A Conserved Mechanism for Gating in an Ionotropic Glutamate Receptor

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Running title: Gating Mechanism of GluA2

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Background: Ligand binding to ionotropic glutamate receptors opens the channel gate to control synaptic activity.

Results: Placing glycine residues at pore-facing positions in the M3 domain of GluA2 confers a more readily activated channel.

Conclusion: Like K channels, GluA2 uses bending of a pore-lining helix to open its gate and conduct ions.

Significance: Flexibility of M3 domain in iGluRs is critical to their function.

SUMMARY

Ionotropic glutamate receptor (iGluR) channels control synaptic activity. The crystallographic structure of GluA2, the prototypical iGluR, reveals a clamshell-like ligand-binding domain (LBD) that closes in the presence of glutamate to open a gate on the pore lining \( \alpha \)-helix. How LBD closure leads to gate opening remains unclear. Here, we show that bending the pore helix at a highly conserved alanine residue (A621) below the gate is responsible for channel opening. Substituting A621 to the smaller more flexible glycine resulted in a basally active, non-desensitizing channel with ~ 39-fold increase in glutamate potency without affecting surface expression or binding. On GluA2(A621G), the partial agonist kainate showed efficacy similar to a full agonist, and competitive antagonists CNQX and DNQX acted as a partial agonists. Met629 in GluA2 sits above the gate and is critical in transmitting LBD closure to the gate. Substituting M629 with the flexible glycine resulted in reduced channel activity and glutamate potency. The pore regions in potassium channels are structurally similar to iGluRs. While potassium channels typically use glycines as a hinge for gating, iGluRs use the less flexible alanine as a hinge at a similar position in order to maintain low basal activity allowing for ligand-mediated gating.

Ionotropic glutamate receptors (iGluRs), including the AMPA receptor family GluA1-4 (also called GluR1-4 or GluRA-D) play critical roles in controlling neuronal activity at the synapse (1). These receptors have extracellular N-termini and intracellular C-termini with 4 membrane domains (M1-4), one of which forms a re-entrant loop. Crystal structures of GluA2 with intact membrane domains (2) and ligand binding domain structures (3) show tetrameric channels with 2-fold symmetry in the ligand-binding domain (LBD) which forms an open cleft that closes upon ligand binding. This Venus flytrap closure model accommodates binding of full agonists, partial agonists as well as antagonists with each causing a different degree of closure (4). Glutamate for instance closes the GluA2 LBD cleft by ~20°, while the partial agonist kainate closes it by ~12°. The competitive antagonist DNQX closes the cleft by ~2.5°(4). It is speculated that the degree of domain closure translates to channel gating by specific ligands.

The transmembrane domains (TMD) of iGluRs have 4-fold symmetry and resemble inverted potassium channels (1, 2, 5). Bending of a pore lining \( \alpha \)-helical domain around a conserved glycine in potassium channels opens their gate (6, 7). We previously showed that in Kir3 channels mutation of this glycine to larger residues impaired channel gating while an alanine could substitute for the glycine and retain near full
activity (7). Detailed requirements for the hinge were subsequently examined to show permissible substitutions at the hinge depend on the local environment of the channel pore and the interactions among neighboring residues (8, 9). Approximately 15% of potassium channels use small residues other than glycine, mostly alanine, serine or asparagine, at the hinge position.

In iGluRs, the Ala to Thr, mutation at the lurcher site in the conserved SYTANLAAF region results in a highly active channel (10-12). Cysteine substitutions in this motif can alter channel activity after modification with MTS reagents (13). While, a previous study suggested that iGluRs do not use a hinge for their gating (14), structural information for any full-length iGluR was not available for those studies, and glycines were the only residues considered as a potential hinge. Overall, these types of approaches have shed some light on channel pore behavior but have fallen short in elucidation the gating mechanism.

In this study, we set out to examine whether glutamate receptors use a pivoted bending mechanism for gating. Since there are no obvious glycines that can play the pivot role in the pore helix, we reasoned that a bulkier residue may serve as pivot, therefore substitution with glycine at such a critical position would improve gating. In the conserved SYTANLAAF motif located below the channel gate in the M3 of GluA2, we find that glycine substitutions at several pore-facing residues results in highly active channels. Among these, the A621G mutation resulted in non-desensitizing channels with large agonist-independent basal activity, and increased potency for glutamate. Glycine substitution at M629 above the channel gate impaired channel activity. Together these results suggest a bending mechanism for channel opening that requires a hinge, most likely at amino acid position 621, on the pore lining face of the helix below the gate.

