1465-Pos Board B375
Mechanistic Studies on Ionotropic Glutamate Receptors Using Tethered Photoswitchable Ligands
Andreas Reiner, Ehud Y. Isacoff.
Several mechanistic aspects of ligand-gated ion channels remain poorly understood, such as the allosteric coupling between different subunits. The development of photoswitchable ligands that can be covalently attached to specifically introduced cysteine residues [1, 2] enables us to overcome some limitations of conventional studies.
Using a glutamate analogue combined with an azobenzene photoswitch, we can study activation, deactivation and desensitization of ionotropic glutamate receptors (iGluRs). With this approach, ligand binding and unbinding can be precisely controlled using short light pulses, and are not limited by the time required to apply and remove the ligand. Furthermore, ligand binding can be restricted to a specific subset of the four subunits constituting the tetrameric channel. Here we used voltage-clamp recordings to measure the time courses of channel opening and closing upon full and partial activation with short pulses of light. Even low light doses lead to opening of iGluR6 on the submillisecond timescale followed by rapid desensitization. Extending the photoswitching experiments to single channel recordings and complexes with defined subunit stochiometries will provide further information on how single subunits contribute to the function of this important class of signaling molecules.
1. Volgraf M. et al., Nat. Chem. Biol. (2006) 2: 47.
2. Gorostiza P. et al., Proc. Natl. Acad. Sci. USA (2007) 104: 10865.
This work was supported by a Deutsche Forschungsgemeinschaft fellowship to A. Reiner and the NIH Nanomedicine Development Center for the Optical Control of Biological Function (5PN2EY018241).

1466-Pos Board B376
Potent and Selective Inhibition of the Open-Channel Conformation of AMPA Receptors by an RNA Aptamer
Zhen Huang, Yan Han, Congzhou Wang, Li Niu.
Inhibitors targeting the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are useful as biochemical probes for structure-function studies and as drug candidates for a number of neurological disorders and diseases. Here we describe the identification of an RNA inhibitor or aptamer by an in vitro evolution approach, i.e., systematic evolution of ligands by exponential enrichment (SELEX), with the $GluA2Q_{flip}$ AMPA receptor subunit as the selection target. This aptamer inhibits only the open-channel, but not the closed-channel, conformation of the AMPA receptors with a nanomolar affinity (for example, the K_I value was found to be 0.95 ± 0.2 μM with $GluA2Q_{flip}$. Furthermore, the aptamer has no effect on either kainate or NMDA receptors. By using a laser-pulse photolysis technique and a caged glutamate, we also determined that the aptamer inhibited the channel-closing rate constant or k_{cl}, which reflects the lifetime of the open channel, but it did not affect the channel opening rate constant or k_{op}, which reflects the closed-channel state. In a homologous competitive binding experiment, the aptamer was found to bind to both the open-channel and the closed-channel state with K_d of 80 ± 23 nM and 68 ± 40 nM respectively. Taken together, these results are consistent with the aptamer being a noncompetitive inhibitor. Our results further suggest the possibility of developing RNA aptamers that are conformation-selective, potent and water-soluble as a new type of inhibitors against AMPA receptors.

1467-Pos Board B377
Structural and Functional Characterization of Reconstituted Glua2 Tetramers
Jelena Baranovic, Chandra S. Ramanujan, Nahoko Kasai, Dean R. Madden, Keiichi Torimitsu, John F. Ryan.
α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs) are glutamate activated ion channels present throughout the vertebrate central nervous system. AMPARs are located primarily at postsynaptic membranes where they act as the main mediators of fast excitatory neurotransmission. Their proper functioning is vital for memory formation and learning.
To tackle the challenge of observing full-length membrane-embedded receptors in near physiological conditions, we have focused on the reconstitution of purified AMPARs into artificial membranes which are then imaged in liquid using atomic force microscopy (AFM) and whose activity is studied through electrical recordings of lipid bilayers.
We report successful reconstitution of GluA2 homotetramers. CHAPS has proven to be the most suitable detergent for liposome destabilization in the current protocol resulting in the most efficient (i.e. highest protein density) reconstitution. The influence of lipid composition of membranes on the reconstitution process was also tested: no clear difference in efficiency was observed between phosphatidylcholine membranes and membranes formed from porcine brain total lipid extract, hence, most of our studies were performed with liposomes composed of brain lipids. High-resolution AFM images reveal closed receptors protruding up to 14 nm from the membrane with lateral dimensions of approximately 20 nm - dimensions that closely resemble those reported for the full-length receptors by EM and X-ray diffraction. Tetrameric structure was clearly resolved for many but not all receptor molecules. Activity of reconstituted GluA2 receptors was tested by fusing proteoliposomes with a bilayer formed on the tip of a patch pipette. Single-channel currents of variable conductances were elicited by addition of L-glutamate into a bath solution containing proteoliposomes and cyclothiazide.
The results indicate that high spatial and temporal resolution AFM measurements can reveal functional dynamics of GluA2 whereas bilayer recordings offer the possibility of studying functional integrity of the receptors under various conditions.

