum of the decreases caused by DOPC and colesteryl hemisuccinate equalled that obtained in the presence of 18:1 alone. From this series of experiments we reached the conclusions that (i) there are independent sites for phospholipid and sterol, both accessible to fatty acid, in the vicinity of the AChR²⁸, in agreement with previous ESR work^{12,19} and (ii) fatty acids of different chain length

and saturation share a common site(s) but produce different effects on the physical properties of AChR-associated lipid belt region and bulk lipids, respectively²⁹.

1. Barrantes, F. J. (ed.), *The Nicotinic Acetylcholine Receptor: Current Views and Future Trends*, Springer Verlag, Berlin, 1998, pp. 1–226.
2. Corringer, P. J., Le Novère, N. and Changeux, J. P., Nicotinic receptors at the amino acid level. *Annu. Rev. Pharmacol. Toxicol.*, 2000, **40**, 431–458.
3. Karlin, A., Emerging structure of the nicotinic acetylcholine receptors. *Nat. Rev. Neurosci.*, 2002, **3**, 102–114.
4. Bocquet, N. *et al.*, A prokaryotic proton-gated ion channel from the nicotinic acetylcholine receptor family. *Nature*, 2007, **445**, 116–119.
5. Lindstrom, J., Merlie, J. and Yogeewaran, G., Biochemical properties of acetylcholine receptor subunits from *Torpedo californica*. *Biochemistry*, 1979, **18**, 4465–4470.
6. Reynolds, J. A. and Karlin, A., Molecular weight in detergent solution of acetylcholine receptor from *Torpedo californica*. *Biochemistry*, 1978, **17**, 2035–2038.
7. Barrantes, F. J., Modulation of nicotinic acetylcholine receptor function through the outer and middle rings of transmembrane domains. *Curr. Opin. Drug Discov. Devel.*, 2003, **6**, 620–632.
8. Barrantes, F. J., Structural basis for lipid modulation of nicotinic acetylcholine receptor function. *Brain Res. Rev. Brain Res. Rev.*, 2004, **47**, 71–95.
9. Marsh, D. and Barrantes, F. J., Immobilized lipid in acetylcholine receptor-rich membranes from *Torpedo marmorata*. *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 4329–4333.
10. Abadji, V. C., Reines, D. E., Dalton, L. A. and Miller, K. M., Lipid-protein interactions and protein dynamics in vesicles containing the nicotinic acetylcholine receptor: a study with ethanol. *Biochim. Biophys. Acta*, 1994, **1194**, 25–34.
11. Arias, H. R., Sankaram, M. B., Marsh, D. and Barrantes, F. J., Effect of local anaesthetics on steroid-nicotinic acetylcholine receptor interactions in native membranes of *Torpedo marmorata* electric organ. *Biochim. Biophys. Acta*, 1990, **1027**, 287–294.
12. Dreger, M., Krauss, M., Herrmann, A. and Hucho, F., Interactions of the nicotinic acetylcholine receptor transmembrane segments with the lipid bilayer in native receptor-rich membranes. *Biochemistry*, 1997, **36**, 839–847.
13. Ellena, J. F., Blazing, M. A. and McNamee, M. G., Lipid-protein interactions in reconstituted membranes containing acetylcholine receptor. *Biochemistry*, 1983, **22**, 5523–5535.
14. Horváth, L. I., Arias, H. R., Hankovszky, H. O., Hideg, K., Barrantes, F. J. and Marsh, D., Association of spin-labeled local anaesthetics at the hydrophobic surface of acetylcholine receptor in native membranes from *Torpedo marmorata*. *Biochemistry*, 1990, **29**, 8707–8713.
15. Marsh, D., Watts, A. and Barrantes, F. J., Phospholipid chain immobilization and steroid rotational immobilization in acetylcholine receptor-rich membranes from *Torpedo marmorata*. *Biochim. Biophys. Acta*, 1981, **645**, 97–101.
16. Barrantes, F. J., Cholesterol effects on nicotinic acetylcholine receptor. *J. Neurochem.*, 2007, **103**, Suppl. 1, 72–80.
17. Ipsen, J. H., Karlström, G., Mouritsen, O. C. and Zuckermann, M. J., Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta*, 2003, **905**, 162–172.
