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# Distinct Functional Roles of Subunits within the Heteromeric Kainate Receptor

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**Kainate receptors (KARs) have been implicated in a number of neurological disorders, including epilepsy. KARs are tetrameric, composed of a combination of GluK1–GluK5 subunits. We examined the contribution of GluK2 and GluK5 subunits to activation and desensitization of the heteromeric receptor. Heteromeric GluK2/K5 receptors expressed in HEK-293T cells showed markedly higher glutamate sensitivity than GluK2 homomers and did not desensitize at low glutamate concentrations. Mutation of residue E738 in GluK2 substantially lowered its glutamate sensitivity. However, heteromeric KARs containing this mutant GluK2 [GluK2(E738D)] assembled with wild-type GluK5 showed no change in glutamate $EC_{50}$ compared with wild-type heteromeric KARs. Instead, higher concentrations of glutamate were required to produce desensitization. This suggested that, within the heteromeric receptor, glutamate binding to the high-affinity GluK5 subunit alone was sufficient for channel activation but not desensitization, whereas agonist binding to the low-affinity GluK2 subunit was not necessary to open the channel but instead caused the channel to enter a closed, desensitized state. To test this hypothesis in wild-type receptors, we used the competitive antagonist kynurenate, which has higher affinity for the GluK2 than the GluK5 subunit. Coapplication of kynurenate with glutamate to heteromeric receptors reduced the onset of desensitization without affecting the peak current response, consistent with our hypothesis. Our results suggest that GluK2 and GluK5 subunits can be individually activated within the heteromeric receptor and that these subunits serve dramatically different functional roles.**

## Introduction

Kainate receptors (KARs) are tetramers composed of combinations of low-affinity GluK1–GluK3 (GluR5–GluR7) and high-affinity GluK4–GluK5 (KA1–KA2) subunits. Each subunit contains a glutamate binding site, and all subunits contribute to the formation of the ionic pore. However, only the GluK1–GluK3 subunits can produce functional homomeric receptors. GluK4 and GluK5 subunits do not assemble as functional homomeric receptors but rather form heteromeric receptors with GluK1–GluK3 subunits. These heteromeric assemblies comprise the majority of KARs in the CNS. In particular, GluK5 subunits are the most prevalent KAR subunit, and GluK2/K5 receptors are the most common KAR (Petralia et al., 1994). Despite their prevalence, the relative roles of the GluK2 and GluK5 subunits in gating the heteromeric receptor are poorly understood.

Studies of GluK4- or GluK5-containing KARs have revealed important differences in biophysical and pharmacological properties of homomeric and heteromeric receptors (Herb et al., 1992; Swanson et al., 1996, 1998, 2002; Contractor et al., 2003; Mott et al., 2003, 2008, 2010; Barberis et al., 2008). Several possible mechanisms could explain these differences. For example, the inability of GluK4-GluK5 subunits to gate current when expressed in homomeric configuration has led to the hypothesis that GluK1–GluK3 subunits control the response to glutamate, whereas GluK4–GluK5 subunits act as accessory proteins, modifying the physiological and pharmacological properties of current mediated by GluK1–GluK3 subunits. Alternately, GluK1–GluK3 and GluK4–GluK5 subunits may independently gate current with unique properties. Thus, KAR gating may occur in a manner similar to AMPA receptors in which each subunit produces current independent of the other subunits in the tetramer (Rosenmund et al., 1998). Recent studies have lent support to this latter hypothesis by showing that both GluK4 and GluK5 subunits can contribute a distinct conductance on agonist binding (Swanson et al., 2002; Mott et al., 2010). Based on modeling studies and steady-state glutamate currents at recombinant GluK2/K4 receptors in *Xenopus laevis* oocytes, we have suggested previously that activation of GluK4 or GluK2 subunits in the heteromeric receptor produces unique channel responses, with binding to GluK4 activating the receptor and GluK2 responsible for desensitization (Mott et al., 2010). However, these studies were limited by the slow agonist application rate in the *Xenopus* oocyte system and, as a result, focused on the characteristics of the steady-state response. Thus, confirmation of these findings as well as assessment of the applicability of the findings to other KAR subunit combinations awaited more detailed examination.

