desensitization, while the type 2 PAMs enhance the current amplitude but also slow the desensitization of the receptor. In order to understand the mechanism of action of the type 1 and type 2 PAM's, we tested three modulators on the QPatch automated patch-clamp system using GH4C1 cells stably expressing the rat α-7 receptor. We also explored the effect of repeated applications of these agents on their modulatory activity. Consistent with previously published data, PNU-120596 showed type 2 PAM activity accompanied with a decreasing magnitude of potentiation with repeated applications. Estimated EC50 values for PNU-120596 were stable with repeated compound application. NS-1738 produced a type 1 PAM activity, and a cumulatively increased potentiation following repeated applications, accompanied by an approximate 3-fold increase in EC50. In contrast, SB-206553 had similar potency and effects on the potentiation with repeated application. These results indicate that the different α-7 receptor PAMs have different rates of activation and desensitization, in addition to their type 1 or 2 effects on receptor desensitization.

859-Pos Board B738

Channel Blocking Properties Of Tetramethylammonium At The Human Muscle Acetylcholine Receptor

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Agonists at muscle acetylcholine receptors (AChR) all seem to be able to block the channel as well as activate it. In particular, many partial agonists block the channel pore at concentrations close to those that activate it, e.g. choline or tetramethylammonium (TMA). We recorded TMA-activated single- channel currents in the cell-attached configuration from HEK293 cells expressing human adult AChR. The amplitude of openings recorded at -80 mV appears to decrease progressively with agonist concentration because of fast channel block. The equilibrium constant, K_B, for open channel block was about 9 mM as estimated from the reduction of apparent single channel amplitude. This is comparable with the EC_{50} of 2 mM. Several records obtained at different TMA concentrations were fitted simultaneously with the HJCfit program[1]. Because the blockages were undetectable, the open state and the open-blocked state were treated as a single compound open state in the analysis. In the first instance fits were done with a mechanism that allows block of channels only when they are open. The predicted distribution of apparent open times at the lower concentrations TMA matched the observations quite well, but at the higher concentration the prediction was poor (the predominant mean apparent open time was about 1.5 times smaller than predicted). Then fits were done of a mechanism in which the block is not selective for the open state, but can occur from any state. In this case the distributions of apparent open times were predicted accurately at both low and high concentrations of TMA. The present data suggest that TMA does not act as a pure open channel blocker, but AChRs blocked by TMA can close and return to the resting state without re-opening.

1. Colquhoun *et al. J Physiol* 547, 699, 2003.

860-Pos Board B739

Temperature Dependence And Activation Energy of nAChR Gating

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Neuromuscular acetylcholine receptors (AChRs) are ion channels that alternately adopt conformations that either allow or prohibit the flow of ions across the membrane. These two stable end states are separated by an energy barrier, the peak of which is called the transition region. The energy of the transition region relative to the end states is the reaction activation energy (E_{act}). To quantify E_{act}, we studied the temperature dependence of single-channel gating rate constants (k_o, opening; k_c, closing) for wt and mutant AChRs, activated by different ligands or without any added ligand, over a range of temperatures (5-35 ^oC, HEK cells, cell attached, -110 mV, mouse $\alpha_2\beta\delta\epsilon$). The results were fitted by the Arrhenius equation: $k(T)=A*\exp(-E_{act}/RT)$. Increasing the temperature from 5^0 C to 35^0 C increased k_c for wt and δL265T AChRs activated by choline, each by ~35-fold (E_{act}=20.5 and 23.6 kcal/mol, respectively). We also estimated the temperature sensitivity of k_o and k_c in four more constructs with one or more point mutations in both α subunits. For all four, k_o and k_c increased with temperature: E_{act} (k_o and k_c; kcal/mol)=21 and 23 (Y127E activated by ACh); 24 and 27 (D97A + Y127F + S269I, unliganded); 29 and 23 (D97A + Y127F + S269I + W149F, unliganded); 29 and 25 (G153S activated by choline). For these six constructs the average activation energy was ~24.6 kcal/mol for both closing and opening. This quantity did not change with the agonist (including water) or the mutations. This suggests that the energy barrier for the gating isomerization is not significantly determined by the ligands at the transmitter binding sites or by the gating motions of the mutated residues, and that unliganded and diliganded AChR gating likely proceed by similar reaction pathways.

