

To directly test this model, we altered the tension of the GluN1 and GluN2A M3-S2 linkers through residue insertions (to decrease tension) or deletions (to increase tension). Based on single-channel analyses, we find that these manipulations specifically affect pore opening (as opposed to ligand binding) and that they alter gating more dramatically in GluN2A than in GluN1. All-atom molecular dynamics simulations on a modeled GluN1/GluN2A receptor showed that this subunit-specific difference may arise, in part, from the disparate extensions and orientations of the GluN1 vs GluN2A M3-S2 linkers. Our functional data also suggests that the GluN1 M3-S2 linker gates primarily through tension. Using rate equilibrium free energy relationship (REFER) analysis and length-tension analysis, we find that for the GluN1 M3-S2 linker, tension arises primarily during the C1-O1 transition (assuming a linear kinetic scheme) with a spring constant of ~ 7.2 pN/nm, agreeing well with other biological springs. In contrast, our functional data suggests that mechanisms other than tension mainly mediate the role of the GluN2A M3-S2 linker in gating. Alternative mechanisms may include twisting and changes in electrostatic interactions.

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Unique Conformational Distributions for NMDA Receptor Glycine and Glutamate Ligand-Binding Domains

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Ionotropic glutamate receptors (iGluRs) mediate communication at most excitatory synapses in the brain. iGluRs are organized into three major families—the AMPA, kainate, and NMDA receptors. NMDA receptors are obligate heteromeric assemblies of glycine- and glutamate-binding subunits. Unexpectedly, crystal structures of the glycine-binding GluN1 and GluN3A ligand binding domains (LBDs) in their apo states reveal open and closed cleft conformations, respectively. Computed conformational free energy landscapes also exhibit minima at both open and closed cleft conformations for apo GluN1 and GluN3A LBDs. The minimum at the closed cleft conformation is preserved for the glycine-bound LBDs. In contrast, the free energy landscapes for the NMDA and AMPA receptor glutamate-binding subunits GluN2A and GluA2 show a shift in the minimum upon glutamate binding. Principal component analysis reveals a spectrum of conformational transitions that differ for the GluN1, GluN3A, GluN2A, and GluA2 LBDs. This variation highlights the structural complexity of signaling by iGluRs.

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Gating of GluA2 Receptors is Mediated by a Pivot in the M3 Helix

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Closure of the ligand-binding domain (LBD) of glutamate receptor channels opens their gate by a mechanism that is not fully clear. We find that glycine substitutions of pore facing residues in the conserved SYTANLAAF region in the transmembrane M3 helix of GluA2 improve gating, notably, replacing alanine 621 with glycine, two turns of the helix below the gate, resulted in a non-desensitizing channel with significant agonist-independent basal activity and ~ 36 -fold increase in glutamate potency without changes in expression or binding. On GluA2(A621G), the partial agonist kainate acted as a full agonist and the antagonist CNQX acted as a partial agonist. In contrast, a glycine mutation above the channel gate, reduced activity and glutamate potency. Therefore, closure of the LBD opens the channel by pulling apart the M3 helix around a pivot at small flexible amino acids in the pore facing region below the gate, in a mechanism similar to potassium channel gating.

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Structural Mechanisms underlying AMPA Receptor Oligomerization

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Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that play critical roles in excitatory neurotransmission. A functional iGluR is composed of four subunits, each containing an amino-terminal domain (ATD), a ligand-binding domain (LBD), a transmembrane domain (TMD) composed of three transmembrane helices (M1, M3 and M4) and a re-entrant loop (M2), as well as an intracellular carboxyl-terminal domain (CTD). Formation of the tetrameric complex, which is a prerequisite for the receptor's surface expression and function, involves a dimeric intermediate. Nevertheless, the biophysical mechanisms underlying iGluR oligomerization remain largely unknown. We studied the relative energetic contributions of the different structural domains to the oligomerization of iGluRs using blue native PAGE and fluorescent size-exclusion chromatography (FSEC). We find that deletion of the M4 transmembrane helix renders AMPA receptors incapable of tetramerization and

traps them in a dimeric form, suggesting that the crucial dimer-to-tetramer transition is driven by molecular interactions within the TMD. Further highlighting the importance of TMD to AMPA receptor tetramerization, the isolated TMD of the GluA1 subunit, which lacks ATD and LBD, still form tetramers. On the other hand, the GluA1 ATD is essential for the stability of homo-dimer intermediates, although its absence does not prevent tetramerization. Interestingly, the prokaryotic glutamate receptor GluR0, which lacks both the ATD and the M4 helix, forms both dimers and functional tetramers. In contrast, GluA1 subunits lacking the ATD and the M4 helix (and thus topologically similar to GluR0) do not oligomerize, forming monomers only. Our results indicate that the mechanisms for tetrameric assembly differ significantly between GluA1 and GluR0 in terms of the energetic contributions of each structural domain. Such differences may have important implications in the evolution of iGluRs and their roles in fast excitatory neurotransmission.