1468-Pos Board B378
Molecular Mechanisms of Ca^{2+} Selectivity and Mg^{2+} Block of NMDA Receptors
Lea Veras, Michael Younkunas, Jane Hwang, Erik Kallenbach, Igor Kurnikov, Jon W. Johnson, Maria Kurnikova.
Glutamate receptors are membrane proteins activated by the neurotransmitter glutamate that mediate synaptic excitation in the mammalian brain. NMDA receptors constitute a glutamate receptor subfamily specifically activated by N-methyl-d-aspartate. Due to their high Ca^{2+} permeability and voltage-dependent channel block by Mg^{2+}, NMDA receptors play a central role in development through stabilization of synaptic connections, as well as in learning and memory by mediating many forms of synaptic plasticity. The mechanisms by which the ion channel of NMDA receptors selects Ca^{2+} for permeation over all other physiological ions, while binding and restricting Mg^{2+} permeation, are not well understood. We hypothesize that the slightly different radii of Mg^{2+} and Ca^{2+} ions result in drastically different free energy barriers for movement of the ions from a binding site in the selectivity filter to water. We applied quantitative theoretical "bottom up" approaches to this complex system by combining methods of computational chemistry, molecular mechanics, and bioinformatics. We performed high accuracy quantum chemical (QC) calculations to determine the energy of the transition state of the ligand exchange reaction that occurs when divalent ions transition from the selectivity filter of NMDA receptors to water. The results of QC calculations are used to parameterize accurately the polarizable molecular mechanics force fields for divalent ion-ligand interactions. Molecular dynamics simulations with polarizable and non-polarizable force fields are used to predict quantitatively ion interactions with the heterogeneous environment of the pore. The structure of the NMDA receptor channel is constructed and refined using a combination of experimental studies, homology modeling, molecular dynamics, and an optimized structure of a Mg^{2+} - Asn ligand complex. A homology model of NMDA receptors is refined through extensive equilibration and all-atom molecular dynamics simulations on the multi-nanosecond time-scale to acquire a stable model structure.

1469-Pos Board B379
Contributions of Carboxy-Terminal Domains to NMDA Receptor Gating
Bruce E. Maki, Gabriela K. Popescu.
The intracellularly located carboxy terminal domain (CTD) of NMDA receptors represents almost half of these receptors' mass; it interacts with a wide variety of regulatory proteins; and it serves to control receptor targeting and function. Calcium-activated proteins such as calmodulin, actinin and calcineurin, bind to or modify residues on CTDs and results in run-down, inactivation or desensitization of macroscopic responses. The mechanisms by which these kinetic changes occur are unknown. As a prelude into investigations of calcium-dependent modulation mechanism, we first asked how do CTDs of GluN1 and GluN2A subunits contribute to the receptor's gating reaction. We used cell-attached single-channel recordings, kinetic analyses and modeling to characterize the stationary gating kinetics of NMDA receptors lacking CTDs: GluN1(K838Stop) and/or GluN2(K844Stop). We found that compared to wild-type receptors (Po, .53 ± 0.03; MOT, 6.0 ± 0.6 ms; MCT, 5.4 ± 0.7; n = 17) receptors lacking CTD of GluN1 subunits had similar open probabilities but with slightly longer openings and closures (Po, .5 ± 0.05 , MOT, 8.5 ± 1.2 ms, MCT, 11.0 ± 2.4 ms; n = 9). In contrast, receptors lacking the CTD of GluN2A subunits were ~2.5-fold less active (Po, .2 ± 0.06; n = 9), with slightly longer openings (MOT, 10.7 ± 1.3 ms,) and ~17-fold longer closures. Receptors completely devoid of CTD, were much less active then either of the single-subunit CTD deletions, mainly due to substantially longer closures (Po, 0.1 ± 0.03, MOT, 6.5 ± 1.4 ms, MCT, 175.2 ± 53.4; n=8). These results demonstrate that the CTDs of both the GluN1 and GluN2A subunits contribute to normal

gating kinetics, with the CTD of GluN2A receptors playing a major role in maintaining high open probabilities.