EXPERIMENTAL PROCEDURES

Expression of recombinant receptors in Xenopus oocytes. All constructs for oocyte expression were subcloned into the pGEMHE plasmid vector (15). The flop splice variant of GluA2 was used for all experiments. Throughout the paper, the residue numbering of the mature protein without the 21 amino acid signal peptide is used. An HA-epitope was inserted following glycine 382 in the N-terminal domain. As previously reported, addition of this epitope does not interfere with receptor function (16). Two mutations, L483Y and R586Q were introduced in order to remove desensitization (17) and increase channel expression (18). The point mutants were generated using the Quickchange site-directed mutagenesis kit (Stratagene) and the HA-epitope was inserted using splicing by overlap PCR as described (19). The resulting construct is hereafter referred to as GluA2**HA. All other point mutations were made in this background unless noted. The full sequences of all constructs were confirmed by automated DNA sequencing. All constructs were linearized with appropriate restriction enzymes and cRNAs were transcribed in vitro using mMessage mMachine (Ambion). Oocytes were isolated and microinjected with 2 ng of cRNA. All oocytes were maintained at 18 °C in OR2 solution containing (in mM): 82.5 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES (pH 7.5) supplemented with penicillin and streptomycin. Electrophysiological recordings were performed 1-3 days, following injection.

Two-electrode voltage-clamp recording and analysis. Whole-oocyte currents were measured by conventional two-microelectrode voltage clamp with a GeneClamp 500 amplifier (Molecular Devices). Agarose-cushioned microelectrodes with resistances of 0.1 to 1.0 MΩ were used (20). Oocytes were constantly superfused with normal frog Ringer’s (NFR) with barium containing (in mM): 125 NaCl, 2.5 KCl, 0.5 MgCl₂, 1.8 BaCl₂, 10 HEPES (pH 7.4). In some experiments, N-Methyl-D-glucamine replaced NaCl in NFR. To block or activate currents, the oocyte chamber was perfused with NFR with various drugs illustrated by bars above tracings in the Figures. Typically, oocytes were held at -60 mV and recorded at 1 KHz, filtered at 500 Hz. Data were collected using voltage ramps from -100 to +50 or in the gap-free mode at -60 mV. Dose-responses were measured using single oocytes expressing each construct as noted in the Figure. EC₅₀ values for glutamate were determined from dose-response curves constructed using GraphPad Prism 5.0 (GraphPad Software Inc.).

Cell surface expression assay. Live oocytes were incubated in OR2 with 1% BSA for 60 minutes at 4°C. An HRP-conjugated anti-HA
antibody was then added to the oocytes and incubated for 60 minutes at 4 °C. A 96 well plate was loaded with 50 μL of ECL reagent mixture per well and background luminescence was read using a plate reader. After 3 washes with OR2+1%BSA and 3 washes with OR2, individual oocytes were transferred into the wells of the 96-well plate containing the ECL reagent and the plate was read again. Background luminescence for each well was subtracted to determine total surface signal. Un-injected, H2O injected, and oocytes expressing non-HA tagged GluA2**served as controls.

**Ligand binding.** For ligand binding experiments, GluA2**HA and mutants were subcloned into pcDNA3.1(-). HEK293T cells from ATCC were grown on 100 mm dishes and maintained in DMEM with 10% FBS. Each construct was transfected using FugeneHD (Roche) and cells were collected 48-hours post transfection. Crude membranes were prepared using homogenization in hypotonic lysis buffer (10mM Tris, 5mM EDTA, and 250 mM sucrose pH7.4) with protease inhibitors. Membranes were collected by centrifugation and protein amounts determined using a BCA protein assay (Thermo). To determine binding affinity for glutamate for each mutant a competition assay was used displacing 3H-AMPA. Briefly, equal amounts of membrane from each group were incubated with 12 nM 3H-AMPA in the presence of varying concentrations of glutamate. After incubating with 3H-AMPA and glutamate for 90 minutes on ice, membranes were washed and collected on nitrocellulose GF/C filters using a Brandell harvester. Non-specific binding was determined using supersaturating concentration of glutamate. Total binding was determined in the absence of glutamate. Filters were counted in scintillation fluid and GraphPad Prism 5.0 was used to determine IC50 for displacement of 3H-AMPA by glutamate for each mutant.