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Re-Evaluating a Proposed Mechanism for Ion Modulation in Kainate Receptors

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Glutamate is the major excitatory neurotransmitter in the brain, activating ionotropic receptors named for their selectivity to the agonists NMDA, AMPA, and kainate (KARs). The KAR subunit GluK2 requires external ions, in addition to glutamate, to produce a detectable current, making it unique among iGluRs. Interestingly, the non-decaying GluK2 mutant Y521C/L783C (YC/LC) functions in the absence of external ions, and has been used to suggest that ions initiate desensitization. We investigated this hypothesis through electrophysiological recordings performed on outside-out patches excised from HEK 293 cells. Unitary openings of YC/LC were extremely brief and sporadic, suggesting that desensitization is intact. Meanwhile, its agonist potency relationship was consistent with the equilibrium current of wild-type (WT) receptors, which is comprised of channels cycling between active and desensitized states. Molecular dynamics simulations of YC/LC showed that sodium quickly departs the cation binding pocket, in contrast to WT GluK2, where sodium is more stable. As a result, we decided to study GluK2 E524G, a mutant that disrupts the cation pocket. In most cases, glutamate only elicited measurable responses from E524G following application of the allosteric modulator concanavalin-A (conA), a lectin that potentiates the WT equilibrium current, and YC/LC to a lesser extent. Surface expression studies relying on TIRF microscopy and a pH-sensitive probe were used to confirm that poor functionality, not trafficking defects, account for the small responses of E524G and YC/LC. Moreover, WT GluK2 exposed to conA exhibits increased channel openings during equilibrium, suggesting both mutants principally reside in desensitized states. Our data argues that a reduction in cation binding coincides with desensitization, rather than inhibition of desensitization, supporting the perspective that external ions serve as KAR co-activators.

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Atomistic Simulations Explain Mutational Effects on Ion Modulation and Kainate Receptor Activity

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The most important excitatory neurotransmitter in the central nervous system is glutamate. Ionotropic glutamate receptors (iGluRs) thus play key roles in the brain; for example they are associated with learning and memory at the molecular level and implicated in various diseases. Binding of glutamate to the iGluR ligand-binding domain (LBD) triggers opening of the transmembrane ion channel, facilitating influx of cations. As opposed to other iGluRs, kainate-selective iGluRs (KARs) require binding of extracellular sodium and chloride ions to the LBD dimer interface in addition to agonist binding to obtain normal activity. We have examined the structural/dynamical effects of the presence/absence of these ions in conjunction with mutations at the dimer interface of GluK2 receptors. Electrophysiological studies of a double cysteine mutant (Y521C/L783C), cross-linking the LBD dimer, indicate that this mutant cannot be activated to wildtype(WT)-like high-conductance states but, as opposed to previous interpretations, does in fact reside mainly in a desensitized state. Atomistic molecular dynamics simulations of the WT and the cross-linked mutant reveal that whereas ions remain stably bound to the WT LBD dimer, they tend to leave early in the simulations of the cross-linked mutant. Interestingly, the mutant is also found to relax quickly to give a more open dimer interface than the WT, consistent with the current model of the desensitized state. Similar

ion unbinding and interface opening is observed for a mutant (L783S) where the cation site is indirectly disrupted by disturbing a hydrophobic plug shielding the site. In the electrophysiological studies, this mutant appears non-functional. Overall, the results are consistent with the view that maximum conductance depends on a tightly packed dimer-interface regulated by the presence of extracellular ions.

1412-Pos Board B304

Details of GABA Binding to the GABA-A Receptor Revealed by Molecular Dynamics

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Through the analysis of two rare-event molecular dynamics simulations, we were able to observe the complete dissociation of a GABA molecule from the GABA_A-receptor, the diffusion of that molecule into bulk water, and then the subsequent re-binding of GABA into its binding pocket.

