unliganded gating, and the mutation αW149F reduces the ACh affinity of **C** only by 13-fold, but of **O** by 190-fold. Rate-equilibrium free energy relationships for different regions of the protein show similar slopes (Φ-values) for un- vs. diliganded gating. The mechanisms of the gating conformational change and of desensitization are similar with and without ligands at the transmitter binding sites.

864-Pos Board B743

Detection and Trapping of Elusive Priming Intermediates Towards Open Nicotinic Receptor Channel

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Acetylcholine receptors (AChRs) mediate rapid synaptic transmission by transducing a chemical signal into an electrical impulse. Transduction comprises binding of agonist followed by opening of the AChR ion channel, and in the classical view both processes depend on the agonist. However previous studies suggest the ultimate channel opening step is agonist-independent[1,2], and is preceded by a priming step facilitated by the agonist[3]. Here, by studying mutant AChRs, we detect two such priming steps; the first generates a closed state that elicits brief openings, and the second generates a closed state that elicits long-lived openings. Long-lived openings and the associated priming step are detected in the absence of agonist and in its presence, and show identical kinetics under each condition. By covalently locking the agonist binding sites in the bound conformation, we show that each site initiates a priming step. Thus a change in binding site conformation primes the AChR for channel opening in a process that determines the maximum response to agonist and functional consequences of disease-causing mutations.

865-Pos Board B744

Single Channel Current Through Nicotinic Receptor Produced By Closure Of The Binding Site C-loop

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We investigated the initial coupling of agonist binding to channel gating of the nicotinic acetylcholine receptor (nAChR) using Targeted Molecular Dynamics (TMD) simulation. Following TMD to accelerate closure of the C-loops at the agonist binding sites, the region of the pore that passes through the cell membrane expands. To determine whether the structural changes in the pore result in ion conduction, we used a coarse-grained ion conduction simulator, called Biology Boltzmann Transportor Monte Carlo (BioMOCA) simulation, and applied it to two structural frames taken from before and after the TMD simulation. The structural model of the pre-TMD simulation represents the channel in the proposed ''resting'' state, whereas the model of the post-TMD simulation represents the proposed "active" state. Under external voltage biases, the channel in the ''active'' state was permeable to cations. Our simulated ion conductance approaches that obtained experimentally and recapitulates several known functional properties of the nAChR. Thus, closure of the C-loop triggers a structural change in the channel pore that is sufficient to account for the open channel current. This approach of applying BioMOCA in computational studies of ion channels can be used to uncover the binding to gating transduction mechanism and the structural bases for ion selection and translocation.

866-Pos Board B745

Electrical Fingerprinting Reveals Agonist Binding Sites Required for Activation of Homo-pentameric Cys-loop Receptors

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Ancestral neurotransmitter Cys-loop receptors were homo-pentamers harboring five identical agonist binding sites but most present day receptors are hetero-pentamers with only two binding sites. To understand why Cys-loop receptors evolved to utilize fewer than five binding sites, we disabled different numbers of sites and developed a method to monitor lifetimes of individual active receptors and the corresponding number of functional binding sites. We find that maximal open-channel lifetime is achieved when the neurotransmitter occupies three non-consecutive binding sites. Occupancy of one site allows receptor activation, although the open state is unstable; occupancy of two non-consecutive sites produces a much longer-lived open state appropriate for efficient activation. However, occupancy of a third site further increases channel lifetime, thus providing optimal stabilization of the active state. Maximal activation of homomeric receptors by agonist occupancy of less than the five potential sites enhances the rate of channel opening and increases agonist sensitivity.
The results reveal that allosteric requirements dictated the number and location of the agonist binding sites, and provide an indispensable framework for further progress in drug design.