18. Bhushan, A. and McNamee, M. G., Correlation of phospholipid structure with functional effects on the nicotinic acetylcholine receptor. A modulatory role for phosphatidic acid. *Biophys. J.*, 1993, **64**, 716–723.
19. Jones, O. T. and McNamee, M. G., Annular and nonannular binding sites for cholesterol associated with the nicotinic acetylcholine receptor. *Biochemistry*, 1988, **27**, 2364–2374.

20. Rousselet, A., Devaux, P. F. and Wirtz, K. W., Free fatty acids and esters can be immobilized by receptor rich membranes from *Torpedo marmorata* but not phospholipid acyl chains. *Biochem. Biophys. Res. Commun.*, 1979, **90**, 871–877.
21. Epstein, M. and Racker, E., Reconstitution of carbamylcholine-dependent sodium ion flux and desensitization of the acetylcholine receptor from *Torpedo californica*. *J. Biol. Chem.*, 1978, **253**, 6660–6662.
22. Criado, M., Eibl, H. and Barrantes, F. J., Effects of lipids on acetylcholine receptor. Essential need of cholesterol for maintenance of agonist-induced state transitions in lipid vesicles. *Biochemistry*, 1982a, **21**, 3622–3629.
23. Criado, M., Eibl, H. and Barrantes, F. J., Functional properties of the acetylcholine receptor incorporated in model lipid membranes. Differential effects of chain length and head group of phospholipids on receptor affinity states and receptor-mediated ion translocation. *J. Biol. Chem.*, 1984, **259**, 9188–9198.
24. Ochoa, E. L., Dalziel, A. W. and McNamee, M. G., Reconstitution of acetylcholine receptor function in lipid vesicles of defined composition. *Biochim. Biophys. Acta*, 1983, **727**, 151–162.
25. Jones, O. T., Eubanks, J. H., Earnest, J. P. and McNamee, M. G., A minimum number of lipids are required to support the functional properties of the nicotinic acetylcholine receptor. *Biochemistry*, 1988, **27**, 3733–3742.
26. Parasassi, T., De Stasio, G., d'Ubaldo, A. and Gratton, E., Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. *Biophys. J.*, 1990, **57**, 1179–1186.
27. Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R. M. and Gratton, E., Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. *Biophys. J.*, 1991, **60**, 179–189.
28. Antollini, S. S. and Barrantes, F. J., Disclosure of discrete sites for phospholipid and sterols at the protein–lipid interface in native acetylcholine receptor-rich membrane. *Biochemistry*, 1998, **37**, 16653–16662.
29. Antollini, S. S. and Barrantes, F. J., Unique effects of different fatty acid species on the physical properties of the *Torpedo* acetylcholine receptor membrane. *J. Biol. Chem.*, 2002, **277**, 1249–1254.
30. Antollini, S. S., Soto, M. A., Bonini de Romanelli, I. C., Gutierrez-Merino, C., Sotomayor, P. and Barrantes, F. J., Physical state of bulk and protein-associated lipid in nicotinic acetylcholine receptor-rich membrane studied by laurdan generalized polarization and fluorescence energy transfer. *Biophys. J.*, 1996, **70**, 1275–1284.
31. Unwin, N., Nicotinic acetylcholine receptor at 9 Å resolution. *J. Mol. Biol.*, 1993, **229**, 1101–1124.
32. Zingsheim, H. P., Barrantes, F. J., Frank, J., Haenicke, W. and Neugebauer, D. C., Direct structural localization of two toxin-recognition sites on an ACh receptor protein. *Nature*, 1982a, **299**, 81–84.
33. Zingsheim, H. P., Neugebauer, D. C., Frank, J., Hanicke, W. and Barrantes, F. J., Dimeric arrangement and structure of the membrane-bound acetylcholine receptor studied by electron microscopy. *EMBO J.*, 1982b, **1**, 541–547.
34. Mitra, A. K., McCarthy, M. P. and Stroud, R. M., Three-dimensional structure of the nicotinic acetylcholine receptor and location of the major associated 43-kD cytoskeletal protein, determined at 22 Å by low dose electron microscopy and x-ray diffraction to 12.5 Å. *J. Cell Biol.*, 1989, **109**, 755–774.
35. Toyoshima, C. and Unwin, N., Three-dimensional structure of the acetylcholine receptor by cryoelectron microscopy and helical image reconstruction. *J. Cell Biol.*, 1990, **111**, 2623–2635.
