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Agonist binding to the GluK5 subunit is sufficient for functional surface expression of heteromeric GluK2/GluK5 kainate receptors

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Abstract

Trafficking of ionotropic glutamate receptors to the plasma membrane commonly requires occupation of the agonist binding sites. This quality control check does not typically involve receptor activation, as binding by competitive antagonists or to non-functional channels may also permit surface expression. The tetrameric kainate receptors can be assembled from five different subunits (GluK1-GluK5). While the 'low-affinity' GluK1-3 subunits are able to produce functional homomeric receptors, the 'high-affinity' GluK4 and GluK5 subunits require co-assembly with GluK1, 2 or 3 for surface expression. These two different types of subunits have distinct functional roles in the receptor. Therefore, we examined the relative importance of occupancy of the agonist site of the GluK2 or GluK5 subunit for surface expression of heteromeric receptors. We created subunits with a mutation within the S2 ligand binding domain which decreased agonist affinity. Mutations at this site reduced functional surface expression of homomeric GluK2 receptors, but surface expression of these receptors could be increased with either a competitive antagonist or co-assembly with wild-type GluK5. In contrast, mutations in the GluK5 subunit reduced the production of functional heteromeric receptors at the membrane, and could not be rescued with either an antagonist or wild-type GluK2. These findings indicate that ligand binding to only the GluK5 subunit is both necessary and sufficient to allow trafficking of recombinant GluK2/K5 heteromers to the cell membrane, but that occupancy of the GluK2 site alone is not. Our results suggest a distinct role for the GluK5 subunit in regulating surface expression of heteromeric kainate receptors.

Introduction

The ionotropic glutamate receptors (iGluRs) are the primary mediators of fast excitatory neurotransmission in the mammalian brain. These ligand-gated ion channels are divided into three classes, the AMPA, NMDA and kainate receptors (Traynelis et al., 2010). All of the iGluRs are tetrameric in structure, and each subunit contains a ligand-binding site and contributes to the channel pore. Assembly and surface expression of the oligomers is highly regulated by both intrinsic and cell-specific processes (Coussen et al., 2009; Henley et al., 2011; Mayer, 2011). One common requirement for surface expression of the receptors is occupancy of the ligand-binding site(s). This was first demonstrated for a *C. elegans* glutamate receptor (Grunwald and Kaplan, 2003), and later shown for AMPA (Coleman et al., 2009), kainate (Fleck et al., 2003; Mah et al., 2005; Valluru et al., 2005) and NMDA receptors (Kenny et al., 2009; She et al., 2012). The mechanisms underlying this process are

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not clear, but functional activation of the receptor does not appear to be necessary, as competitive antagonists are effective chaperones and non-functional channels can be trafficked to the membrane.

The kainate receptors regulate synaptic transmission in both pre- and post-synaptic locations (Jane et al., 2009; Contractor et al., 2011) and can be assembled from five different subunits (GluK1-5). The GluK1-3 subunits (formerly GluR5-7) have relatively low affinity for kainate and glutamate, and are able to form homomeric receptors. The GluK4 and K5 subunits (formerly KA1 and KA2) have high-affinity agonist binding sites, but can only contribute to functional receptors in heteromeric assemblies with GluK1-3. The GluK5 subunits contain several ER retention motifs which prevent surface expression of homomeric receptors, and that can be masked by interaction with the GluK1-3 subunits (Gallyas et al., 2003; Ren et al., 2003; Nasu-Nishimura et al., 2006). This process normally permits forward trafficking only of heteromeric receptors, and not the non-functional GluK5 homomers.

For homomeric kainate receptors, the importance of ligand binding for receptor assembly and surface expression has been clearly demonstrated. Mutations that reduce agonist affinity cause subunits and assembled oligomers to be retained in the ER (Mah et al., 2005; Gill et al.,2009). However, surface expression of receptors containing these low-affinity subunits can be rescued by co-assembly with wild-type subunits (Mah et al., 2005). This suggested that full occupancy of all four subunits may not necessary for efficient surface expression of homomeric kainate receptors. Interestingly, this is not the case for the NMDA receptors, which are obligate heteromers. NMDA receptors are assembled from two types of subunits, which have distinct agonist binding sites. The GluN1 subunits bind to glycine while the GluN2/N3 subunits bind to glutamate (Traynelis et al., 2010). Recent studies showed that occupancy of both the glycine and the glutamate sites are required for surface expression of the NMDA receptors (Kenny et al., 2009; She et al., 2012). Like the NMDA receptors, GluK5-containing kainate receptors are obligate heteromers, and earlier studies showed that expression of mutated GluK5 subunits unable to bind agonist could not be rescued through co-assembly with wild-type GluK1 or GluK2 subunits (Valluru et al., 2005). This suggests differential mechanisms regulating surface expression of heteromeric, GluK5-containing receptors. Therefore, we examined the importance of ligand binding to each subunit for functional surface expression of GluK2/K5 kainate receptors.

We used mutations at a conserved glutamate residue within the ligand binding site to reduce agonist affinity of either the GluK2 or GluK5 subunit. We then co-expressed these low-affinity subunits with wild-type partners and measured functional receptors with patch-clamp recordings. We found that surface expression of the mutated GluK2 subunits could be enhanced either with wild-type GluK5 subunits or by incubation with a competitive antagonist. In contrast, neither the GluK2 subunit nor the antagonist was able to rescue expression of the mutated GluK5 subunit. Our results suggest that the GluK2 and GluK5 subunits have distinct roles in this process, and that activation of the GluK5 subunit, but not the GluK2 subunit, is necessary for surface expression of heteromeric receptors.