1470-Pos Board B380
Role of Salt Bridges within the Ligand Binding Domains of NMDA Receptors During Receptor Activation
Meaghan Paganelli, Gabriela K. Popescu.
Glutamatergic synaptic transmission mediated by the *N*-methyl-*D*-aspartate (NMDA) receptor plays a fundamental role in numerous central nervous system processes, but how the receptor becomes active is insufficiently understood. Structural studies of isolated ligand-binding domains (LBDs) revealed that agonists bind between two flexible lobes (D1 and D2) and by causing the lobes to move closer together initiate the activation reaction. In many cases, the efficacy of the agonist correlates with how narrow the cleft becomes and/or how stable the closed-cleft conformation is. In NMDA receptors, full-agonists induce cleft-closure and the formation of new stabilizing electrostatic interactions across the lobes of soluble LBDs. However, how these interactions contribute to the kinetics of NMDA receptors is not known. To evaluate the roles of putative cross-cleft interactions to the activation mechanism of intact receptors, we eliminated these salt bridges in individual subunits and quantified their effect on gating by kinetic analyses of single channel traces. We were surprised to find that: receptors containing GluN1(K483A) and GluN2A(K487A) had activities that were ~10% higher than wild-type controls; receptors containing GluN1(K483M) had wild-type activity; and those containing GluN2(N687L) had activities that were ~30% lower than controls. These results suggest that at these positions, residue-size as well as the ability to form cross-cleft interactions affect the gating process. They also reveal subtle distinctions between how residues in the GluN1 and GluN2A LBDs contribute to gating.

1471-Pos Board B381
Constraining Gating Actions of Specific Subunits During NMDA Receptor Activation
Iehab Talukder, Rashek Kazi, Lonnie P. Wollmuth.
NMDA receptor activation gating initiates at its extracellular ligand-binding domain (LBD) and culminates in opening of the associated ion channel. Kinetically, this gating transition occurs through sequential intermediaries with at minimum three pre-open and two open states. The overall gating transition occurs in a concerted manner only after each subunit has bound its respective ligand. Functional NMDA receptors, however, are obligate heterotetramers, typically composed of two glycine-binding GluN1 and two glutamate-binding GluN2 subunits. To study subunit-specific contributions to gating kinetics, we constrained relative movements of the M3/M3-S2 central channel gating axis through intra-subunit disulfide cross-linking of partner positions in the M3-S2 and S2-M4 LBD-ion channel linkers, GluN1(C,C) and GluN2A(C,C). Analysis of single-channel recordings revealed the cross-linked receptors to be inefficient in activation gating, with a significantly lower P_o [mean SEM; 0.02 $\pm$ 0.005 for GluN1(C,C)-GluN2A vs. 0.61 $\pm$ 0.05 for GluN1-GluN2A]. This 30-fold reduction in P_o is manifested by both a 6-fold decrease in mean open duration (1.3 $\pm$ 0.2 vs. 8.3 $\pm$ 0.7 ms) and an 18-fold increase in mean closed duration (94 $\pm$ 20 vs. 5.1 $\pm$ 0.7 ms). The cross-linked receptors undergo only flickery openings to a short-lived open state (τ_{O1}, 0.25 $\pm$ 0.05 vs. 0.12 $\pm$ 0.01 ms) and virtually do not visit the main long-lived (~ 8.7 ms) open state. Instead, cross-linked receptor activity is locked into two long-lived pre-open states (τ_{C3}, 38 $\pm$ 9 vs. 4.2 $\pm$ 0.4; τ_{C4}, 208 $\pm$ 45 vs. 52 $\pm$ 3 ms). Thus, GluN1 subunit-specific constraint of its M3/M3-S2 gating axis most strongly affects the kinetic end states of activation gating, with minimal, if any, perturbation of the intermediate states. These results support a tightly coupled LBD to ion channel gating machinery in the NMDA receptor.

1472-Pos Board B382
Atp^{4-} Activates P2x Receptors
Mufeng Li, Shai D. Silberberg, Kenton J. Swartz.
P2X receptors are non-selective cation channels that are involved in many important physiological processes, including neurotransmission and inflammation. Extracellular ATP released from synaptic vesicles or damaged tissues has been demonstrated to be the ligand for activating P2X receptors. However, it is not clear if ATP activates P2X receptors in its free anionic form as ATP^{4-}, or in complex with divalent cations such as magnesium $(MgATP)^{2-}$. Here we examined if ATP can open the channels in the absence of divalent cations, and tested the effects of different concentrations of Mg^{2+} on the dose response curve for channel activation by ATP. The results show that all seven subtypes of P2X receptors can be activated by ATP^{4-}, and on all but one subtype of P2X receptor, Mg^{2+} has a modulatory effect. In the one exception, we found that Mg^{2+} competes with P2X receptors for ATP^{4-}, suggesting that $(MgATP)^{2-}$ is not the agonist. In summary, our results suggest that free ATP, but not magnesium bound ATP, is the ligand for opening P2X receptors.