The route for this re-binding event was not linear in fashion, but rather adopted 'bind, slide, and find' approach. We found that the GABA molecule BINDS to the protein surface outside of the binding pocket, SLIDES along the protein surface into the pocket itself, and then FINDS the correct binding orientation once it is within the binding pocket.

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GABA_A Receptor Subunit Rare Variants Identified in Patients with Idiopathic Generalized Epilepsy Alter Receptor Gating and Assembly

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Exome sequencing of ion channel genes from cases with well characterized idiopathic generalized epilepsies (IGEs) has identified many rare, nonsynonymous single nucleotide polymorphisms (nSNPs) in the human non-epilepsy genes *GABRA4*, *GABRA5*, *GABRA6*, *GABRB1*, *GABRB2*, *GABRG1* and *GABRG3*. Structural homology modeling predicts that the rare variants are mainly localized between the agonist-binding domain (N-terminus) and the channel gate (transmembrane domain, TM) of GABA_A receptor subunits. We sought to investigate the effects of the rare nSNPs on assembly and function of GABA_A receptors containing one of seven separate IGE-associated rare variants identified in $\alpha 5$ and $\beta 2$ subunits ($\alpha 5V204I$, $W280R$, $S402A$, $P453L$, $A459T$; $\beta 2R293W$, $R354C$), which are located within the well-defined structural GABA_A receptor domains. We studied gating properties and surface expression of wild-type (wt) $\alpha 5\beta 3\gamma 2$, $\alpha 1\beta 2\gamma 2$ and mutant (mut) $\alpha 5(\text{mut})\beta 3\gamma 2$, $\alpha 1\beta 2(\text{mut})\gamma 2$ receptors expressed in HEK293T cells. We found that variants located within the N-terminal domain and in the juxtamembrane interface displayed gating or mixed gating and trafficking defects. Furthermore, variants within TMs displayed trafficking defects, but those within the cytoplasmic loop had no defect. Thus, variant subunits $\alpha 5V204I$ and $\beta 2R293W$ displayed a mixed profile, causing both gating and trafficking defects of $\alpha 5\beta 3\gamma 2$ and $\alpha 1\beta 2\gamma 2$ receptors, whereas variant $\alpha 5W280R$ subunit caused primarily impaired trafficking of $\alpha 5\beta 3\gamma 2$ receptors. Interestingly, homology modeling predicted that $\alpha 5W280R$ and $\beta 2R293W$ variants stabilize new hydrogen bonds across the β/α subunit interface, which seems to be essential for inter-subunit interactions in assembled receptors. These findings suggest that gating and/or trafficking defects might suggest that a specific variant may be a susceptibility gene and may help to predict functional risk for loss of GABAergic function in individual IGE cases.

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Mechanistic Basis of Partial Agonism at 5-HT_{3A} Receptors

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Partial agonists are unable to elicit full maximal responses. The characterization of a genuine partial agonist is complex because other mechanisms, such as channel block, may also limit maximum open probability. Taking advantage of the high conductance form of the 5-HT_{3A} receptor, we evaluated at the single-channel level its activation by 2-Me-5HT and tryptamine, which have been classically considered as partial agonists of 5-HT₃ receptors. For all ligands, activation appears as openings in quick succession grouped in clusters showing high open probability (Popen > 0.9), and open time distributions show three components. The slowest open component is 6.5- and 3.5-fold briefer for 2-Me-5HT and tryptamine, respectively, than for 5-HT. The duration of this component decreases as a function of agonist concentration due to

open-channel block. For 2-Me-5HT, the forward blocking rate is 10-fold faster than for tryptamine and 5-HT. Single-channel kinetic analysis shows that 2-Me-5HT is actually a full agonist, its maximum response being limited by channel block. In contrast, tryptamine is a genuine partial agonist and its low efficacy is mainly due to a slow transition from the fully-liganded closed state to a pre-open state. After reaching the latter state, activation proceeds similarly as in the presence of 5-HT. Molecular docking shows that interactions at the binding site are similar for 2-Me-5HT and 5-HT. In contrast, the potential to form the cation- π interaction with W183 seems to be reduced for tryptamine. The mechanism by which ligands produce non maximal responses has implications for the design of partial agonists for therapeutic use.