Materials and Methods

Transfection of mammalian cells

Full-length cDNAs in mammalian expression vectors were transfected into the human embryonic kidney cell line HEK-293T (GenHunter). The rat GluK2(Q) and GluK5 subunits in mammalian expression vectors were generous gifts from C. Mulle (Université Bordeaux) and R. Dingledine (Emory University), respectively. The GluK2(R) editing variant was generated through site-directed mutagenesis of GluK2(Q). The GluK2(R)-myc subunit has 6

copies of the myc epitope between residues 3 and 4 of the mature subunit and the GluK5-flag subunit has the flag sequence inserted between residues 42 and 43 of the GluK5 structure. For selection of transfected cells for electrophysiological recordings, 1 μg of the plasmid pHook™-1 (Invitrogen) containing cDNA encoding the surface antibody sFv was also transfected into the cells (Chesnut et al., 1996). Cells were maintained in Dulbecco's modified Eagle medium (DMEM)(Mediatech) plus 10% fetal bovine serum (Atlanta Biologicals), 100 IU/ml penicillin and 100 μg/ml streptomycin (Mediatech). Cells were passaged by a 5 min. incubation with 0.05% trypsin/0.02% EDTA solution (Mediatech) in phosphate buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH=7.3).

The cells were transfected using calcium phosphate precipitation. Plasmids encoding kainate receptor subunit cDNAs were added to the cells in a 1:3 ratio (GluK2:GluK5) to ensure incorporation of the GluK5 subunit into heteromeric receptors (Barberis et al., 2008). Following a 4-6 hr. incubation at 3% CO_2, the cells were treated with a 15% glycerol solution in BBS buffer (50 mM BES(N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5 mM Na_2HPO_4) for 30 sec. All chemicals were obtained from Sigma-Aldrich unless otherwise noted. The selection procedure for pHook expression was performed 18-28 hrs after transfection. The cells were passaged and mixed with 3-5 μl of magnetic beads coated with antigen for the pHook antibody (approximately 6×10^5 beads) (Chesnut et al., 1996). Following a 30-60 min. incubation to allow the beads to bind to positively transfected cells, the beads and bead-coated cells were isolated using a magnetic stand. The selected cells were resuspended into DMEM and allowed to attach to collagen-coated glass coverslips. DMEM containing either 100 μM CNQX (Tocris Biosciences) or an equivalent volume of DMSO was then added and the cells were used for recordings 18-28 hrs. later. The number of beads bound to a cell generally correlates with the amplitude of the response from recombinant ligand-gated channels (Greenfield et al., 1997). Only cells decorated with at least 3 beads were used for these experiments.

Electrophysiological recording solutions and techniques

For all recordings the external solution consisted of (in mM): 150 NaCl, 3 KCl, 0.4 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with pH = 7.4 and osmolarity adjusted to 295-305 mOsm. Recording electrodes were filled with an internal solution of (in mM); 130 CsGluconate, 5 CsCl, 2 $MgCl_2$, 2 Cs-BAPTA, 0.5 $CaCl_2$, 2 MgATP, 0.3 NaGTP, and 10 HEPES with pH = 7.4 and osmolarity adjusted to 295-305 mOsm. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments) on a two-stage puller (Narishige, Japan) to a resistance of 5-10 MΩ. Glutamate was diluted into external solution from freshly made or frozen stocks in water and applied to cells using a stepper solution exchanger with a complete exchange time of <50 msec (open tip, SF-77B, Warner Instruments). There was a continuous flow of external solution through the chamber. Currents were recorded with an Axon 200B patch clamp amplifier. Whole-cell currents were analyzed using the programs Clampfit (pClamp9 suite, Axon Instruments) and Prism (Graphpad).

Fluorescence Microscopy

After treatment with CNQX or vehicle, transfected cells on coverslips were fixed in fresh 3% paraformaldehyde. Some samples were permeabilized with 0.2% Triton X-100 before blocking with 10% normal equine serum. Samples were incubated with either Anti-FLAG M2 antibody (Sigma) or rabbit anti-myc tag antibody (Upstate), followed by Cy3-conjugated donkey anti-mouse IgG or Cy2-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch), respectively. For some conditions, DAPI (Invitrogen) was added at a final concentration of 1 μg/ml. Cells were analyzed by confocal scanning laser microscopy

with an LSM 510 Meta instrument (Zeiss). Samples were scanned at an appropriate depth to distinguish between the surface and intracellular fluorescence signals.

Construction of mutated subunit cDNAs

Point mutations were generated using the QuikChange procedure and products (Agilent). Oligonucleotide primers were synthesized by Integrated DNA Technologies and DNA sequencing was performed by the University of South Carolina Environmental Genomics core facility (Columbia, SC).

Statistical analysis

Statistical comparisons were performed using Tukey-Kramer multiple comparisons and Student's unpaired t-tests using the Instat program (Graphpad) with a significance level of $p<0.05$.