36. Prince, R. J. and Sine, S. M., The ligand binding domains of the nicotinic acetylcholine receptor. In *The Nicotinic Acetylcholine Receptor: Current Views and Future Trends* (ed. Barrantes, F. J.), Landes Bioscience, Austin, Texas, 1998, pp. 31–59.
37. Valenzuela, C. F., Weign, P., Yguerabide, J. and Johnson, D. A., Transverse distance between the membrane and the agonist binding sites on the *Torpedo* acetylcholine receptor: a fluorescence study. *Biophys. J.*, 1994, **66**, 674–682.
38. Unwin, N., Neurotransmitter action: opening of ligand-gated ion channels. *Cell*, 1993a, **72**, 31–41.
39. Unwin, N., The Croonian Lecture 2000. Nicotinic acetylcholine receptor and the structural basis of fast synaptic transmission. *Philos. Trans. R. Soc. London. B Biol. Sci.*, 2000, **355**, 1813–1829.
40. Changeux, J. P. and Edelstein, S. J., Allosteric receptors after 30 years. *Neuron*, 1998, **21**, 959–980.
41. Opella, S. J., Marassi, F. M., Gesell, J. J., Valente, A. P., Kim, Y., Oblatt-Montal, M. and Montal, M., Structures of the M2 channel-lining segments from nicotinic acetylcholine and NMDA receptors by NMR spectroscopy. *Nat. Struct. Biol.*, 1999, **6**, 374–379.
42. Pashkov, V. S., Maslennikov, I. V., Tchikin, L. D., Efremov, R. G., Ivanov, V. T. and Arseniev, A. S., Spatial structure of the M2 transmembrane segment of the nicotinic acetylcholine receptor alpha-subunit. *FEBS Lett.*, 1999, **457**, 117–121.
43. Miyazawa, A., Fujiyoshi, Y. and Unwin, N., Structure and gating mechanism of the acetylcholine receptor pore. *Nature*, 2003, **423**, 949–955.
44. Unwin, N., Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution. *J. Mol. Biol.*, 2005, **346**, 967–989.
45. Unwin, N., Acetylcholine receptor channel imaged in the open state. *Nature*, 1995, **373**, 37–43.
46. Blanton, M. P. and Cohen, J. B., Mapping the lipid-exposed regions in the *Torpedo californica* nicotinic acetylcholine receptor. *Biochemistry*, 1992, **31**, 3738–3750.
47. Blanton, M. P. and Cohen, J. B., Identifying the lipid–protein interface of the *Torpedo* nicotinic acetylcholine receptor: secondary structure implications. *Biochemistry*, 1994, **33**, 2859–2872.
48. Blanton, M. P., McCarty, E. A., Huggins, A. and Parikh, D., Probing the structure of the nicotinic acetylcholine receptor with the hydrophobic photoreactive probes [¹²⁵I]TID-BE and [¹²⁵I]TIDPC/16. *Biochemistry*, 1998, **37**, 14545–14555.
49. Baezinger, J. E. and Methot, N., Fourier transform infrared and hydrogen/deuterium exchange reveal an exchange-resistant core of α -helical peptide hydrogens in the nicotinic acetylcholine receptor. *J. Biol. Chem.*, 1996, **270**, 29129–29137.
50. Corbin, J., Methot, N., Wang, H. H., Baenziger, J. E. and Blanton, M. P., Secondary structure analysis of individual transmembrane segments of the nicotinic acetylcholine receptor by circular dichroism and fourier transform infrared spectroscopy. *J. Biol. Chem.*, 1998a, **273**, 771–777.
51. Lugovskoy, A. A., Maslennikov, I. V., Utkin, Y. N., Tsetlin, V. I., Cohen, J. B. and Arseniev, A. S., Spatial structure of the M3 transmembrane segment of the nicotinic acetylcholine receptor α subunit. *Eur. J. Biochem.*, 1998, **255**, 455–461.
52. Williamson, P. T. F., Zandomeneghi, G., Barrantes, F. J., Watts, A. and Meier, B. H., Structural and dynamic studies of the γ -M4 transmembrane domain of the nicotinic acetylcholine receptor. *Mol. Membr. Biol.*, 2005, **22**, 485–496.
53. Brejc, K., van Dijk, W. J., Klaassen, R. V., Schuurmans, M., van Der, O. J., Smit, A. B. and Sixma, T. K., Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature*, 2001, **411**, 269–276.