In the present study, we have examined the role of GluK1–GluK3 and GluK5 subunits in gating glutamate current at recombinant KARs. Using both a point mutation and a subunit-selective competitive antagonist, our findings suggest that GluK5 subunits play a

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primary role in channel gating. Because of their high glutamate affinity, they are activated first and gate the channel in a non-desensitizing manner. Rapid desensitization occurs only when the lower-affinity GluK1–GluK3 subunit is activated by agonist. These findings demonstrate that unique kinetic behavior is associated with GluK5-containing KARs and suggest that GluK1–GluK3 and GluK5 subunits serve different functional roles in the heteromeric receptor.

## Materials and Methods

*Culture and transfection of HEK-293T cells.* HEK-293T cells (GenHunter) were cultured in DMEM plus 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged by a 5 min incubation with 0.05% trypsin/0.02% EDTA solution in PBS (10 mM $Na_2HPO_4$ and 150 mM NaCl, pH 7.3) and dissociated further by gentle trituration.

Cells were transfected with full-length cDNAs for the GluK subunits in JG3.6, pCDNA1amp, or pCIneo expression vectors using calcium phosphate precipitation according to Mott et al. (2010). For expression of GluK1 or GluK2 homomers, 2 μg of cDNA was used. For expression of heteromeric receptors 1 μg of GluK1–GluK3 was combined with 3 μg of GluK5. Formation of heteromeric receptors was confirmed by measuring the rectification ratio of the peak response to 1 mM glutamate (+70 mV/−70 mV ratio). This ratio was >0.9 for both GluK1(Q)/K5 (0.98 ± 0.04, $n = 5$) and GluK2(Q)/K5 (0.91 ± 0.06, $n = 5$), as expected for heteromeric receptors containing GluK5. We also tested GluK2(Q)/K5 and GluK3(Q)/K5 receptors for sensitivity to 30 μM AMPA and found that all cells that responded to glutamate also responded to AMPA ($n = 11$ and 4, respectively). For selection of transfected cells, 1 μg of a cDNA encoding a single-chain antibody recognizing the hapten 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (phOx) was also transfected into the cells (Chesnut et al., 1996). Cells were incubated 4–6 h at 3% $CO_2$, followed by treatment with a 15% glycerol solution in BES buffer (50 mM *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, 280 mM NaCl, and 1.5 mM $Na_2HPO_4$) for 30 s. After 18–28 h, cells underwent a selection procedure in which they were passaged and then incubated with 3–5 μl of magnetic beads (~$6 \times 10^5$ beads) conjugated with phOx–BSA for 30-60 min (Chesnut et al., 1996). Positively transfected cells were isolated with a magnetic stand and plated onto glass coverslips treated with poly-L-lysine and collagen and used for recording 18–28 h later. To increase surface expression of GluK2(E738D) homomeric receptors, 100 μM CNQX was added to the medium overnight (Fleck, 2006).

*Whole-cell and excised patch recording.* Transfected HEK-293T cells plated on a glass coverslips were transferred to a perfusion chamber on the stage of an inverted microscope (Olympus IX50) and continually perfused at a rate of 0.5 ml/min with 23°C media containing the following (in mM): 150 NaCl, 3 KCl, 10 HEPES, 1 $CaCl_2$, and 0.4 $MgCl_2$, at pH 7.4 (osmolarity, 295–305 mOsm). Whole-cell recordings of agonist-evoked membrane currents were performed under voltage-clamp conditions with pipettes containing the following (in mM): 130 Cs-gluconate, 5 CsCl, 10 HEPES, 5 Cs-BAPTA, 2 $MgCl_2$, 2 MgATP, and 0.3 NaGTP, pH 7.3 (osmolarity, 290–300 mOsm). Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments) on a two-stage puller (Narishige) to a resistance of 5–10 MΩ. For whole-cell recordings, glutamate was applied to cells using a stepper solution exchanger (SF-77B; Warner Instruments) containing a three-barrel square glass application pipette in which control solution continuously flowed through two barrels and glutamate solution flowed through the third barrel. The solution flowing through the application pipette could be changed by means of a valve connected to each barrel. The time course of drug application was 20–30 ms in the whole-cell recording configuration. For macropatch recordings, the three-barrel square glass was pulled to a final size near 200 μm. Rise times (10–90%) of the junction potential at the open tip were consistently faster than 400 μs and were tested using a diluted external solution. Current recordings were amplified (Axopatch 200B; Molecular Devices), filtered (1 kHz) and digitized at 10 kHz using a Digitata 1320 analog-to-digital board (Molecular Devices), and stored on a computer hard drive for off-line analysis.

