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).

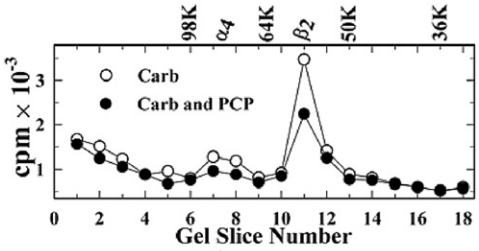

Fig. 1: Inhibition of [^{3}H]chloropromazine labeling of α4β2 receptor by PCP.

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

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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.
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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.