RESULTS

Functional expression of GluK2 homomeric receptors with reduced glutamate sensitivity can be enhanced with a competitive antagonist

Each kainate receptor subunit contains an agonist binding site, which is formed from domains S1 in the extracellular N-terminal domain and S2 in the M1/M2 linker (Contractor et al., 2011). A highly conserved glutamate residue within the S2 domain plays a key role in agonist binding of AMPA and kainate receptors (Armstrong and Gouaux, 2000; Mayer, 2005) (Figure 1), and even conservative mutations at this site reduce glutamate affinity dramatically (Mah et al., 2005). Studies with homomeric kainate receptors have demonstrated that occupancy of the agonist binding site by a ligand (either agonist or antagonist) is necessary for forward trafficking of oligomers to the cell membrane (Mah et al., 2005). To determine if each subunit has an equivalent role in this quality control step for surface expression, we compared the effect of mutations within the binding sites of GluK2(R) and GluK5 subunits. We used the R variant of GluK2 as most GluK2 subunits are in this edited form in the mature brain (Bernard et al., 1999).

The GluK2(R) subunits readily produce functional homomeric receptors which respond to 10 mM glutamate with rapidly desensitizing currents (Figure 2). The E738D mutation in the S2 domain of these subunits reduces glutamate sensitivity nearly 300-fold (Mah et al, 2005; Fisher and Mott, 2011) and decreased the current amplitude, but had little effect on desensitization kinetics (Figure 2). When a less conservative mutation to glycine was made, we found no response to 10 mM glutamate. This is consistent with earlier reports that this mutation eliminates surface expression of the subunits (Mah et al., 2005).

Previous studies have found that a competitive antagonist such as CNQX or DNQX can substitute for agonist binding and allow surface expression of kainate or AMPA receptor subunits with low agonist affinity (Fleck, 2006; Coleman et al., 2009). We incubated transfected cells in media containing 100 μM CNQX for 18-28 hours prior to recording and found that this treatment significantly increased the peak amplitude of the response of $GluK2_{(E738D)}$ receptors to 10 mM glutamate (Figure 2). CNQX treatment did not significantly change the response of wild-type GluK2 receptors nor did it allow measurement of a response from $GluK2_{(E738G)}$ receptors, although some surface expression could be detected with immunofluorescence following CNQX treatment (Figure 2C), suggesting that 10 mM glutamate may not be sufficient to activate these receptors. As expected, transfection of the wild-type GluK5 subunit alone did not produce a functional response or show surface expression (Herb et al.,1992; Gallyas et al., 2003; Ren et al., 2003), and this was unaffected by addition of CNQX to the media (Figure 2). These results

confirm that mutations which reduce agonist affinity of GluK2 subunits also reduce surface expression of homomeric receptors, but that membrane trafficking can be enhanced through occupancy of ligand binding site.

Co-assembly with wild-type GluK5 subunits allows functional surface expression of mutated GluK2 subunits in the absence of a high affinity ligand

Next we examined the effect of these mutations within the GluK2 subunits on the surface expression of heteromeric GluK2/K5 receptors. It is well known that GluK5 subunits require a GluK(1-3) partner to process through the ER and traffic to the cell membrane. However, it is not known whether ligand binding to the GluK5 subunit would be sufficient for efficient surface expression when combined with a low-affinity partner. We co-expressed the $GluK2_{(E738D/G)}$ mutated subunits with wild-type GluK5 subunits and examined the response to 10 mM glutamate as a measure of functional surface expression (Figure 3). Addition of the GluK5 subunit to produce heteromeric GluK2/K5 increases glutamate sensitivity and slows deactivation and desensitization kinetics compared to GluK2 homomeric receptors (Barberis et al., 2008; Fisher and Mott, 2011). Recent evidence suggests that these two subunits serve different functional roles in the heteromer. Binding of agonist to the GluK5 subunit activates the receptor in a non-desensitizing manner while higher levels of glutamate activate the GluK2 subunits, which then induces desensitization (Mott et al., 2010; Fisher and Mott, 2011). At high glutamate levels a small rebound current can be observed following removal of the agonist from GluK2/K5 receptors (Figure 3B). This has been attributed to the large difference in affinity between the GluK2 and GluK5 subunits, allowing re-activation of the channel when glutamate unbinds from the GluK2 subunits. Mutations that reduce agonist affinity further enhance this difference, producing an even larger rebound current when $GluK2_{(E738D)}$ subunits are co-expressed with wild-type GluK5 subunits (Figure 3B). Little desensitization and no rebound current was observed when the $GluK2_{(E738G)}$ subunit was co-expressed with GluK5, suggesting that this subunit is poorly activated even by 10 mM glutamate.

The whole-cell current amplitude of heteromeric receptors was not decreased by the E738D mutation in GluK2, and incubation with CNQX had no effect on functional expression of these receptors (Figure 3). The less conservative $GluK2_{(E738G)}$ mutation greatly reduced the current amplitude, but co-expression with GluK5 did allow some functional surface expression of heteromeric receptors. Imaging of surface receptors with immunofluorescence supports these findings, showing that surface labeling of the myc-tagged GluK2 mutated receptors is apparent in the presence of GluK5, regardless of the addition of CNQX(Figure 3C). This demonstrates that the GluK5 subunits can serve as effective chaperones for low-affinity subunits, and that agonist binding to the GluK5 is sufficient for forward trafficking of heteromers. These results are consistent with the ability of wild-type GluK2 subunits to rescue surface expression of mutated GluK2 subunits (Mah et al., 2005), but is the first demonstration that the GluK5 subunits can also perform this role. This provides further evidence for the supposition that occupancy of all four binding sites within the tetrameric kainate receptor is not required for efficient surface expression.