**Patch clamp recordings.** Desensitizing GluA2(R586Q)HA and GluA2(R586QA621G)HA mutants were subcloned into pcDNA3.1(-). HEK293T cells from ATCC were grown on fibronectin-treated 12 mm coverslips and maintained in DMEM with 10% FBS. Each construct was transfected using FugeneHD (Roche) and cells were recorded 48-72 hours post transfection. Coverslips with transfected cells were moved to a chamber mounted onto an inverted Nikon microscope. Outside-out patch clamp recordings were performed in successfully transfected HEK cells identified using fluorescence from co-transfected GFP. Electrodes (3-5 MΩ) were filled with intracellular solution (in mM): 15 CsCl, 125 CsMeSO3, 0.5 CaCl2, 3 MgCl2, 2 MgATP, 5 Cs4BAPTA and 10 HEPES, (pH 7.4). The cells were superfused with bath solution (in mM): 140 NaCl, 4 KCl, 2 CaCl2, 0.1 MgCl2, 10 HEPES and 10 glucose, pH 7.4. Cells were held at -60 mV and recorded using gap-free protocol using a Multiclamp700B amplifier, digitized with a Digidata 1322B, sampled at 10KHz, low-pass filtered at 4KHz, and collected using pClamp9.2 (all from Molecular Devices). To achieve fast-perfusion in outside-out recordings, a theta-glass with two streams, one control and one with 10 mM glutamate was used. The patch was placed in the control stream and a fast piezo switcher (Siskiyou Corp.) was used to control the movement of the theta-glass and apply glutamate to the patch. Solution exchange had a τ<500μs determined using changes in junction potential.

**RESULTS**

**Glycine substitution of pore-facing residues in M3 helix below the gate result in highly-active channels.** According to the published crystal structure of GluA2 (2), the highly conserved region 615SYTANLAAF623 occupies the region in GluA2 pore that corresponds to a part of the water filled cavity in potassium channels where a glycine hinge resides (Figure 1A) (6, 7). Using the GluA2**HA (see Methods), we mutated each amino acid to glycine starting with I611 one turn of helix before the conserved region, up to M629, approximately two turns of the helix above this region. Based on the crystal structure, residues 627-629 are part of the helix in two of the four subunits in GluA2 (2) and the helix bundle crossing (HBC) at this position may form the putative channel gate. We expressed each mutant in oocytes and tested for activation with a saturating concentration of glutamate. Figure 1B shows summary data for these recordings. Many of the mutants were inactive, while glycine substitutions at A618, A621, T625, R628 showed activity that was ≥50% of GluA2**HA. All active glycine mutants reside in pore-facing positions in
a short stretch below the HBC. Quantitative cell surface labeling with an anti-HA antibody in live oocytes showed that all mutants, except for I612G, were expressed at detectible levels that were generally similar to or more than the GluA2**HA (Figure 1C). Overall, altered expression cannot account for the pattern of activity observed in the mutants.

**A621G increases ligand sensitivity not affinity.** Dose response experiments showed that glycine substitutions at positions 618, 621 and 625 reduced the glutamate EC$_{50}$ compared to GluA2**HA (sample traces Figure 2A-B, summary Figure 2C). Most notably, the glutamate EC$_{50}$ for the A621G mutant was shifted 39 fold. M629 resides just outside the membrane-spanning region (2) and the HBC (Figure 1A). This residue is critical in the transition from the 2-fold symmetry of the LBD to the 4-fold symmetry in the TMD. In contrast to the pore facing residues below the gate, glycine mutation at M629 reduced channel activity and glutamate sensitivity (Figures 1B and 2C).

In order to determine whether the change in activity is due to corresponding changes in binding, we performed radioligand displacement experiments. We selected three constructs to test for binding. GluA2**HA, A621G and E627G all had similar surface expression (Figure 1C), while GluA2**HA and A621G were active and E627G was completely inactive (Figure 1B). We found no discernible difference in glutamate binding between the three (Figure 2D). Furthermore, the ligand affinities were similar to those reported for the isolated ligand binding domain (21), indicating a lack of substantial effect of the channel pore on ligand binding affinity. The binding data show that improved gating is independent of ligand affinity.

**Increased activity by glycine substitutions is not due to a larger pore.** Due to the small size of glycine, substitutions in the pore may enlarge the channel cavity to increase ion flow. To test this, we substituted alanine 621 with residues of different side chains and determined the glutamate dose-responses for each (Figure 3A). The glutamate EC$_{50}$ values for various substitutions at position 621 of GluA2**HA were as follows (in $\mu$M): Gly:0.12, Ser:1.04, Asn:1.2, Cys:1.8, Asp:1.97, Ala(WT):4.73, Thr:8.1, Val:13.2. We also mutated A621 to two larger amino acids, leucine and phenylalanine. Both mutants were expressed at similar levels as GluA2**HA, but they were less active than the wild-type receptors, A621F was more active than A621L despite its larger size (Data not shown). Together these data show that small substitutions at A621 are permissible while amino acid size *per se* does not determine activity.