867-Pos Board B746

Photoaffinity Labeling the Agonist Binding Sites of nAChRs with [^{3}H]Epibatidine

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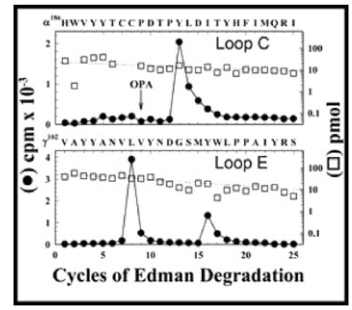
Neuronal nAChR subtype-selective agonists have potential therapeutic uses in many neurological disorders. Determination of structural aspects unique to individual nAChR agonist binding sites (ABS) is important to the development of nAChR subtype-selective agonists/ligands. To this end, we photolabeled *Torpedo*, α4β2 and α4β4 nAChRs with [^{3}H]Epibatidine. [^{3}H]Epibatidine binds to α4β2 and α4β4 nAChRs with high affinity (10-200 pM) and binds with similar affinity at the α1-γ and α1-δ ABS of the *Torpedo* nAChR (~11 nM). At the subunit level, [^{3}H]Epibatidine photoincorporated into the principal component of the ABS (α1 and α4 subunits) and the complementary component of the ABS in γ and β4 subunits but not in the δ or β2 subunits. Since little is known about the photochemistry of [^{3}H]Epibatidine and the stability of UV-induced [^{3}H]Epibatidine-amino acid adducts under Edman degradation conditions, we first established the merit of [^{3}H]Epibatidine as a photoaffinity probe by determining sites of [^{3}H]Epibatidine labeling in the *Torpedo* nAChR. The principal sites of labeling were αTyr^{198} within Loop C and γLeu^{109} and γTyr^{117} within Loop E of ABS (see figure). Studies are currently underway to identify the sites of labeling within the α4 and β4 subunits.



868-Pos Board B747

Hyperfine Splitting Trends in the EPR Spectra of M2δ in Aligned Phospholipid Bilayers

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In Nuclear Magnetic Resonance (NMR) spectroscopic techniques, polarization index slant angle (PISA) wheels, dipolar waves. and residual dipolar coupling waves, demonstrate the relation between the secondary periodic structure of α helices and their magnetic properties. Based on very many of the same principles as NMR, it is anticipated that similar trends will be evident in the information obtained from Electron Paramagnetic Resonance (EPR) studies of spin-labeled α-helical membrane proteins incorporated into aligned lipid bilayers. Towards this end, we have proposed that a rigid spin- labeled transmembrane α-helix exhibits a sinusoidal periodicity in the EPR specific hyperfine splitting values obtained for consecutively labeled residues of the peptide. We have shown that this can be mathematically related to the helical tilt angle at which it is oriented within the membrane and the corresponding static magnetic field. This phenomenon is demonstrated using the M2δ pore lining peptide of the nicotinic acetylcholine (AChR) receptor. Also, the effect of environmental conditions such as motional averaging caused by rotation of the M2δ helix within the membrane. These experimental results are evidence of how a theoretical model can be used to determine the helical tilt angle of M2δ, and by extrapolation, the helical tilt angle of any other membrane protein - verifying a relatively simple, but powerful method of extracting crucial topological information from minimal experimental EPR data.

869-Pos Board B748

Examining the Structure of the Neuronal a4b2 nAChR Transmembrane Domain by Photoaffinity Labeling

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The ability to purify neuronal nAChRs in large quantities allows the use of photoaffinity labeling to study their structure. To characterize the structure of the transmembrane domain of the α4β2 nAChR, we used [^{3}H]chlorpromazine, which has been used to identify amino acids in the *Torpedo* nAChR ion channel, and [^{3}H]TDBzl-etomidate, which acts as a *Torpedo* nAChR positive allosteric modulator by binding at a novel site within the transmembrane domain at the interface between the γ and α subunits. In the presence of agonist, [^{3}H]chlorpromazine and [^{3}H]TDBzl-etomidate incorporated into α4 and β2 subunits

with >70% of the labeling in the β2 subunit. [^{3}H]chlorpromazine subunit labeling was inhibited (~40%) by the non-competitive antagonist PCP (Fig 1).