*Analysis of whole-cell and macropatch currents.* Whole-cell currents were analyzed using the programs Clampfit (pClamp9.2 suite; Molecular Devices) and Prism (GraphPad Software). Concentration–response data were fit with a four-parameter logistic equation: Current = [Minimum current + (Maximum current − Minimum Current)]/1 + (10^(log $EC_{50}$ − log [Glutamate]) * $n$, where $n$ represents the Hill number. All fits were made to normalized data with current expressed as a percentage of the maximum response to glutamate for each cell. Macropatch currents were digitized at 10 kHz and analyzed with the pClamp9.2 suite of programs (Molecular Devices). The desensitization rate was determined by fitting the decay current with the Levenberg–Marquardt least-squares method with increasing numbers of exponential functions until additional components did not significantly improvement of the fit ($F$ test of the sum of squared residuals).

*Materials.* Rat GluK1, GluK2, and GluK5 plasmids (GluK1 in pCIneo, GluK2 in JG3.6) were generously provided by S. Heinemann (Salk Institute, San Diego, CA). For these experiments, the GluK5 plasmid from the Heinemann laboratory was subcloned into the pCIneo vector (Promega). Human GluK3 in pcDNA3.1 (Invitrogen) was provided by A. Srivastava (Greenwood Genetics Center). The GluK2(E738D) mutant was a generous gift from M. Fleck (Albany Medical Center, Albany, NY). Salts, glutamate, and kynurenic acid were purchased from Sigma. All tissue culture reagents were obtained from Thermo Fisher Scientific and Atlanta Biologicals.

## Results

### GluK5 increases agonist sensitivity and decreases desensitization of GluK2-containing KARs

The contribution of GluK2 and GluK5 subunits to KAR activation and desensitization was examined by comparing glutamate currents at GluK2 homomeric and GluK2/GluK5 heteromeric receptors. To induce expression of GluK2/K5 heteromeric receptors, HEK-293T cells were cotransfected with GluK2 and GluK5 cDNAs at a 1:3 ratio. This ratio is optimal for expression of GluK2/GluK5 heteromeric receptors (Nasu-Nishimura et al., 2006; Barberis et al., 2008). Glutamate was applied for 10 s to cells voltage clamped at −70 mV in the whole-cell recording configuration. Compared with the GluK2 homomer, addition of the GluK5 subunit altered both agonist sensitivity and onset of desensitization (Fig. 1*A*). Glutamate concentration–response curves indicated markedly higher glutamate sensitivity for GluK2/K5 receptors than for GluK2 receptors (GluK2, $EC_{50}$ = 294 ± 36 μM, Hill number = 0.88 ± 0.09, $n = 6$; GluK2/K5, $EC_{50}$ = 2 ± 1 μM, Hill number = 1.5 ± 0.17, $n = 5$; Fig. 1*B*). These $EC_{50}$ values are in good agreement with previous reports (Barberis et al., 2008). The average peak current amplitude evoked by a maximally effective concentration of glutamate was similar for both receptors (GluK2, 297 ± 102 pA, $n = 6$; GluK2/K5, 257 ± 97 pA, $n = 5$). Although a high glutamate concentration produced strong desensitization, lowering the glutamate concentration slowed the onset of desensitization for both GluK2 and GluK2/K5 receptors. At glutamate concentrations below 10 μM, substantial differences in desensitization between the two receptors became apparent. The smallest detectable current at GluK2 homomers was produced by 3 μM glutamate and showed complete desensitization. In contrast, at this same concentration, GluK2/K5 receptors exhibited a large current with only partial desensitization. At 1 μM glutamate, GluK2 receptors produced no detectable current, whereas the current at GluK2/K5 receptors was no longer desensitizing. Thus, desensitization at GluK2/K5 receptors was only present at glutamate concentrations sufficient to activate GluK2 homomers. Plotting the glutamate concentration–response curve of the steady-state current for GluK2/K5 receptors revealed a biphasic curve with a peak at 3 μM glutamate, the concentration that produced the smallest detectible response