We also tested amino acid size at two other positions: T625, below the gate and M629, at the extracellular domain-channel interface. The dose-response relationship for glutamate activation T625A showed a similar EC$_{50}$ value to the GluA2**HA (Figure 3B). The glycine mutation at the same site reduced the EC$_{50}$ ~ 5 fold. Mutating M629, to alanine (M629A) actually improved glutamate sensitivity compared to M629G (Figure 3B), suggesting that a less flexible residue between the LBD and the gate accommodates the force transfer similar to that described for potassium channels (7).

**GluA2(A621G) mutant is basally active.** Oocytes expressing the A621G mutants had a leak basal current (See Figure 2B). We therefore reasoned that A621G might be basally open in the absence of exogenous glutamate. To directly test for agonist-independent activity, we used the open channel blocker IEM1460 (22) in the absence of exogenous glutamate to block basal currents. In oocytes expressing the A618G, A621G and T625G mutants, IEM 1460 blocked a significantly larger current compared to oocytes expressing GluA2**HA (Figure 4A, B). We did not observe an effect in uninjected oocytes (not shown). In the presence of glutamate, IEM1460 blocked glutamate induced current in both wild type and mutant channels, however the GluA2**HA receptor block was significantly less than the block of A618G, A621G, T625G and R628G mutants (Figure 4C). To further examine the basal activity in GluA2 pore mutant, we used a recording solution containing the impermeant N-methyl-D-glucamine (NMDG) in place of sodium and measured channel activity. In oocytes expressing the GluA2**HA, there was a negligible channel block when switching from sodium solution to NMDG (Figure 4D), a similar effect was observed in uninjected oocytes (not shown). In oocytes expressing A621G, the large basal current was blocked by NMDG (Figure 4E). Summary data for NMDG sensitive basal current expressed as percent of the glutamate induced...
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Since the EC$_{50}$ for glutamate activation of some of the glycine substitution is significantly reduced, the basal activity that we observe may reflect channel opening by ambient glutamate levels (11, 12). Nevertheless, our data is consistent with a lower barrier for channel gating in these glycine mutants, most prominently in the A621G.

**A621G mutant eliminates receptor desensitization.** GluA2 receptors desensitize rapidly in the presence of glutamate, and cyclothiazide (CTZ) eliminates this desensitization (1). We had been using GluA2**HA which contains the L483Y mutant to eliminate this desensitization. In order to test the effect of the A621G mutation on desensitization, we used a GluA2 construct (carrying R586Q, without the L483Y (GluA2(R586Q)HA)) that readily desensitizes (Figure 5A). While the fast peak current cannot be detected in oocytes due to slow bath exchange kinetics, the steady state desensitized current was readily observed in GluA2(R586Q)HA expressing oocytes (Figure 5B). This current was increased approximately five fold in the presence of cyclothiazide (Figure 5A,C). In contrast in GluA2(R586Q,A621G)HA expressing oocytes, glutamate activated a large steady-state current that was only slightly increased by cyclothiazide (Figure 5B,C). In outside-out recordings from HEK293T cells expressing GluA2(R586Q)HA, ultra-fast perfusion of glutamate gives a brief peak followed by a very small steady state current (Figure 5D). In contrast, patches from cells expressing GluA2(R586Q,A621G)HA did not desensitize (Figure 5E). Summary data for desensitization in outside-patches is shown in Figure 5F.

**Changes in partial agonist and weak antagonist sensitivity of the glycine mutants.** Partial agonists such as kainate do not fully close the LBD clamshell, which translates to partial channel activity. We found that kainate was more efficacious in activating the A621G mutant compared to GluA2**HA (Figure 6A-B). Other active pore-facing glycine mutants were also more kainate activated compared to GluA2**HA (Figure 6B).

The competitive antagonists CNQX and DNQX bind to the same site as glutamate on GluAs but fail to induce adequate structural changes to open the channel (23). CNQX robustly inhibited the glutamate activated currents in GluA2**HA but was less effective on A621G or other pore facing active glycine mutants (Figure 7A). DNQX was also less effective in inhibiting the mutants compared to GluA2**HA (Data not shown). In order to determine CNQX inhibition of each receptor, we stimulated with EC$_{50}$ concentrations of glutamate (12 µM for GluA2**HA and 4µM for A621G). The IC$_{50}$ for CNQX inhibition of GluA2**HA and A621G were 2.78 and 36.7 µM respectively (Figure 7B).