54. Dellisanti, C. D., Yao, Y., Stroud, J. C., Wang, Z.-Z. and Chen, L., Crystal structure of the extracellular domain of nAChR α bound to α -bungarotoxin at 1.94 Å resolution. *Nature Neurosci.*, 2007, **10**, 953–962.
55. Hilf, R. J. and Dutzler, R., X-ray structure of a prokaryotic pentameric ligand-gated ion channel. *Nature*, 2008, **452**, 375–379.
56. Brown, E. R. and Subbiah, P. V., Differential effects of eicosapentaenoic acid and docosahexaenoic acid on human skin fibroblasts. *Lipids*, 1994, **29**, 825–829.

57. Mielke, D. L. and Wallace, B. A., Secondary structural analyses of the nicotinic acetylcholine receptor as a test of molecular models. *J. Biol. Chem.*, 1988, **263**, 3177–3182.
58. Aslanian, D., Grof, P., Galzi, J. L. and Changeux, J. P., A Raman spectroscopic study of acetylcholine receptor-rich membranes from *Torpedo marmorata*. Interaction of the receptor with carbamylcholine and (+)-tubocurarine. *Biochim. Biophys. Acta*, 1993, **1148**, 291–302.
59. McCarthy, M. P. and Stroud, R. M., Conformational states of the nicotinic acetylcholine receptor from *Torpedo californica* induced by the binding of agonists, antagonists, and local anesthetics. Equilibrium measurements using tritium–hydrogen exchange. *Biochemistry*, 1989, **28**, 40–48.
60. Methot, N., Demers, C. N. and Baenziger, J. E., Structure of both the ligand- and lipid-dependent channel-inactive states of the nicotinic acetylcholine receptor probed by FTIR spectroscopy and hydrogen exchange. *Biochemistry*, 1995, **34**, 15142–15149.
61. Barrantes, F. J., Agonist-mediated changes of the acetylcholine receptor in its membrane environment, *J. Mol. Biol.*, **124**, 1978, 1–26.
62. Galzi, J. L., Revah, F., Bouet, F., Ménez, A., Goeldner, M., Hirth, C. and Changeux, J. P., Allosteric transitions of the acetylcholine receptor probed at the amino acid level with a photolabile cholinergic ligand. *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 5051–5055.
63. White, B. H. and Cohen, J. B., Photolabeling of membrane-bound *Torpedo* nicotinic acetylcholine receptor with the hydrophobic probe 3-trifluoromethyl-3-(m-[¹²⁵I]iodophenyl)diazirine. *Biochemistry*, 1988, **27**, 8741–8751.
64. Castresana, J., Fernández-Ballester, G., Fernández, A. M., Laynez, J. L., Arrondo, J. L. R., Ferragut, J. A. and González-Ros, J. M., Protein structural effects of agonist binding to the nicotinic acetylcholine receptor. *FEBS Lett.*, 1992, **314**, 171–175.
65. Fernández-Ballester, G., Castresana, J., Fernandez, A. M., Arrondo, J.-L. R., Ferragut, J. A. and Gonzalez-Ros, J. M., A role for cholesterol as a structural effector of the nicotinic acetylcholine receptor. *Biochemistry*, 1994, **33**, 4065–4071.
66. Methot, N., McCarthy, M. P. and Baenziger, J. E., Secondary structure of the nicotinic acetylcholine receptor: Implications for structural models of a ligand-gated ion channel. *Biochemistry*, 1994, **33**, 7709–7717.
67. Finer-Moore, J. and Stroud, R. M., Amphipathic analysis and possible formation of the ion channel in an acetylcholine receptor. *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 155–159.
68. Methot, N., Ritchie, B. D., Blanton, M. P. and Baenziger, J. E., Structure of the pore-forming transmembrane domain of a ligand-gated ion channel. *J. Biol. Chem.*, 2001, **276**, 23726–23732.
69. Conti-Fine, B. M., Protti, M. P., Bellone, M. and Howard, J. F., *Myasthenia Gravis: The Immunology of an Autoimmune Disease*, Chapman & Hall, 1997, pp. 1–230.
70. Noda, M. *et al.*, Cloning and sequence analysis of calf cDNA and human genomic DNA encoding subunit precursor of muscle acetylcholine receptor. *Nature*, 1983, **305**, 818–823.