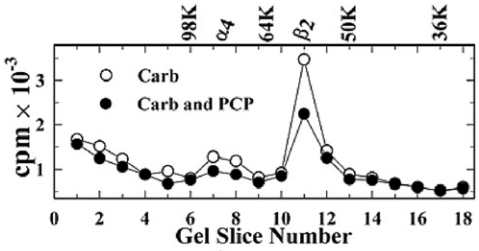

Fig. 1: Inhibition of [^{3}H]chloropromazine labeling of α4β2 receptor by PCP.

When HPLC-purified EndoLys-C digests of [^{3}H]chlorpromazine-labeled β2 subunit was sequenced, a PCP-inhibitable ^{3}H release at cycle 6 was evident, consistent with labeling of β2Ser246 (M2-6), the position photolabeled by [^{3}H]chlorpromazine in the *Torpedo* nAChR ion channel. Sequence analyses of HPLC-purified EndoLys-C/V8 protease digests of α4 and β2 subunits photolabeled by [^{3}H]TDBzl-etomidate indicated the presence of multiple sites of ^{3}H incorporation. Studies are in progress to identify amino acids labeled by [^{3}H]TDBzl-Etomidate and [^{3}H]chlorpromazine.

870-Pos Board B749

Regulation Of The Acetylcholine Receptor Function By Thyroid Hormones

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The basic molecular mechanisms underlying the muscular weakness initiated early during thyroid dysfunction are poorly understood. In this work we have explored how the acute and chronic alterations of thyroid hormones (THs) affect the acetylcholine receptor (AChR) channel function in the neuromuscular junction (NMJ). Acute methimazole (MMI) treatment produced a significant ($p<0.005$) reduction in weight of euthyroid mice of 8.64%, induced by a transient hyperthyroid phase. This result is compatible with the loss of weight produced acutely by T_3 (1μg/g-bw) of 4.11%. The resulting action of these acute treatments is a significantly ($p<0.001$) reduced muscular strength by MMI (58.0%) and T_3 (52.9%) when compared to sham animals. The hypothyroidism induced by the chronic MMI treatment causes a reduced daily weight gain of 76.5%. Focal recordings of miniature endplate currents (MEPCs) in isolated NMJ of chronically MMI-treated mice revealed a significant ($p<0.001$) reduction in the frequency (57.7%), amplitude (59.3%), and decay time (66.7%). The electrophysiological results appear to be related to the membrane-lipid metabolism in particular cholesterol, since NMJ of hypothyroid mice treated with methyl-beta-cyclodextrin (MβCD) recover the MEPCs normal characteristics. This data also agrees with results where the MEPCs' decay time 1.45 ± 0.06 ms (n=8) of fat euthyroid mice differs significantly ($p<0.001$) from those of slim animals 1.01 ± 0.08 ms (n=7). The relationship between weight (w) and decay time (τ) is well-described by the equation: $\tau = -0.00447w + 2.456$. We propose that membrane cholesterol in the NMJ could be an important target for the *in vivo* regulation of synaptic activity during thyroid dysfunction. Importantly, the docking of cholesterol to the AChR occurs in the C418 vicinity, a highly relevant residue in the protein-lipid interface.
NIH 5S06 GM050595 and G12 RR03035 to LVR

871-Pos Board B750

Role of Membrane Cholesterol Levels in the Lateral Diffusion and Function of the Novel Slow Channel Congenital Myasthenia Syndrome αC418W AChR Mutant