**Comparing the active A621G to the A622T lurcher site mutant**

Mutation of the 3rd alanine in the conserved SYTANLAAF region of GluD2 to a threonine In the lurcher mouse results in excess activity and ataxia(24, 25). Substituting the equivalent Ala to Thr in iGluRs has been show to results in channels with increased basal activity and left shifted glutamate dose-response curves(10-12). We made the lurcher site mutant A622T in GluA2**HA and compared it to the active A621G mutant. Figure 8A shows the glutamate dose-response curve for the two mutants compared to GluA2**HA. The EC$_{50}$ value for glutamate activation of GluA2**HA, A621G and A622T were 4.28, 0.12 and 2.2 µM respectively. Figure 8B shows sample tracings from oocytes expressing either A621G or A622T. In the absence of applied glutamate, A622T has a larger current compared to A621G determined by NMDG block. Application of CNQX and DNQX in the absence of glutamate activated small currents in the A621G expressing oocytes but no activation was observed in the A622T expressing oocytes. Summary data for NMDG sensitive current and CNQX and DNQX effects are shown in Figures 8C-D. While there are functional similarities between A621G and the lurcher site A622T mutant, there are quantitative differences in basal activity, glutamate potency and activity of competitive antagonist on the two mutants. CNQX induces a 6.4° closure in the binding cleft and DNQX causes a 2.5° closure, giving both some partial agonist activity in GluA1 in the presence of stargazin (23). The relative activation of A621G by CNQX and DNQX compared to glutamate is similar to those reported for GluA1 co-expressed with stargazin, while NBQX acts as a true antagonist in both (23). Together these data show that with the more
flexible glycine residue in the M3 helix, a smaller closure of the LBD can open the channel.

**DISCUSSION**

The \(6_{150}SYTANLAAF_{623}\) motif in the \(\alpha\)-helix that forms the M3 in GluA2 is highly conserved among all glutamate receptors channels (26). Our data is consistent with a critical role for this region in channel gating. Glycine substitutions in most residues within this region were inactive; however, most mutants were highly expressed and targeted to the cell surface. Four residues (A618, A621, T625 and R628), separated by ~3.5 amino acids tolerated glycine mutation. A618G, A621G and T625G had lower \(EC_{50}\) for activation by glutamate all without significant changes in expression or membrane targeting. The most active glycine mutant, A621G had a 39-fold reduction in glutamate \(EC_{50}\) without any changes in glutamate binding affinity. This data is consistent with a critical role for this residue in translating ligand binding to efficient channel gating. We believe that alanine 621 most likely acts as the hinge for GluA2 gating and its flexibility is critical to channel function. In contrast, placing a glycine residue at position M629 reduced channel activity. Since M629 resides at the interface between the LBD and the pore domain, we speculate that rigidity here may be critical in transmitting the mechanical force of LBD closure to opening the gate. In Kir3.4 channels, placing glycines between the gate and activation domains reduces channel activity (7), providing further evidence for a conserved gating mechanism between iGluRs and potassium channels. That glycine substitution in the GluA2 pore improved ligand-mediated activation suggests a more flexible M3 has improved function, but may be physiologically unfavorable due to significant basal activity. A previous study showed that substitutions at a corresponding position on NMDA receptors can also affect their channel gating, but the hinging mechanism was not explicitly tested (13).

Three possible effects of glycine replacements in the M3 helix may explain their functional consequences:

First, Glycine residues can enhance close packing of adjacent \(\alpha\)-helices (27, 28). Closer packing of the membrane domains may enlarge the pore. The side chain of the residues in the M3 that allowed glycine substitutions (A618, A621, T625) all face the channel pore, therefore these glycine substitutions would not affect helix packing.

A second possibility is replacing bulkier side chains in the pore with hydrogen, in glycine mutants, widens the pore cavity. If this were the case there would be a graded response based on the size of the side chain. We tested this explicitly by replacing A621 with various residues. An asparagine substitution that is ~29% larger than the native alanine has glutamate \(EC_{50}\) that is 3.6 times lower. A phenylalanine substitution that is 23 Å\(^3\) larger than a leucine resulted in much higher activity. Therefore, the size of the side chain per se was not the determining factor for activity in A621 substituted mutants.