71. Chavez, R. A. and Hall, Z. W., Expression of fusion proteins of the nicotinic acetylcholine receptor from mammalian muscle identifies the membrane-spanning regions in the alpha and delta subunits. *J. Cell Biol.*, 1992, **116**, 385–393.
72. Tobimatsu, T., Fujita, Y., Fukuda, K., Tanaka, K., Mori, Y., Konno, T., Mishina, M. and Numa, S., Effects of substitution of putative transmembrane segments on nicotinic receptor function. *FEBS. Lett.*, 1987, **222**, 56–62.
73. Görne-Tschelnokow, U., Strecker, A., Kaduk, C., Naumann, D. and Hucho, F., The transmembrane domains of the nicotinic acetylcholine receptor contain α -helical and β -structures. *EMBO J.*, 1994, **13**, 338–341.
74. Chattopadhyay, A. and McNamee, M. G., Average membrane penetration depth of tryptophan residues of the nicotinic acetylcholine receptor by the parallax method. *Biochemistry*, 1991, **30**, 7159–7164.
75. Ortells, M. O., Barrantes, G. E. and Barrantes, F. J., Molecular modeling of the nicotinic acetylcholine receptor. In *The Nicotinic Acetylcholine Receptor: Current Views and Future Trends* (ed. Barrantes, F. J.), Landes Bioscience, Austin, Texas, 1998, pp. 85–108.
76. London, E. and Feigenson, G. W., Fluorescence quenching in model membranes. 2. Determination of the local lipid environment of the calcium adenosinetriphosphatase from sarcoplasmic reticulum. *Biochemistry*, 1981, **20**, 1939–1948.
77. Marsh, D., ESR spin label studies of lipid–protein interactions. In *Progress in Protein–Lipid Interactions* (eds Watts, A. and De Pont, J. H. H. M.), Elsevier, Amsterdam, 1985, pp. 143–172.
78. Fong, T. M. and McNamee, M. G., Stabilization of acetylcholine receptor secondary structure by cholesterol and negatively charged phospholipids in membranes. *Biochemistry*, 1987, **26**, 3871–3880.
79. Andreasen, T. J. and McNamee, M. G., Inhibition of ion permeability control properties of acetylcholine receptor from *Torpedo californica* by long-chain fatty acids. *Biochemistry*, 1980, **19**, 4719–4726.
80. Bonini, I. C., Antollini, S. S., Gutierrez-Merino, C. and Barrantes, F. J., Sphingomyelin composition and physical asymmetries in native acetylcholine receptor-rich membranes. *Eur. Biophys. J.*, 2002, **31**, 417–427.
81. Mantipragada, S. B., Horvath, L. I., Arias, H. R., Schwarzmann, G., Sandhoff, K., Barrantes, F. J. and Marsh, D., Lipid–protein interactions and effect of local anesthetics in acetylcholine receptor-rich membranes from *Torpedo marmorata* electric organ. *Biochemistry*, 2003, **42**, 9167–9175.
82. Barrantes, F. J., Antollini, S. S., Blanton, M. P. and Prieto, M., Topography of nicotinic acetylcholine receptor membrane-embedded domains. *J. Biol. Chem.*, 2000a, **275**, 37333–37339.
83. Marsh, D. and Horváth, L. I., Structure, dynamics and composition of the lipid–protein interface. Perspectives from spin-labeling. *Biochim. Biophys. Acta*, 1998, **1376**, 267–296.
84. Chattopadhyay, A. and London, E., Parallax method for direct measurement of membrane penetration depth utilizing fluorescence quenching by spin-labeled phospholipids. *Biochemistry*, 1987, **26**, 39–45.
85. Zanello, L. P., Aztiria, E., Antollini, S. and Barrantes F. J., Nicotinic acetylcholine receptor channels are influenced by the physical state of their membrane environment. *Biophys. J.*, 1996, **70**, 2155–2164.
86. Barrantes, F. J., Antollini, S. S., Bouzat, C. B., Garbus, I. and Massol, R. H., Nongenomic effects of steroids on the nicotinic acetylcholine receptor. *Kidney Int.*, 2000b, **57**, 1382–1389.
87. Ortells, M. O. and Lunt, G. G., A mixed helix-beta-sheet model of the transmembrane region of the nicotinic acetylcholine receptor. *Protein Eng.*, 1996, **9**, 51–59.