1473-Pos Board B383
Application of the Cytopatch Instrument for Compound Screening of Ligand-Gated ion Channels
Thomas Knott, Gesa Rascher-Eggstein, Olaf Scheel.
Ligand-gated ion channels are in the focus of the pharmaceutical research as important drug targets, like the family of GABA receptors, 5-HT receptors, acetylcholine receptors (AChR), P2X receptors or TRP channels. The method of choice to screen for ligand-gated ion channel modulators is the automated patch clamp technique but a combination of high data quality and high throughput is required to carry out successful and efficient compound screens.
Thus, patch clamp screening of ligand-gated ion channels demands of flexible application schemes and a very rapid ligand application below 50 ms. Furthermore, a fast and complete wash out of agonists is a premise for a good data quality, because desensitization of some ligand-gated ion channels can lead to failures in current quantification due to low leftovers of the ligands. This specification is a big challenge for automated patch clamp instruments, as very fast movements of microfluidics must be controlled precisely whilst rupturing of the cell must be excluded.
Here, we show that the CytoPatch Instrument with its unique application technique is well suited for the investigation of ligand-gated ion channels: The application of agonists is accomplished within 25 ms, as shown for the nicotinic AChR in TE672 cells. Furthermore the investigation of the purinergic P2RX7 receptor is presented. Desensitization or rundown has not been detected. Offering a fast and flexible perfusion, the instrument allows to investigate agonistic and antagonistic modulation of ligand-gated channels with a data quality similar to that of the manual patch clamp. With its modular assembly throughput is individually scalable by the numbers of CytoPatch modules within a screening facility.

1474-Pos Board B384
P2X7 Receptor-Mediated Currents in Rat Hypothalamic Neurohypophysial System Terminals
Adolfo E. Cuadra, Thomas K. Knott, Edward Custer, José R. Lemos.
ATP facilitates the process of secretion by stimulation of purinergic P2X receptors (P2XR) in secretory cells and neurons. While the expression and function of the P2X(7)R-subtype in neurons is controversial, studies show that P2x(7)R mRNA is present in the brain, including in the hypothalamic neurohypophysial system (HNS). Our laboratory has reported that HNS arginine-vasopressin (AVP) terminals exhibit P2x(7)R immunoreactivity (IR) along with other P2xR subtypes, while oxytocin (OT) terminals display only P2X(7) IR. Therefore, our objective was to characterize P2x7R function in isolated HNS terminals from rat brain.
The P2x(7)R is distinguished from other P2XR types by low sensitivity to ATP (EC50>100 μM) and potent inhibition by Brilliant Blue-G (BBG; IC50<<μM). In voltage clamp experiments OT terminals showed negligible response to 10 μM ATP, an effective dose for other P2XR. In turn, 300 μM ATP induced a rapidly-activated, slowly-inactivating current that was reversibly inhibited by 300 nM BBG. These results are consistent with P2x(7)R function in OT terminals. We have reported that AVP containing terminals generated maximal current with 100 μM ATP that was blocked by the P2XR inhibitor PPADS (10 μM). Dose-response experiments performed using higher ATP doses (up to 1 mM) revealed increasing current amplitudes with 300 μM and 1 mM application in AVP terminals. Subsequent treatment with PPADS nearly abolished current stimulated at 10 μM ATP. However, 1 mM ATP application revealed a resistant current-component of approximately 20 percent of total. This component was reversibly inhibited by 300 nM BBG. The presence of low ATP-sensitive currents, which is inhibited by BBG, is consistent with P2x(7)R function in OT and AVP containing terminals. To our knowledge this is the first direct evidence of P2x(7)R function shown in neuronal terminals. (supported by NIH grant NS29470 to JRL)

1475-Pos Board B385
Validation of Screening Assays for Ligand Gated ion Channels on Qpatch HTX
Søren Friis, Mats H. Holmqvist, Lars D. Løjkner, M. Knirke Jensen, Morten Sunesen.
QPatch HTX has the highest throughput in the QPatch series. With signature features such as unattended operations, eight pipettes and the integrated microfluidic glass flow channels of the QPlates, the QPatch HTXis well suited for testing ligand gated ion channels. The recent advent of a QPlate with 10 patch holes per measurement site, instead of the classical single patch holehas minimized problems with low expression or problems with stability of the recodings over time. The success rate of recording on QPatch HTX is therefore approaching 100% for a number of different applications. We have previously shown that compound profiling on a wide variety of ligand-gated ion channels is certainly possible on the QPatch. With the increased stability and success rates of