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There is a critical need for understanding the regulation of acetylcholine receptor (AChR) function and dynamics by cholesterol. Indeed, lipid-protein interactions are known to regulate the function and dynamics of ligand-gated ion channels; however, the underlying mechanisms are poorly understood. The novel Slow Channel Congenital Myasthenia Syndrome (SCCMS) AChR mutant αC418W is the first lipid-exposed mutation identified in a patient (Shen et al., 2006). This AChR mutation was shown to be cholesterol-sensitive (Santiago et al., 2001) and to accumulate in caveolin1-positive microdomains (Baez-Pagan et al., 2008). The objective of this study is to gain insight into the mechanism by which lateral segregation into specialized raft membrane microdomains regulates the activatable pool of AChRs. We performed Fluorescent Recovery After Photobleaching (FRAP) experiments and whole-cell patch clamp recordings of GFP-encoding *mus musculus* AChRs transfected into HEK 293 cells under cholesterol enrichment and depletion conditions to assess the role of cholesterol levels in the diffusion and functionality of the AChR (WT and αC418W). Our results further demonstrate the cholesterol-sensitive nature of the αC418W mutant as both lateral diffusion and mobile fraction are modified by either cholesterol enrichment or depletion differently in the αC418W mutant when compared to the WT. Furthermore, the low mobile fraction (<20%) displayed by the AChR provides further evidence of its trafficking to caveolin-positive microdomains. Because our methodological approach combines FRAP and electrophysiological experiments, our results provide a framework to understand the structural and functional basis for the partition of AChRs into specialized membrane microdomains.
This work was supported in part by grants from NIH, RO1GM56371-12, NCRR 1S0RR13705 and SNRP U54NS0430311.

872-Pos Board B751

Modulation Of Nicotinic Acetylcholine Receptor Conformational States By Free Fatty Acids And Steroids

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Steroids and free fatty acids (FFA) are non-competitive antagonists of the nicotinic acetylcholine receptor (AChR). Their site of action is purportedly located at the lipid-AChR interface, but their exact mechanism of action is still unknown. Here we studied the effect of structurally different free fatty acids and steroids on the conformational equilibrium of the AChR in *T. californica* receptor-rich membranes. We took advantage of the higher affinity of the fluorescent AChR open channel blocker, crystal violet (CrV), for the desensitized state than for the resting state. Increasing concentrations of steroids and certain *cis*-unsaturated free fatty acids decreased the K_D of CrV in the *absence* of agonist. The position of the double bond at the hydrocarbon chain of *cis*-monounsaturated fatty acids appears to be critical for their effect on the AChR resting conformation state. All *cis*-unsaturated fatty acids tested, but not *trans*-unsaturated or saturated fatty acids, caused an increase of the KD value in the *presence* of agonist. This latter effect was also observed with membrane treatments that caused opposite effects on membrane polarity (phospholipase A2 treatment or temperature increase, which decreased or increased the membrane polarity, respectively). Quenching by spin-labeled fatty acids of pyrene-labeled AChR reconstituted into model membranes, with the label located at the γM4 transmembrane segment, disclosed the occurrence of conformational changes induced by steroids and *cis*-FFA. These results suggest that the direct contact between exogenous lipids and the AChR transmembrane segments removes the AChR from its resting state and that membrane polarity modulates the AChR activation equilibrium by an independent mechanism.

873-Pos Board B752

Embedded Cholesterol in the Nicotinic Acetylcholine Receptor

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The nicotinic acetylcholine receptor (nAChR) is a cation-selective channel central to both neuronal and muscular processes and is considered the prototype for ligand-gated ion channels, motivating a structural determination effort that spanned several decades. Purified nAChR must be reconstituted in a mixture containing cholesterol to function. Proposed modes of interaction between cholesterol and the protein range from specific binding to indirect membrane-mediated mechanisms. However the underlying cause of nAChR sensitivity to cholesterol remains controversial, in part because the vast majority of functional studies were conducted before a medium resolution structure was reported. We show that the nAChR contains internal sites capable of containing cholesterol, whose occupation stabilizes the protein structure. We detect sites at the protein-lipid interface as conventionally predicted from functional data, as well as deeply buried sites that are not usually considered. Molecular dynamics simulations reveal that occupation of both superficial and deeply buried sites most effectively preserves the experimental structure; the structure collapses in the absence of bound cholesterol. In particular, we find that bound cholesterol directly supports contacts between the agonist binding domain and the pore that are thought to be essential for activation of the receptor. These results likely apply to those other ion channels within the Cys-loop superfamily that are dependent on cholesterol, such as the GABA receptor.