Mutation of the glutamate binding site in the GluK5 subunit reduces functional expression of heteromeric subunits

The previous results demonstrated that agonist binding to the GluK5 subunit was sufficient to allow membrane expression of GluK2/K5 heteromeric receptors. To determine if this was a subunit-dependent process, we made mutations at the homologous location (E722) in the GluK5 subunit, co-transfected with wild-type GluK2, and examined the level of functional receptor expression.

In contrast to the effects with GluK2, we found that even the conservative E722D mutation in the GluK5 subunit greatly reduced the whole-cell current amplitude of heteromeric receptors in response to 10 mM glutamate (Figure 4A, C). In addition, unlike the homomeric GluK2 receptors, incubation with CNQX did not significantly increase the current amplitude, suggesting that occupancy of the GluK5 binding site with an antagonist was not sufficient to allow forward trafficking of the heteromer. These receptors have a glutamate EC_{50} similar to that of the wild-type GluK2 subunit, and are therefore fully activated by 10 mM glutamate. This suggests that the reduced current amplitude is due to a decrease in surface expression. Immunofluorescence also demonstrated that CNQX did not increase appearance of flag-tagged GluK5 subunits at the cell surface, but instead, may decrease receptors containing this subunit in favor of GluK2 homomeric receptors (Figure 4B,D). We verified that the mutations in the GluK2 and GluK5 subunits had no effect on sensitivity to inhibition by 100 μM CNQX, producing >90% reduction of the response to an $EC_{30\text{-}50}$ concentration of glutamate for receptors containing wild-type or mutated subunits. This is consistent with previous work showing that mutations at this site reduce agonist, but not antagonist affinity at the binding site (Fisher and Mott, 2011). Similar properties were observed when the $GluK2_{(E738D)}$ and $GluK5_{(E722D)}$ subunits were co-expressed, with no increase in current amplitude produced by CNQX incubation (Figure 4A). When GluK2 was co-expressed with $GluK5_{(E722G)}$, 10 mM glutamate elicited small currents with a substantial steady-state component, similar to the appearance of $GluK2_{(E738G)}$/K5 receptors (Figure 3B). This suggests that the GluK5 subunit was assembled, at least to some extent, into these receptors. Incubation with CNQX significantly increased the current amplitude, but the resulting currents exhibited a larger, rapidly desensitizing component, more consistent with GluK2 homomeric receptors. Immunofluorescence measurements also suggest an increase in GluK2 homomers, as CNQX caused an increase in surface anti-myc labeling, but a decrease in surface labeling of the mutated GluK5 subunits (Figure 4D).

To determine the level of GluK5 incorporation into these receptors, we examined their voltage-dependent characteristics. Outward current through kainate receptors can be inhibited by cytoplasmic polyamines, producing a pronounced inward rectification. Sensitivity to polyamine block is determined in large part by a residue in pore domain that is subject to RNA editing, which produces an arginine(R) residue rather than the encoded glutamine(Q) (Bowie and Mayer, 1995). Co-assembly with GluK5 subunits also reduces sensitivity to polyamine block, and the decreased inward rectification can be used as a measure of GluK5 incorporation into heteromeric receptors containing GluK2(Q) (Barberis et al., 2008). Since the previous experiments had been performed with GluK2(R), we examined the effect of the mutations and incubation with CNQX on the rectification properties of GluK2(Q) homomers and heteromers to determine relative assembly of the GluK5 subunit (Figure 4B). As expected, we found that GluK2(Q) homomers exhibited pronounced inward rectification, and that co-expression with wild-type GluK5 subunits increased the relative amount of current observed at positive membrane potentials. Incubation with CNQX had no effect on these properties (Figure 4B). The GluK2(Q)/$K5_{(E722D)}$ receptors also had reduced rectification, suggesting incorporation of the mutated GluK5 subunit at levels similar to wild-type. However, incubation with CNQX significantly reduced the rectification ratio, indicative of an increased production of homomeric GluK2(Q) receptors. This is consistent with the increase in surface anti-myc and decrease in surface anti-flag immunofluorescence produced by CNQX (Figure 4D). The GluK2(Q)/$K5_{(E722G)}$ receptors showed a level of inward rectification comparable to the homomers, suggesting that the majority of these receptors did not contain a GluK5 subunit. The Q/R editing site of the GluK2 subunit can influence its surface expression and assembly, and GluK2(Q) subunits are more likely to traffic to the surface as homomers compared to the edited form (Ball et al., 2010). Therefore, our results may overestimate the amount of GluK2(R) homomeric receptors that were generated. Overall, however, our findings with

GluK2(Q) are consistent with the effects of the point mutations and the role of CNQX that we observed with GluK2(R).

These results demonstrate distinct roles for the GluK2 and GluK5 subunits in regulating surface expression of recombinant kainate receptors. This is consistent with earlier studies by Valluru et al. (2005), who reported that mutations at T675 in the GluK5 subunit also reduced surface expression of wild-type GluK1 or GluK2 subunits. Together, this work shows that ligand binding to the GluK5 subunit, but not the GluK2 subunit, is required for expression of heteromeric receptors. In addition, CNQX treatment was unable to enhance surface expression of the GluK5 subunit, in contrast to its effects on GluK2. This suggests a unique requirement of the GluK5 subunits for activation by agonist, rather than simply occupancy of the binding site, for efficient trafficking to the cell membrane.

Discussion

The trafficking of multimeric ionotropic glutamate receptor to the plasma membrane is a highly regulated process which is controlled at a number of steps. A primary checkpoint is the release of properly folded, assembled, and functional oligomers from the ER (Fleck, 2006; Greger et al., 2007). Our results add to the growing evidence that both ligand binding and conformational changes associated with the ligand-binding domain are necessary for forward trafficking of these receptors. In addition, our work clarifies the distinct roles for different kainate receptor subunits in heteromeric receptors.