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Proline Residues at the Nicotinic Acetylcholine Transmitter Binding Sites

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The two neuromuscular acetylcholine receptor (AChR) transmitter binding sites are located in the extracellular domain of the protein at the α - ϵ and α - δ subunit interfaces. At each site there are two vicinal prolines in the complementary ϵ/δ subunit (ProD1 and ProD2). We estimated for dozens of mutations of ϵ/δ ProD1 and ProD2 (single-channels, HEK cells, +100mV, 23 °C) the gating energies with ACh molecules bound (G2 and G1) and without any agonist (G0). From these we calculated the energy for gating arising from the affinity change for ACh (GB1+GB2=G2-G0). Mutation of ProD2 had larger effects than of ProD1. The ϵ ProD2-L mutation, which causes a congenital myasthenic syndrome, increases α GB (+3.4 kcal/mol, throughout) but does not change G0. For the same mutation in the δ subunit the change in GB was smaller (+1.9). All of the side chain substitutions at ϵ/δ ProD2 increased both GB and G0 compared to the wild-type (which has the values -5.1 and +8.4). The largest increase in α GB was for ϵ ProD2-R (+4.7) and in G0 for δ ProD2-Q (+2.5). We used mutant cycle analysis to estimate α GB- coupling between side chains. The two ProD2 residues interact weakly with each other (-0.6), but there is a strong interaction between ϵ ProD2- α GlyB1 (+2.7) but not between δ ProD2- α GlyB2. It is possible that a concerted, Pro-Gly 'switch' at α - ϵ occurs near to the onset of channel-opening. We engineered AChRs having only one functional binding site and quantified GB(ACh) for either the α - ϵ (by using the δ ProD2-R knockout or α - δ (by using the ϵ ProD2-R knockout) site. The sites provide +5.2 and -4.7 kcal/mol with ACh. One-site AChRs will be useful for studying the energy sources for gating by agonists at the two different binding sites.

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Enhancement of $\alpha 9\alpha 10$ Nicotinic Acetylcholine Receptor Desensitization by Nimodipine

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Entry of calcium ions through nicotinic acetylcholine receptors hyperpolarizes outer hair cells (OHCs) by activating colocalized SK potassium channels. This process, termed efferent inhibition, plays a central role in the protective reflex by suppressing the amplification of incoming sound. OHC nicotinic receptors are composed of $\alpha 9$ and $\alpha 10$ subunits that are activated by acetylcholine (ACh) release from efferent neurons originating within the medial olivocochlear nucleus. Fast application of ACh to GH₄C₁ cells transiently transfected with plasmids encoding mouse $\alpha 9$ and $\alpha 10$ subunits, produced an inward current that displayed a concentration-dependent amplitude, with an EC₅₀ of $41 \pm 5.4 \mu\text{M}$ and Hill slope (n_H) of 0.89 ± 0.1 ($n=5$). This current was antagonized by nicotine, displaying a K_B of $9.0 \pm 1.0 \mu\text{M}$ ($n=8$), consistent with the ACh-mediated current arising from activation of heteromeric $\alpha 9\alpha 10$ receptor channels. Inward ACh currents in the presence of the dihydropyridine antagonist nimodipine (10 μM) were reduced in amplitude and displayed faster decay kinetics. Exponential decay rates (τ) increased from $647.5 \pm 66.0 \text{ ms}$ ($n=6$) in control, to $160.6 \pm 25.8 \text{ ms}$ ($n=6$) in the presence of nimodipine (10 μM) ($p < 0.0005$). The concentration-response relationship to ACh was not significantly affected by nimodipine, displaying an EC₅₀ of $28 \pm 6.7 \mu\text{M}$ and Hill slope (n_H) of 1.28 ($n=5$) ($p > 0.05$), suggesting that the increased decay rate of ACh currents did not result from a decrease in agonist binding affinity. Using a double-pulse protocol, ACh (300 μM)-mediated responses recovered from desensitization with an exponential time-course ($\tau = 3.81 \pm 0.67 \text{ s}$, $n=3$). Recovery was slowed in the presence of nimodipine ($\tau = 8.88 \pm 2.54 \text{ s}$; $n=3$), indicating that nimodipine stabilizes the desensitization state of the $\alpha 9\alpha 10$ receptor and suggests that these receptors can be modulated to fine-tune efferent inhibition.