88. del Camino, D., Holmgren, M., Liu, Y. and Yellen, G., Blocker protection in the pore of a voltage-gated K⁺ channel and its structural implications. *Nature*, 2000, **403**, 321–325.
89. Grant, M. A., Gentile, L. N., Shi, Q. L., Pellegrini, M. and Hawrot, E., Expression and spectroscopic analysis of soluble nicotinic acetylcholine receptor fragments derived from the extracellular domain of the alpha-subunit. *Biochemistry*, 1999, **38**, 10730–10742.
90. Kim, J. and McNamee, M. G., Topological disposition of Cys 222 in the alpha-subunit of nicotinic acetylcholine receptor analyzed by fluorescence-quenching and electron paramagnetic resonance measurements. *Biochemistry*, 1998, **37**, 4680–4686.
91. Barrantes, F. J., Fluorescence studies of the acetylcholine receptor: Structure and dynamics in the membrane environment. *J. Fluorescen.*, 2001, **11**, 273–285.
92. De Almeida, R. F., Loura, L. M., Prieto, M., Watts, A., Fedorov, A. and Barrantes, F. J., Cholesterol modulates the organization of

- the gammaM4 transmembrane domain of the muscle nicotinic acetylcholine receptor. *Biophys. J.*, 2004, **86**, 2261–2272.
93. Butler, D. H. and McNamee, M. G., FTIR analysis of nicotinic acetylcholine receptor secondary structure in reconstituted membranes. *Biochim. Biophys. Acta Bio-Membr.*, 1993, **1150**, 17–24.
 94. Dietrich, C., Bagatolli, L. A., Volovyk, Z. N., Thompson, N. L., Levi, M., Jacobson, K. and Gratton, E., Lipid rafts reconstituted in model membranes. *Biophys. J.*, 2001, **80**, 1417–1428.
 95. Vaz, W. L., Criado, M., Madeira, V. M. C., Schoellmann, G. and Jovin, T. M., Size dependence of the translational diffusion of large integral membrane proteins in liquid-crystalline phase lipid bilayers. A study using fluorescence recovery after photobleaching. *Biochemistry*, 1982, **21**, 5608–5612.
 96. Criado, M., Vaz, W. L., Barrantes, F. J. and Jovin, T. M., Translational diffusion of acetylcholine receptor (monomeric and dimeric forms) of *Torpedo marmorata* reconstituted into phospholipid bilayers studied by fluorescence recovery after photobleaching. *Biochemistry*, 1982b, **21**, 5750–5755.
 97. Baukrowitz, T. *et al.*, PIP2 and PIP as determinants for ATP inhibition of KATP channels. *Science*, 1998, **282**, 1141–1144.
 98. Shyng, S. L. and Nichols, C. G., Membrane phospholipid control of nucleotide sensitivity of KATP channels. *Science*, 1998, **282**, 1138–1141.
 99. Bouzat, C. and Barrantes, F. J., Acute exposure of nicotinic acetylcholine receptors to the synthetic glucocorticoid dexamethasone alters single-channel gating properties. *Mol. Neuropharmacol.*, 1993a, **3**, 109–116.
 100. Bouzat, C. and Barrantes, F. J., Hydrocortisone and 11-desoxycortisone modify acetylcholine receptor channel gating. *NeuroReport*, 1993b, **4**, 143–146.
 101. Bouzat, C. and Barrantes, F. J., Modulation of muscle nicotinic acetylcholine receptors by the glucocorticoid hydrocortisone. Possible allosteric mechanism of channel blockade. *J. Biol. Chem.*, 1996, **271**, 25835–25841.
 102. Bouzat, C., Roccamo, A. M., Garbus, I. and Barrantes, F. J., Mutations at lipid-exposed residues of the acetylcholine receptor affect its gating kinetics. *Mol. Pharmacol.*, 1998, **54**, 146–153.
 103. Bouzat, C., Barrantes, F. J. and Sine, S., Nicotinic receptor fourth transmembrane domain. Hydrogen bonding by conserved threonine contributes to channel gating kinetics. *J. Gen. Physiol.*, 2000, **115**, 663–672.
 104. Curtis, L., Buisson, B., Bertrand, S. and Bertrand, D., Potentiation of human alpha4beta2 neuronal nicotinic acetylcholine receptor by estradiol. *Mol. Pharmacol.*, 2002, **61**, 127–135.