While a requirement for ligand binding has been clearly demonstrated, the mechanism underlying this requirement is not known. For homomeric GluK2 receptors, occupancy of the agonist site was shown to be important for proper folding of the subunit within the ER, while further conformational changes in the ligand-binding domain are important for oligomerization (Gill et al., 2009). Several lines of evidence indicate that opening of the ion channel by this ligand is not necessary. Non-functional channels and isolated extracellular domains share the same requirement for binding site occupancy and competitive antagonists are generally able to enhance surface expression (Mah et al., 2005; Gill et al., 2009; Coleman et al., 2009). Recent work identified critical structural differences within the ligand-binding domain that influence assembly and trafficking, but not other functional properties of the receptor (Coleman et al., 2010). However, other studies have demonstrated that restricting movement of the ligand binding domain reduces surface trafficking of receptors, suggesting that conformational changes induced by ligand binding, or at least flexibility within these domains, is required (Valluru et al., 2005; Gill et al., 2009). Similarly, evidence from AMPA receptors suggests that the ability of a receptor to undergo ‘gating motions’ is required for forward trafficking (Penn et al., 2008) and that mutations which disrupt interactions between the ligand-binding domain and transmembrane regions of kainate receptors also slow tetramerization and release from the ER (Vivithanaporn et al. 2007). Our finding that the competitive antagonist CNQX does not enhance surface expression of receptors containing GluK5 subunits with low affinity produced by a mutation, while it does increase expression of wild-type or mutated GluK2 homomers with low affinity, suggests a unique requirement for agonist-induced conformational changes in the GluK5 subunit. One possibility is that agonist binding induces structural changes in the subunit or oligomer that allow the GluK2 subunit to shield the GluK5-associated ER retention domains.

Previous work with mutations in GluK5 subunits suggested that ligand binding to each subunit in the tetrameric kainate might be required for forward trafficking from the ER (Valluru et al., 2005). In contrast, our results are more consistent with studies that utilized mutations in GluK2 subunits, and concluded that all four binding sites need not be occupied

for efficient surface expression (Mah et al., 2005). However, our results demonstrate distinct roles for the GluK2 and the GluK5 subunits within the oligomer, providing an explanation for the differing conclusions from these earlier studies. The heteromeric GluK5-containing receptor may be regulated in a similar fashion to the NMDA receptor, which is also an obligate heteromer. As with the GluK5 subunit, co-expression with wild-type counterparts was unable to permit surface expression of either GluN1 or GluN2B subunits carrying mutations in their ligand-binding sites (Kenny et al., 2009; She et al., 2012).

The requirement for ligand binding is only one of many regulatory mechanisms that control assembly and trafficking of ionotropic glutamate receptors. A large number of chaperone proteins have been identified that aid in proper folding, assembly, and synaptic localization of these receptors (Coussen et al., 2000; Henley et al., 2011). These processes can be modulated by cellular signaling pathways, including phosphorylation and other post-translational modifications of both the pore-forming subunits and the accessory proteins. In addition to the chaperone proteins, which interact only transiently with ionotropic glutamate receptors, auxiliary subunits form more stable associations, and regulate trafficking, membrane localization, and functional characteristics of the receptors (Jackson and Nicoll, 2011; Copits and Swanson, 2012). The surface expression of AMPA receptors is increased by co-assembly with some TARP (transmembrane-AMPA receptor regulatory proteins) subunits, which are also critical for synaptic targeting and maintenance of these receptors in post-synaptic locations (Coleman et al., 2009; Sager et al., 2009; Straub and Tomita, 2012). The Neto1/2 proteins have recently been identified as auxiliary subunits of kainate receptors, and may play a similar role in the regulation of membrane expression and synaptic targeting (Tang et al., 2012; Copits and Swanson, 2012). Auxiliary subunits may aid trafficking by stabilizing oligomer interactions (Shanks et al., 2010), and could also increase agonist affinity, increasing the binding site occupancy by endogenous glutamate.

In cells and neurons in which both GluK(1-3) and GluK5 subunits are expressed, formation of GluK5-containing heteromeric receptors appears to be preferred. The dominant kainate receptor isoform in neurons is likely to be the GluK2/K5 heteromer (Lerma, 2003). Initial dimerization of kainate receptors subunits is mediated by interactions between the extracellular amino-terminal domains and these domains associate with higher affinity as GluK2/K5 heteromers than as GluK2/K2 homomers, providing one mechanism for the preferential assembly of heteromers (Kumar et al., 2011). Our results provide another potential mechanism for this observation, with the higher agonist affinity of the GluK5 ligand-binding domain increasing the likelihood for occupancy of the required minimum of binding sites within the tetramer. Thus, the requirement for agonist occupancy may preferentially increase the formation and expression of high-affinity receptors, which would include GluK4 or GluK5 subunits. Since these subunits also regulate channel kinetics, changing their proportion at the synapse would influence the characteristics of excitatory neurotransmission.