Finally, glycine residues introduce flexibility in an \(\alpha\)-helix (29). While natively an alanine occupies the most likely hinge position at residue 621, substitutions with glycine result in channels where a smaller stimulus results in a larger activation, denoted by the 39-fold reduction for glutamate \(EC_{50}\). Substitutions with different sized amino acids resulted in activity that is consistent with the tolerance for small residues and the flexibility that they afford. Substitution with serine, cysteine, asparagine and aspartate were highly active with low glutamate \(EC_{50}\). Substitution with valine resulted in a channel with low activity and elevated glutamate \(EC_{50}\). Substitution with a large amino acid such as phenylalanine, resulted in channels that would open, however they needed larger stimulus (more glutamate). This model is consistent with structural and functional data in potassium channels in that open and closed potassium channel structures clearly show a kink in the alpha helical structure around a conserved glycine (6, 30). Closer analysis shows that in ~81% of potassium channels the glycine at this position is conserved (8). Another ~15% of the channels have other small residues such as alanine, serine or asparagine. Interestingly, among the A621 mutants, after A621G, A621S and A621N had the lowest glutamate \(EC_{50}\). Substituting the conserved glycine with alanine in potassium channels can be tolerated but may result in reduced function, while substitutions with larger residues result in channel that can only be opened with excessive stimulus such as co-expressed.
exogenous Gβγ(7). Whereas potassium channels are more easily gated due to their native glycine residues, glutamate receptors require external stimulus for gating. Coupled with a clamshell model for ligand binding, the semi-flexible membrane gate fine-tunes response to stimuli.

A previous study attempted to describe iGluR gating in terms of the glycine hinge requirement in potassium channels (14). No glycine residues on pore region of GluRs mimic conserved glycines in potassium channels. This led to the conclusion that iGluR gating is different from potassium channel gating. However, we now know that a glycine is not an absolute requirement for gating in potassium channels, and any residue that maintains some flexibility and does not have deleterious interactions with adjacent residues can serve as a pivot (7, 9, 31). Alanine was initially shown as a good replacement for glycine in Kir3.4 (7). Others have shown that in Kv channels, a mutation of the pivot glycine can be overcome by placing other flexible residues close to the pivot (32). Overall structural and functional data point to a channel pore that needs some flexibility to open the gate. Moreover, while glycine seems to be the preferred residue at the hinge in voltage gated channels or channels that receive cytoplasmic stimulus, in glutamate receptors, which receive extracellular stimulus, alanine may play a role in tempering activity. The naturally occurring threonine mutation found in the δ2 glutamate receptor in lurcher mice results in significant neuronal defects (24). This threonine replaces the third alanine in the SYTANLAAF region in the M3. Mutating the conserved A622 in GluA2**HA resulted in a channel that showed characteristic changes similar but not equivalent to what we observed with the A621G mutation. The basal activity of A622T was more than A621G while the glutamate EC50 was higher and the antagonists CNQX and DNQX acted as weak partial agonists on A621G and not on A622T. In GluA1, the lurcher site mutation, equivalent to A622T in GluA2, causes increase constitutive basal activity, increased agonist potency, changes in antagonist activity, changes in partial agonist activity, and desensitization (10-12). These changes are similar to our observations in the A622T mutant but may highlight the difference in the specific experimental parameters for each study. How placing a threonine in the lurcher position leads to structural alterations that cause these functional changes is not fully clear. A threonine can increase the helix bend angle and slightly open the helix at the turn preceding its position (33). Based on our model of pivoted bending around A621 for GluA2 gating, change in helix bending angle by a threonine may explain the activity of the lurcher mutants. Interestingly a threonine at position 621 of GluA2 was not well tolerated.

The increased efficacy of the partial agonist kainate and partial agonist activity of the competitive antagonists CNQX and DNQX on the glycine pore mutants is consistent with a channel that opens more easily. Relative efficacy and antagonism in iGluRs has been very well described with the degree of clamshell closure in LBD (26). The LBD is directly connected to the M3 helix, whose rigidity may influence the state of domain closure. When there is sufficient closure due to binding of a full agonist, the energy associated with clamshell closure overcomes the pulling of the closed pore leading to channel gating. If the ligand mediated clamshell closure is partial, the pulling force may not overcome the closed channel gate. Glycines make the pore domain more flexible allowing a smaller closure of the clamshell to gate the channel.

GluA2 desensitization was described as a “rupture” in the extensive interactions below the LBD and above the M3 helix (34). A rigid M3 helix provides torsional pull back required for molecular rupture. We suggest that placing the more flexible glycine residue at the pivot point in the transmembrane domain renders a non-desensitizing channel by eliminating the somewhat rigid anchor needed for the interface rupture. This scenario may also happen when GluAs are co-expressed with TARPs such as stargazin. Interestingly, in the presence of stargazin, CNQX and DNQX act as weak partial agonists (23) similar to our findings with the A621G mutant. We speculate that TARPs change the flexibility and or packing of the membrane domains in iGluRs to increase agonist potency and reduce desensitization.

While the mechanism for ligand interaction with GluRs has been well established, the exact mechanism for gating has remained elusive. Our data point to bending in the pore facing domain of M3 as a mechanism for channel opening. This
follows an evolutionary conserved model first described in potassium channels. While in most potassium channels the flexibility is afforded by glycine residues lining the pore, an alanine occupies the most likely pivot point in glutamate receptors. A more rigidly hinged gate may be critical to glutamate receptor function to closely regulate activity upon proper stimulation and interaction with accessory proteins in neurons.