 105. Garbus, I., Bouzat, C. and Barrantes, F. J., Steroids differentially inhibit the nicotinic acetylcholine receptor. *NeuroReport*, 2001, **12**, 227–231.
 106. Garbus, I., Roccamo, A. M. and Barrantes, F. J., Identification of threonine 422 in transmembrane domain α M4 of the nicotinic acetylcholine receptor as a possible site of interaction with hydrocortisone. *Neuropharmacology*, 2002, **43**, 65–73.
 107. Ke, L. and Lukas, R. J., Effects of steroid exposure on ligand binding and functional activities of diverse nicotinic acetylcholine receptor subtypes. *J. Neurochem.*, 1996, **67**, 1100–1112.
 108. Nurowska, E. and Ruzzier, F., Corticosterone modifies the murine muscle acetylcholine receptor channel kinetics. *NeuroReport*, 1996, **8**, 77–80.
 109. Paradiso, K., Sabey, K., Evers, A. S., Zorumski, C. F., Covey, D. F. and Steinbach, J. H., Steroid inhibition of rat neuronal nicotinic alpha4beta2 receptors expressed in HEK 293 cells. *Mol. Pharmacol.*, 2000, **58**, 341–351.
 110. Paradiso, K., Zhang, J. and Steinbach, J. H., The C terminus of the human nicotinic alpha4beta2 receptor forms a binding site required for potentiation by an estrogenic steroid. *J. Neurosci.*, 2001, **21**, 6561–6568.
 111. Dalziel, A. W., Rollins, E. S. and McNamee, M. G., The effect of cholesterol on agonist-induced flux reconstituted acetylcholine receptor vesicles. *FEBS Lett.*, 1980, **122**, 193–196.
 112. Baezinger, J. E., Morris, M. L., Darsaut, T. E. and Ryan, S. E., Effect of membrane lipid composition on the conformational equilibria of the nicotinic acetylcholine receptor. *J. Biol. Chem.*, 2000, **275**, 777–784.
 113. Rankin, S. E., Addona, G. H., Kloczewiak, M. A., Bugge, B. and Miller, K. W., The cholesterol dependence of activation and fast desensitization of the nicotinic acetylcholine receptor. *Biophys. J.*, 1997, **73**, 2446–2455.
 114. daCosta, C. J., Ogrel, A. A., McCarty, E. A., Blanton, M. P. and Baenziger, J. E., Lipid-protein interactions at the nicotinic acetylcholine receptor. A functional coupling between nicotinic receptors and phosphatidic acid-containing lipid bilayers. *J. Biol. Chem.*, 2002, **277**, 201–208.
 115. Sunshine, C. and McNamee, M. G., Lipid modulation of nicotinic acetylcholine receptor function: The role of membrane lipid composition and fluidity. *Biochim. Biophys. Acta Bio-Membr.*, 1994, **1191**, 59–64.
 116. Barrantes, F. J., Structural-functional correlates of the nicotinic acetylcholine receptor and its lipid microenvironment. *FASEB J.*, 1993a, **7**, 1460–1467.
 117. Barrantes, F. J., The lipid annulus of the nicotinic acetylcholine receptor as a locus of structural-functional interactions. In *New Comprehensive Biochemistry* (ed. Watts, A.), Elsevier, Amsterdam, 1993b, pp. 231–257.
 118. Barrantes, F. J., Recent developments in the structure and function of the acetylcholine receptor. *Int. Rev. Neurobiol.*, 1983, **24**, 259–341.
 119. Hakomori, S., Glycosphingolipids in cellular interaction, differentiation and oncogenesis. *Annu. Rev. Biochem.*, 1981, **50**, 733–764.
 120. Koval, M. and Pagano, R. E., Intracellular transport and metabolism of sphingomyelin. *Biochim. Biophys. Acta*, 1991, **1082**, 113–125.
 121. Hanada, K., Nishijima, M. and Akamatsu, Y., A temperature-sensitive mammalian cell mutant with thermolabile serine palmitoyltransferase for the sphingolipid biosynthesis. *J. Biol. Chem.*, 1990, **265**, 22137–22142.
 122. Devaux, P. F., Protein involvement in transmembrane lipid asymmetry. *Annu. Rev. Biophys. Biomol. Struct.*, 1992, **21**, 417–439.