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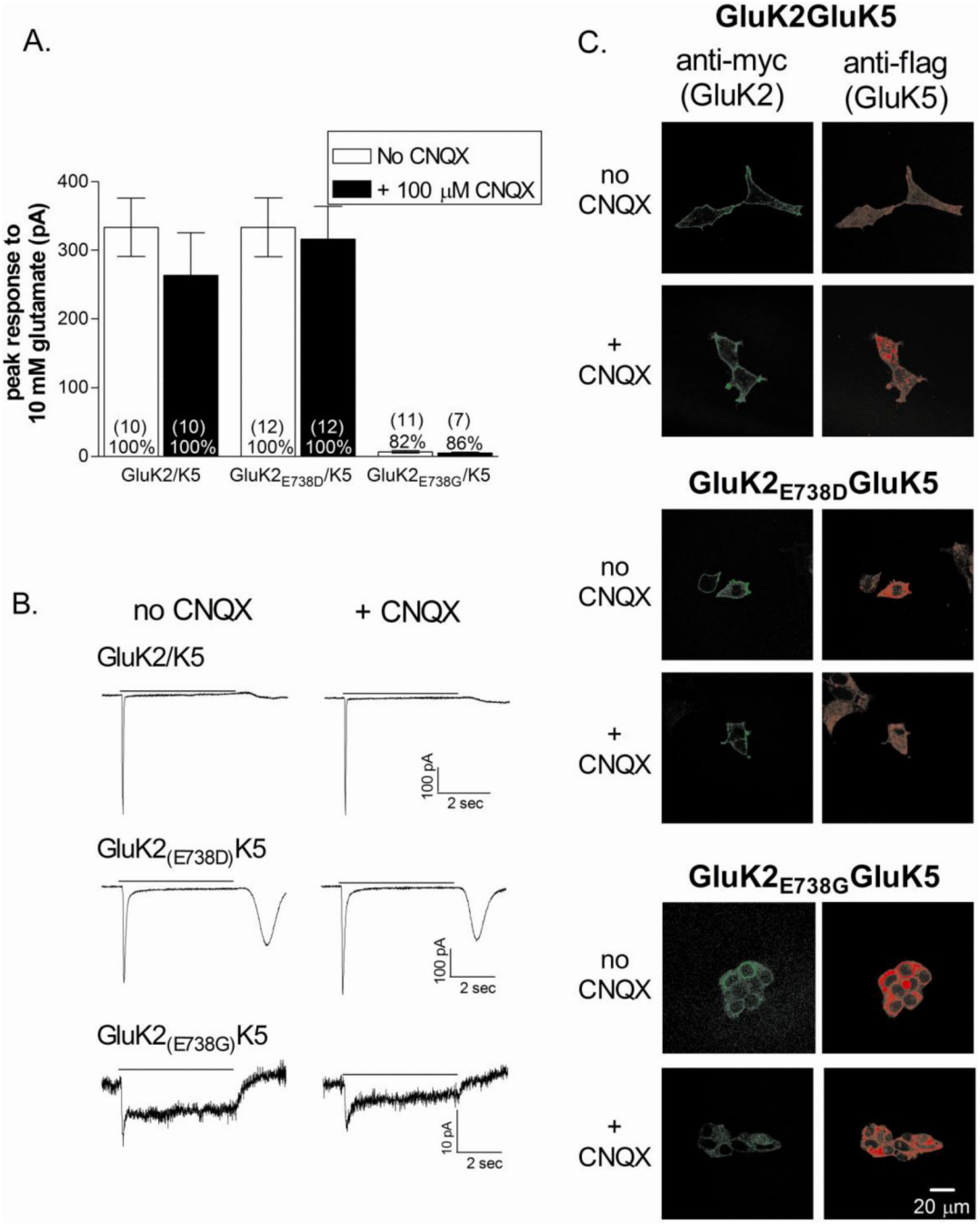

Figure 1. Location of mutation site

Kainate receptor subunits have an extracellular N-terminal domain, three full transmembrane domains (M1, M3 and M4) and a re-entrant pore forming domain (M2). The extracellular S2 domain of ionotropic glutamate receptors is located between the M3 and M4 transmembrane domains, and contributes to the agonist binding site. A partial sequence alignment from this region is shown for the five kainate receptor subunits. The glutamate residue mutated in these studies is bolded and indicated by the box.

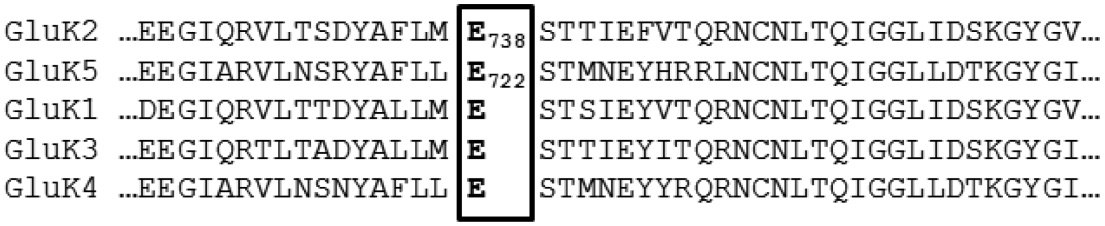

Figure 2. Effect of CNQX on surface expression of homomeric kainate receptors

Wild-type or mutated GluK2(R) or GluK5 kainate subunits were transiently transfected into HEK-293T cells. The peak response to 5 sec applications of 10 mM glutamate was measured from cells voltage-clamped at −70 mV.

A. Mean (±SEM) peak responses from cells transfected with the wild-type or mutated subunit indicated. The number in parentheses indicates the number of cells tested, with the percentage of those cells that gave a measurable current response shown below. 100 μM CNQX (black bars) or an equivalent volume of DMSO (white bars) was added to the culture media 24 hours before recording. ** ($p \leq 0.01$) indicates a significant effect of CNQX (unpaired t-test). No cells transfected with either $GluK2_{(E738G)}$ or GluK5 alone responded to 10 mM glutamate.