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FIGURE LEGENDS

FIGURE 1. Glycine mutations in pore lining residues of M3 α-helix maintain channel activity.
A. Structures from the M1-M3 domain of GluA2 (2) and a closed potassium channels (30). Two opposing subunits are depicted for each protein. In GluA2, M1 region is shown in green, the re-entrant M2 region in purple, and M3 in gold. GluA2 pore region clearly resembles an upside down potassium channel. A glycine located in the middle of the pore lining α-helix for most potassium channels acts as a hinge to pivot and open the channel gate at the helix-bundle crossing (HBC). On the right, the structure of the 3rd transmembrane domain (M3) of GluA2 is viewed from the side. Two opposing subunits (A&C) of the tetramer are depicted. Pore lining residues in and above the conserved 615SYTANLAAF623 region are shown in red; M629, which sits just above the channel gate, is shown in blue. Side chains are depicted for some of the residues with molecular distance for α-carbons and side chains for the same residues in opposing subunits in purple and orange respectively.
B. Glutamate (100 μM) activation of the glycine substituted M3 residues in the GluA2**HA backbone. Activity of each mutant is expressed as percentage of the activity of GluA2**HA recorded in the same batch of the oocytes on the same day.
C. Surface expression for each M3 glycine mutant expressed as percent of the GluA2**HA surface expression determined in the same batch of oocytes (RLU, relative luminescence units).

FIGURE 2. Effects of glycine substitutions on glutamate potency are independent of affinity.
Sample traces from oocytes expressing GluA2**HA (A.) or A621G (B.) recorded at -60 mV using varying concentrations of glutamate (Glu). Dotted line depicts zero current. Note large current in the absence of glutamate for A621G.
C. Glutamate dose response curves for GluA2**HA, the active mutants, and M629G. EC50 values for each are shown.
D. Glutamate binding determined by displacing 12 nM 3H-AMPA in membranes from HEK293T cells expressing GluA2**HA, A621G or E627G. There is no difference in IC50 values for ligand displacement in GluA2**HA and the highly active A621G or the inactive E627G (n=4).

FIGURE 3. The size of the pore facing residues does not dictate activity.
A. The critical A621 residue was mutated to amino acids of different size to test the effects of residue size at the pivot on channel activity. Glutamate dose-response curves for these mutants and corresponding EC50 values are shown.
B. Glutamate dose response curves for GluA2**HA, T625A, T625G, M629A and M629G. EC50 values are shown.
FIGURE 4. The A621G mutant has basal, agonist-independent activity.
A. Sample traces showing that the pore channel blocker IEM1460 (10 μM) has little effect on GluA2**HA in the absence of glutamate and blocks the channels after glutamate (100 μM) induced activation. In contrast, the A621G channel has a large basal current that is blocked by IEM1460, and after glutamate activation, the channel is robustly blocked by IEM1460.
B. Summary data for IEM1460 effect on basal current in pore lining glycine mutants (n=7-26, ** denotes p<0.01 compared to GluA2**HA, One-way ANOVA with Dunnett’s post-hoc).
C. Summary data for IEM1460 effect on glutamate induced currents in pore lining glycine mutants (n=7-26, ** denotes p<0.01 compared to GluA2**HA, One-way ANOVA with Dunnett’s post-hoc).
D. Sample trace showing that replacing sodium with NMDG does not significantly affect activity in the absence of agonist in GluA2**HA.
E. Replacing sodium in the recording buffer with the impermeant NMDG blocks the current in the absence of glutamate for oocytes expressing A621G.
F. Summary data showing the extent of NMDG-blocked basal current expressed as percentage of the glutamate induced current in the same oocyte for GluA2**HA and the pore glycine mutants (n=5-7, * denotes p<0.05 compared to GluA2**HA, One-way ANOVA with Dunnett’s post-hoc).

FIGURE 5. The A621G mutant does not desensitize.
A. Sample tracing showing glutamate activated currents in an oocyte expressing the desensitizing receptor GluA2(R586Q). The current increased significantly once cyclothiazide was co-applied. Due to slow solution exchange, the peak current is not recorded.
B. Sample recording from an oocyte expressing GluA2(R586Q,A621G). Currents increased slightly once cyclothiazide was co-applied.
C. Summary data showing the ratio of the desensitized glutamate induced steady state currents (Glu_{ss}) to the total current recorded in the presence of cyclothiazide (Glu_{ct}). The glutamate steady state activation was significantly larger in receptors with the A621G mutation (*** denotes p<0.01, unpaired t-test, n=10-15).
D. Sample recording for glutamate-activated current in an outside-out patch from a HEK293 cell expressing GluA2(R586Q). A substantial peak current (pk) followed by a very small steady state (ss) current is observed.
E. The glutamate-activated current in an outside-out patch from a HEK293 cell expressing GluA2(R586Q,A621G) did not desensitize.
F. Summary data expressing the desensitized glutamate-induced steady state currents (Glu_{ss}) as percent of peak current in the same patch (Glu_{pk}). (*** denotes p<0.01, unpaired t-test, n=3)