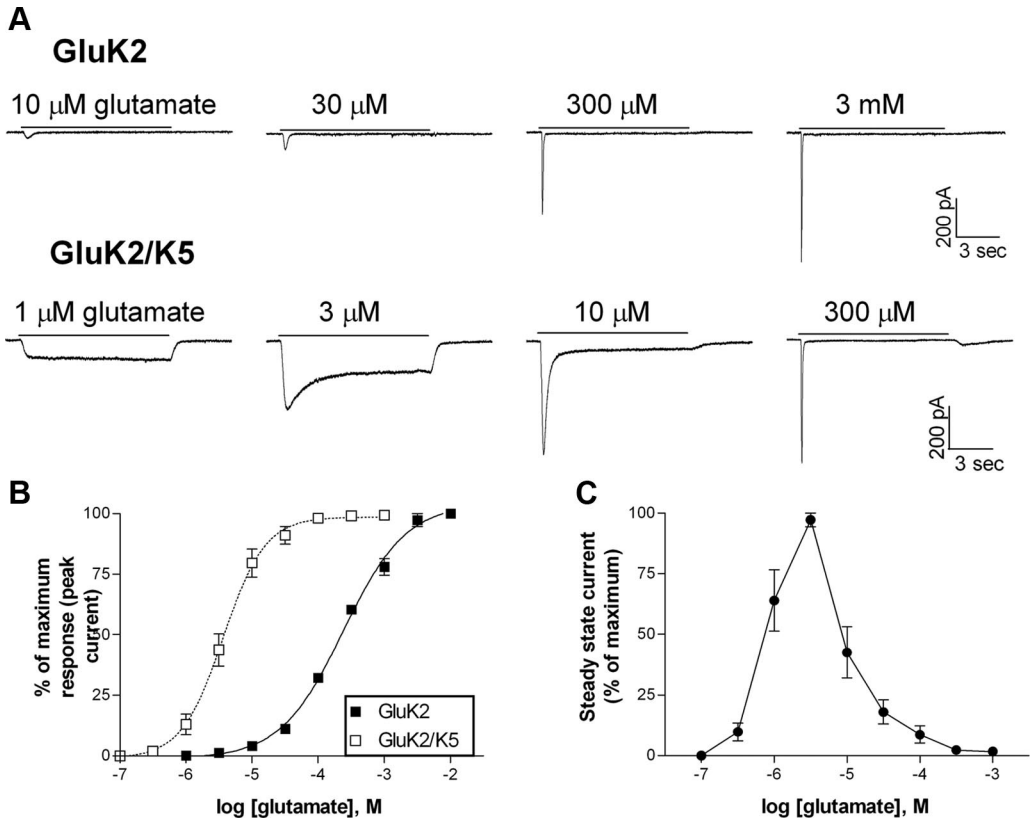


**Figure 1.** Response of recombinant GluK2 and GluK2/K5 receptors to glutamate. ***A***, Representative traces at GluK2 and GluK2/K5 receptors in response to a 10 s application of the indicated concentration of glutamate. ***B***, Peak glutamate concentration–response curves at GluK2 (filled squares; $n = 6$) and GluK2/K5 (open squares; $n = 5$) receptors. ***C***, Glutamate concentration–response relationship for steady-state current (end of 10 s application) for heteromeric GluK2/K5 receptors.

at GluK2 homomers (Fig. 1*C*). Glutamate concentrations above 3 $\mu$M produced decreasing steady-state responses attributable to increased receptor desensitization.

At high glutamate concentrations (>300 $\mu$M), GluK2/K5 receptors also exhibited an inward tail current in response to removal of glutamate (Fig. 1*A*). Solution exchange in our system (20–30 ms) is much faster than the duration of this inward tail current (>2 s), indicating that this current cannot be explained by slow solution exchange. In addition, agonist removal from GluK2 receptors never elicited this tail current. As we suggested previously (Mott et al., 2010), the presence of this tail current can be explained by the rapid unbinding of agonist from a low-affinity site responsible for channel closing, before complete dissociation from a high-affinity site responsible for channel opening. Together, these observations suggest that GluK2/K5 receptors contain a high-affinity agonist binding site responsible for channel activation and a low-affinity agonist binding site responsible for channel closure.