B. Representative whole-cell current traces in response to 5 sec applications of 10 mM glutamate (solid line). 100 μM CNQX or vehicle (no CNQX) was included in the culture media 24 hours prior to recording.

C. Immunofluorescence from $GluK2_{myc}$ or $GluK5_{flag}$ subunits in transiently transfected HEK-293T cells. Cells were incubated for 24 hours with 100 μM CNQX (+CNQX) or vehicle (no CNQX) and then treated with anti-myc or anti-flag antibodies under permeabilizing (+Triton X100) or non-permeabilizing conditions. DAPI staining was performed for those conditions in which no surface receptors were detected to allow visualization of cells.

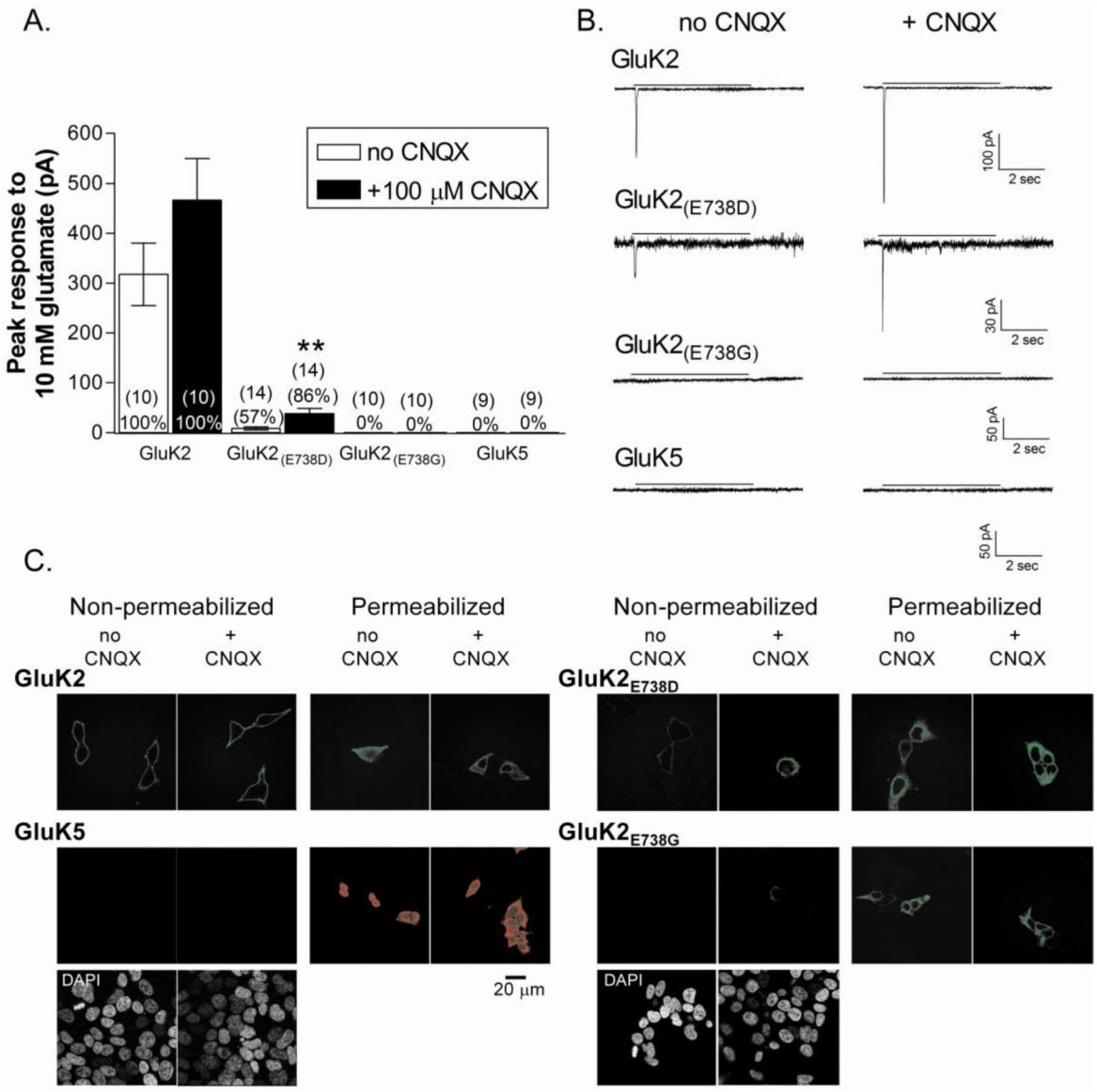

Figure 3. Assembly with wild-type GluK5 subunits permits functional surface expression of low affinity GluK2 subunits

A. Mean (±SEM) peak responses from cells transfected with the wild-type or mutated subunit indicated. The number in parentheses indicates the number of cells tested, with the percentage of those cells that gave a measurable current response shown below. 100 μM CNQX (black bars) or an equivalent volume of DMSO (white bars) was added to the culture media 24 hours before recording.

B. Representative whole-cell current traces in response to 5 sec applications of 10 mM glutamate (solid line). 100 μM CNQX or vehicle (no CNQX) was included in the culture media 24 hours prior to recording.