FIGURE 6. The partial agonist kainate acts as a nearly full agonist on A621G.
A. Sample recordings of glutamate (100 μM) and kainate (500 μM) induced current in oocytes expressing GluA2**HA or A621G.
B. Summary data for kainate activation of GluA2**HA and the active pore mutants expressed as percent of glutamate-induced current from the same oocyte (n=5-18, ** denotes p<0.01 compared to GluA2**HA, one-way ANOVA with Dunnett’s post-hoc).

FIGURE 7. Reduced potency of CNQX on glycine-substitute pore-facing residue.
A. Summary data for inhibition of 100μM glutamate induced current by 10μM CNQX (n=7-17, ** denotes p<0.01 compared to GluA2**HA, one-way ANOVA with Dunnett’s post-hoc).
B. Dose response for CNQX inhibition of glutamate-activated current for GluA2**HA and A621G. For each construct varying doses of CNQX were applied in the presence of ~EC_{50} concentration of glutamate. IC_{50} values for inhibition are shown in the legend.
FIGURE 8. Comparison of A621G and A622T for glutamate potency, basal activity, and conversion of antagonist to partial agonists
A. Glutamate dose response curves for GluA2**HA, A621G and A622T that correspond to the lurcher mutation. EC$_{50}$ values for each are shown.
B. Sample traces for effect of NMDG, CNQX and DNQX on basal current for A621G and A622T. NMDG blocked a larger basal current (as percent of glutamate-activated current) in A622T expressing oocytes compared to A621G. In the absence of agonist, CNQX and DNQX consistently activated small currents in oocytes expressing A621G but not A622T.
C. Summary for NMDG-sensitive basal current of GluA2**HA, A621G and A622T expressed as percentage of the glutamate induced current in the same oocyte (** denotes p<0.01, one-way ANOVA with Tukey’s post-hoc n=5-11, ).
D. Summary for CNQX activation of GluA2**HA, A621G and A622T expressed as percentage of the glutamate-activated current in the same oocyte (** denotes p<0.01, one-way ANOVA with Tukey’s post-hoc n=3-12).
E. Summary for DNQX activation of GluA2**HA, A621G and A622T expressed as percentage of the glutamate-activated current in the same oocyte (* denotes p<0.05 compared to GluA2**HA, one-way ANOVA with Tukey’s post-hoc n=3-7).
Figure 1

A. K⁺ channel

Helix Bundle Crossing (HBC)

B. GluA2

C. Helix Bundle Crossing (HBC)
Figure 2

A. GluA2**HA

B. A621G

C. D.
Figure 3

A. [Graph showing the activation of different GluA2 variants at various concentrations of glutamate.]

B. [Graph showing the activation of different GluA2 variants at various concentrations of glutamate.]
Figure 4

A. GluA2**HA

B. Basal, IEM1460 sensitive current

C. IEM1460 block

D. GluA2**HA

E. A621G

F. NMDG sensitive current
Figure 5

A. GluA2(R586Q)HA

B. GluA2(R586Q/A621G)HA

C. %Glu/GluCTZ

D. GluA2(R586Q)HA 10 mM Glu

E. GluA2(R586Q/A621G)HA 10 mM Glu

F. % Glu/Glu pk
Figure 6

A. GluA2**HA

B. A621G

Kainate current (500 μM)
Figure 7

A. CNQX (10 μM)

B. % Inhibition of Glu-induced current

GluA2**HA (IC₅₀=2.78 μM)
A621G (IC₅₀=36.7 μM)

[CNQX], μM

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Figure 8

A. [Graph showing maximal activation of Glutamate receptors with various concentrations of Glutamate and EC50 values for different mutants.]

B. [Graph showing current responses to different concentrations of NMDG, CNQX, DNQX, and Glu over time.]

C. [Bar graph showing NMDG sensitive current for GluA2*HA, A621G, and A622T.]

D. [Bar graph showing CNQX sensitive basal current for GluA2*HA, A621G, and A622T.]

E. [Bar graph showing DNQX sensitive basal current for GluA2*HA, A621G, and A622T.]
A Conserved Mechanism for Gating in an Ionotropic Glutamate Receptor
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