### A mutation that reduces glutamate binding at GluK2 does not alter agonist sensitivity of GluK2/K5 KARs

The above results are consistent with the hypothesis that glutamate binding to high-affinity GluK5 subunits is responsible for activation of GluK2/K5 heteromers without substantial desensitization, whereas subsequent binding of glutamate to low-affinity GluK2 subunits promotes channel closure through desensitization. Alternatively, an interaction between GluK2 and GluK5 subunits may substantially alter the agonist affinity and desensitization of the receptor. To examine the role of GluK2 and GluK5 subunits in the heteromer, a method was needed to increase the separation between glutamate concentrations that activate the GluK2 and GluK5 subunits. To do this, we made use of a mutation within the glutamate binding site of the GluK2 mutant that substantially reduces its agonist affinity.

The polar side chain of glutamate residue E738 in the S2 segment of the S1S2 binding pocket of GluK2 is necessary for ligand binding and agonist-induced channel opening (Mah et al., 2005). Mutation of this residue to glycine abolishes current at the mutant GluK2(E738G) receptor. In contrast, the more conservative mutation to aspartate [GluK2(E738D)] retains activity at the receptor but produces a 260-fold decrease in the apparent affinity for glutamate with little change in macroscopic desensitization characteristics (Mah et al., 2005). We confirmed that GluK2 (E738D) homomers exhibited reduced sensitivity to glutamate (Fig. 2*A*). Currents at GluK2(E738D) receptors were only detectable at glutamate concentrations in excess of 30 $\mu$M and were nonsaturating at 30 mM glutamate. The onset of desensitization was very similar between wild-type and mutated receptors, although a small steady-state current was apparent in all responses from the GluK2(E738D) receptors (Fig. 2*A*). Because the highest glutamate concentration was nonsaturating for this receptor, maximum current for each cell was estimated by constraining the fit of the concentration–response relationship to the Hill number from the fit of data from wild-type GluK2. The measured responses were then normalized to this estimated maximum to produce the data shown (Fig. 2*B*). This analysis revealed that the glutamate $EC_{50}$ for the mutant receptor was right-shifted 290-fold compared with the wild-type GluK2 receptor [GluK2(E738D), $EC_{50} = 86 \pm 32$ mM, $n = 6$; GluK2, $EC_{50} = 294 \pm 36$ $\mu$M, $n = 6$]. This compares well with the 260-fold shift previously reported