C. Immunofluorescence from $GluK2_{myc}$ or $GluK5_{flag}$ subunits in transiently transfected HEK-293T cells. Cells were incubated for 24 hours with 100 μM CNQX (+CNQX) or vehicle (no CNQX) and then treated with anti-myc or anti-flag antibodies under non-permeabilizing conditions.

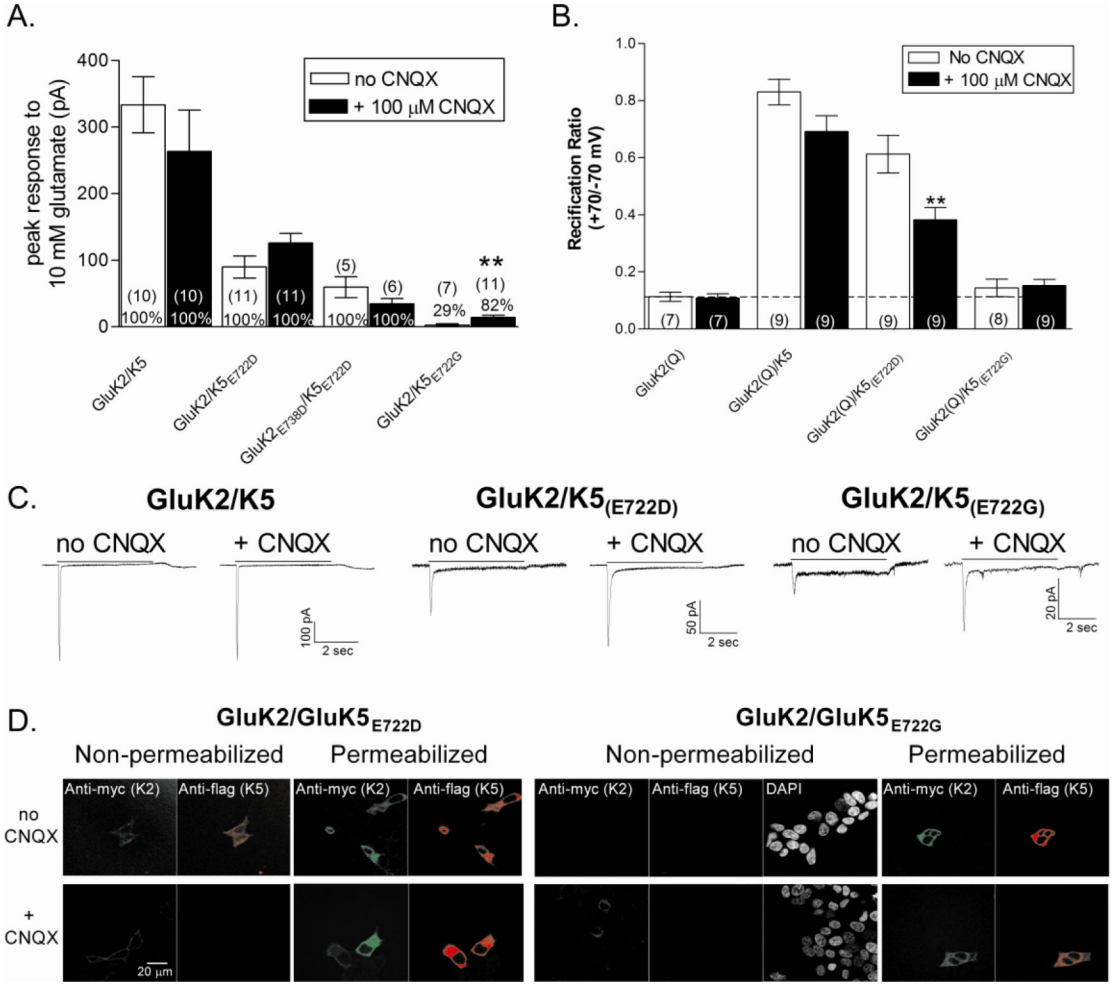

Figure 4. Neither GluK2 nor CNQX allows surface expression of mutated, low affinity GluK5 subunits in heteromeric receptors

A. Mean (±SEM) peak responses from cells transfected with GluK2(R) and a wild-type or mutated GluK5 subunit. The number in parentheses indicates the number of cells tested, with the percentage of those cells that gave a measurable current response shown below. 100 μM CNQX (black bars) or an equivalent volume of DMSO (white bars) was added to the culture media 24 hours before recording.

B. Mean (±SEM) rectification ratios from cells transfected with GluK2(Q) and the wild-type or mutated GluK5 subunit indicated. Peak current measured at a membrane potential of +70 mV was divided by the response at −70 mV. The reversal potential was near 0 mV. The number in parentheses indicates the number of cells tested. CNQX or vehicle treatments as previously described. ** ($p \leq 0.01$) indicates a significant effect of CNQX (unpaired t-test).

C. Representative whole-cell current traces in response to 5 sec applications of 10 mM glutamate (solid line). Cells were voltage-clamped at −70 mV. 100 μM CNQX or vehicle (no CNQX) was included in the culture media 24 hours prior to recording.

D. Immunofluorescence from $GluK2_{myc}$ or $GluK5_{flag}$ subunits in transiently transfected HEK-293T cells. Cells were incubated for 24 hours with 100 μM CNQX (+CNQX) or vehicle (no CNQX) and then treated with anti-myc or anti-flag antibodies under permeabilizing (+Triton X100) or non-permeabilizing conditions. DAPI staining was performed for those conditions in which no surface receptors were detected to allow visualization of cells.