 123. Hannun, Y. A., Functions of ceramide in coordinating cellular responses to stress. *Science*, 1996, **274**, 1805–1976.
 124. González-Ros, J. M., Llanillo, M., Paraschos, A. and Martínez-Carrión, M., Lipid environment of acetylcholine receptor from *Torpedo californica*. *Biochemistry*, 1982, **21**, 3467–3474.
 125. Bonini de Romanelli, I. C., Roccamo de Fernandez, A. M. and Barrantes, F. J., Extraction of peripheral proteins is accompanied by selective depletion of certain glycerophospholipid classes and changes in the phosphorylation pattern of acetylcholine-receptor-rich-membrane proteins. *Biochem. J.*, 1987, **245**, 111–118.
 126. Baier, C. J. and Barrantes, F. J., Sphingolipids are necessary for nicotinic acetylcholine receptor export in the early secretory pathway. *J. Neurochem.*, 2007, **101**, 1072–1084.
 127. Fernandez Nieves, G. A., Barrantes, F. J. and Antollini, S. S., Modulation of nicotinic acetylcholine receptor conformational state by free fatty acids and steroids. *J. Biol. Chem.*, 2008, **283**, 21478–21486.
 128. Froud, R. J., East, J. M., Rooney, E. K. and Lee, A. G., Binding of long-chain alkyl derivatives to lipid bilayers and to $(\text{Ca}^{2+}\text{--Mg}^{2+})\text{-ATPase}$. *Biochemistry*, 1986, **25**, 7535–7544.
 129. Simmonds, A. C., East, J. M., Jones, O. T., Rooney, E. K., McWhirter, J. and Lee, A. G., Annular and non-annular binding sites on the $(\text{Ca}^{2+}\text{+Mg}^{2+})\text{-ATPase}$. *Biochim. Biophys. Acta*, 1982, **693**, 398–406.
 130. Ortells, M. O., Cockcroft, V. B., Lunt, G. G., Marsh, D. and Barrantes, F. J., The nicotinic acetylcholine receptor and its lipid micro-

- environment. In *Membrane Proteins: Structures, Interactions and Models* (eds Pullman, A., Jortner, J. and Pullman, B.), Kluwer, Dordrecht, 1992, pp. 185–198.
131. Addona, G. H., Sandermann, H., Jr., Kloczewiak, M. A., Husian, S. S. and Miller, K. W., Where does cholesterol act during activation of the nicotinic acetylcholine receptor? *Biochim. Biophys. Acta*, 1998, **1370**, 299–309.
132. Schiebler, W. and Hucho, F., Membranes rich in acetylcholine receptor: characterization and reconstitution to excitable membranes from exogenous lipids. *Eur. J. Biochem.*, 1978, **85**, 55–63.
133. Middlemas, D. S. and Raftery, M. A., Identification of subunits of acetylcholine receptor that interact with a cholesterol photoaffinity probe. *Biochemistry*, 1987, **26**, 1219–1223.
134. Fernandez, A. M., Fernandez-Ballester, G., Ferragut, J. A. and Gonzalez-Ros, J. M., Labeling of the nicotinic acetylcholine receptor by a photoactivatable steroid probe: effects of cholesterol and cholinergic ligands. *Biochim. Biophys. Acta*, 1993, **1149**, 135–144.
135. Corbin, J., Wang, H. H. and Blanton, M. P., Identifying the cholesterol binding domain in the nicotinic acetylcholine receptor with [125I]azido-cholesterol. *Biochim. Biophys. Acta*, 1998b, **1414**, 65–74.
136. Blanton, M. P., Xie, Y., Dangott, L. J. and Cohen, J. B., The steroid promegestone is a noncompetitive antagonist of the *Torpedo* nicotinic acetylcholine receptor that interacts with the lipid–protein interface. *Mol. Pharmacol.*, 1999, **55**, 269–278.
137. Liu, X., Xu, Y., Li, H., Wang, X., Jiang, H. and Barrantes, F. J., Mechanics of channel gating of the nicotinic acetylcholine receptor. *PLoS Computat. Biol.*, 2008, **4**, 100–110.

ACKNOWLEDGEMENTS. F.J.B. was supported by grants from the Universidad Nacional del Sur, the Argentinian Scientific Res. Council (CONICET), and the Agencia Nacional de Promoción Científica y Tecnológica (FONCYT), Argentina.