861-Pos Board B740

Interaction Between Two Domains in the AChR Gating Reaction

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The AChR is a large ion channel that isomerizes between non-conducting and conducting conformations. Residue αA96 is located in loop 5 (loop A) near the agonist-binding site, which moves at the outset of the channel-opening process (the Φ value for the adjacent residue αD97 is 0.93). Side chain substitutions at nearby (<0.45 nm, 2QC1.pdb) residue αY127 (β-strand 6) change the gating equilibrium constant (K_{eq}) by up to 290,000-fold (Φ=0.77). This suggests that αY127 moves in concert with the lower part of the extracellular domain, after the motion of loop 5. αD97 and αY127 are not coupled energetically. We tested the hypothesis that αA96 and αY127 energetically link the first two Φ-blocks, to propagate the opening conformational wave from the upper to the lower part of the extracellular domain. We mutated residue αA96 (C, F, K, L, N, Q) and measured single-channel gating kinetics (mouse $\alpha_2\beta\delta\epsilon$, cell-attached, -100 mV, 20 mM choline, PBS, 23°C). The Φ-value for αA96 is 0.90, indicating that it moves at the onset of channel gating along with other residues in loop 5. αA96N showed the largest change in K_{eq} (~900-fold) and markedly increased unliganded gating. Next, we performed mutant cycle analysis to test for energetic coupling between αA96 and αY127. K_{eq} for the double mutant αA96K+αY127E is 18-fold *greater* than the wt, where the effects of the single mutants, if additive, predict one that is 9.2-fold *smaller*. This corresponds to a coupling free energy of -3.1 kcal/mol. Similarly, K_{eq} for the double mutant αA96C+αY127C is 213-fold *greater* than the wt, whereas a value 1.7-fold *smaller* is predicted assuming independence (coupling free energy of -3.6 kcal/mol). These are large interaction energies that suggest αA96 and αY127 form a key energetic link between the first and second Φ-blocks.

862-Pos Board B741

βM2 of The Neuromuscular AChR: Gating, Desensitization and Orientation

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The M2 helix of each of the five acetylcholine receptor (AChR) subunit forms the narrow region of the ion conduction pathway. As part of an overall project of trying to understand the mechanisms that underlie two reactions - the C(losed)↔O(pen) conformational change ('gating') and desensitization - we studied single-channel currents from AChRs with a point mutation in the β subunit (mouse $\alpha_2\beta\delta\epsilon$, HEK cells, cell-attached, -100 mV, 23^{o}C, activated by 30 μM ACh). From measurements of cluster open probabilities (P_o) and durations (τ) we could make *qualitative* inferences about the effects of the mutations on gating (P_o; increase, decrease, no effect) and desensitization ($\tau_{cluster}$; altered, no effect). So far, 58 different mutations of 14 different βM2 residues have been examined. For some of these we also quantified the single-channel current amplitude of the R substitution (i_R; small, no effect). The results are as follows. 1) P_o (by mutation): 26 increased, 13 decreased, 18 no effect. The increases were most apparent in the equatorial 9′-12′ region. 2) $\tau_{cluster}$ (by residue): 6 altered and 8 no effect. The altered bursts were mostly prolonged, with the effects being largest at 9′-12′ and 14′-15′. 3) i_R (by residue): 5 small (8′-10′, 13′, 15′), 3 no effect (6 positions not tested). By examining mutants of all βM2 positions, using a saturating concentration of either choline or ACh, we hope to build maps of the energetic consequences with regard to gating and desensitization, and learn the orientation of residues in the Open conformation of the protein.

863-Pos Board B742

The Unliganded Gating Mechanism Of Nicotinic Acetylcholine Receptors

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The nicotinic acetylcholine receptor (AChR) switches between C (low agonist affinity and low conductance) and O (high agonist affinity and high conductance) conformations ('gating'). The probability of channel opening is very low in the absence of agonist, but when agonists are present at the two transmitter-binding-sites opening increases rapidly (~20μs), transiently to a high probability (~0.95). We observe that 'gain-of-function' mutants that increase the diliganded gating equilibrium constant (without affecting agonist binding to C) also increase the frequency of spontaneous openings. Unliganded openings occur in clusters in AChRs having several of such mutations. We analyzed the intra-cluster interval durations to estimate that the unliganded gating equilibrium constant is $\sim 1.15 \times 10^{-7}$ (mouse, $\alpha_2\beta\delta\epsilon$, -100 mV). The agonist affinity ratios (C vs. O) for acetylcholine, carbamylcholine, tetramethylammonium and choline are ~15,600, ~6700, ~6700 and ~600. The monoliganded (with ACh) gating equilibrium constant is $\sim 1.7 \times 10^{-3}$. Acetylcholine provides only ~0.9 k_BT more binding energy per site than tetramethylammonium, but ~3.1 k_BT more than choline. Mutations of binding site residue αW149 *increase*

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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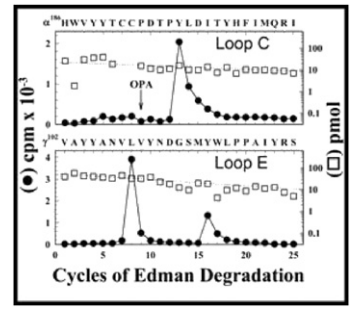
Neuronal nAChR subtype-selective agonists have potential therapeutic uses in many neurological disorders. Determination of structural aspects unique to individual nAChR agonist binding sites (ABS) is important to the development of nAChR subtype-selective agonists/ligands. To this end, we photolabeled *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