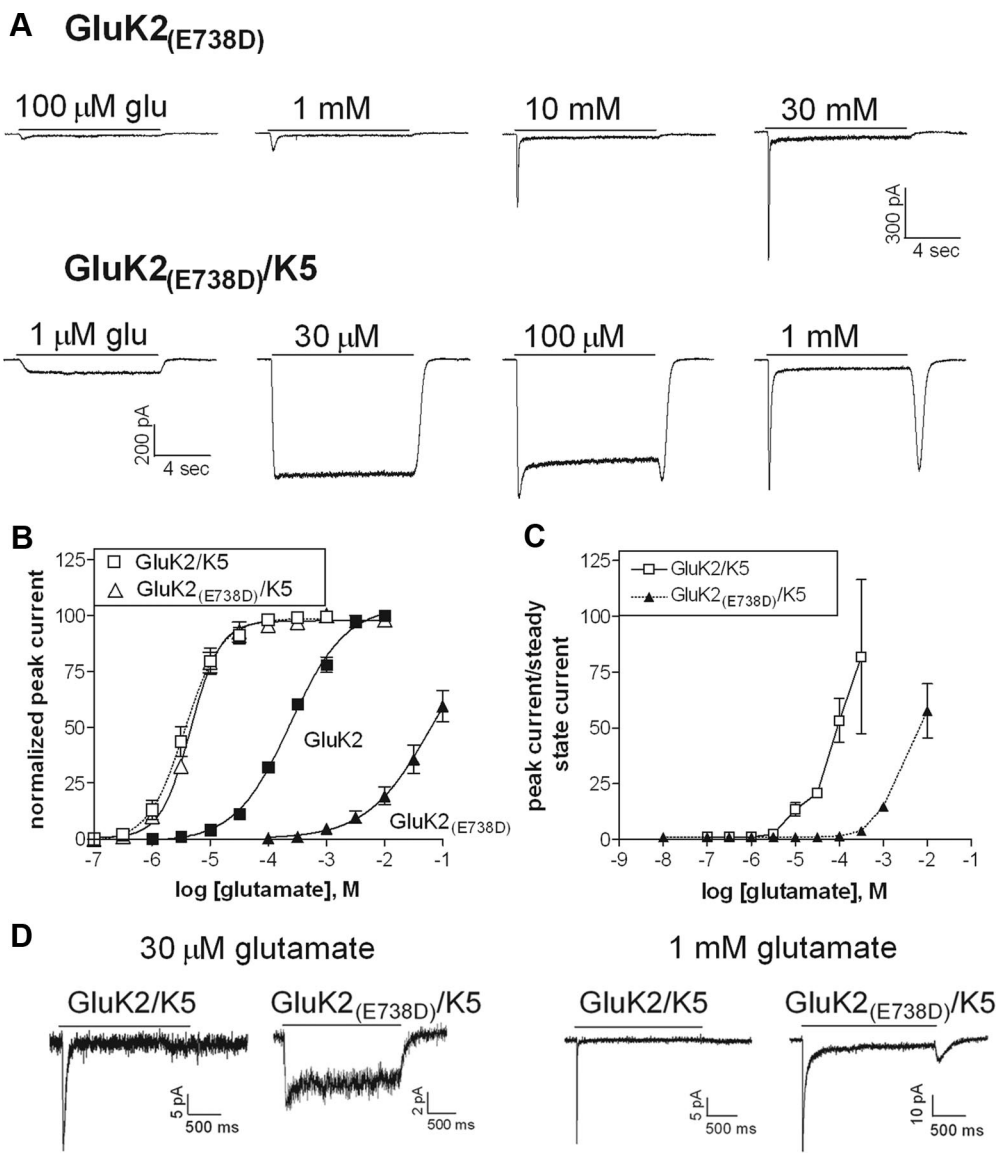


**Figure 2.** Response of recombinant GluK2(E738D) and GluK2(E738D)/K5 receptors to glutamate. ***A***, Representative traces at GluK2(E738D) and GluK2(E738D)/K5 receptors in response to a 10 s application of the indicated concentration of glutamate. ***B***, Peak glutamate concentration–response curves at GluK2(E738D) (filled triangles; $n = 6$) and GluK2(E738D)/K5 (open triangles; $n = 5$) receptors. For comparison, concentration–response curves at GluK2 (filled squares; $n = 6$) and GluK2/K5 (open squares; $n = 5$) are also shown. ***C***, Concentration–response curve of peak to steady-state current ratio for GluK2/K5 (open squares) and GluK2(E738D)/K5 (filled triangles) receptors. ***D***, Representative current evoked by a 2 s application of 30 $\mu$M or 1 mM glutamate in outside-out patches expressing GluK2/K5 or GluK2(E738D)/K5 receptors.

by Mah et al. (2005). This mutation therefore appears to substantially alter glutamate sensitivity with minor effects on desensitization.

Like wild-type GluK2/K5 receptors, heteromeric receptors containing the mutant GluK2 strongly desensitized at high (millimolar) glutamate concentrations. During removal of glutamate, substantially larger tail currents were observed for GluK2(E738D)/K5 channels compared with wild type (Fig. 2*A*). These larger tail currents could be explained if the more rapid dissociation of glutamate from the lower-affinity, mutated binding site of GluK2 enhanced the rising phase of the tail current. Despite exhibiting similar desensitization at high glutamate concentrations, desensitization of mutant heteromeric receptors at lower glutamate concentrations ($<300$ $\mu$M) was dramatically reduced (Fig. 2*A*). In particular, at concentrations of 30 $\mu$M or below, at which glutamate produced no detectable current at GluK2(E738D) homomers, glutamate produced a large and completely non-desensitizing response at GluK2(E738D)/K5 heteromeric receptors. These observations suggest that glutamate binding to GluK5 subunits alone is sufficient to produce channel activation but not desensitization. This would also predict that mutation of the GluK2 subunit, despite producing a 290-fold decrease in the glutamate sensitivity of the homomer, would have little effect on the ability of glutamate to activate the heteromeric receptor. Indeed, the concentration–response curve for peak currents at GluK2(E738D)/K5 receptors was indistinguishable from that of the wild-type heteromer [GluK2(E738D)/K5, $EC_{50} = 5 \pm 0.3$ $\mu$M, $n = 5$; GluK2/K5, $EC_{50} = 2 \pm 1$ $\mu$M, $n = 5$; Fig. 2*B*]. Thus, glutamate binding to high-affinity GluK5 subunits produced channel activation but not desensitization.

In contrast, we suggest that agonist binding to the low-affinity GluK2 subunits is not necessary to open the channel but instead causes the channel to enter a closed, desensitized state. This suggestion predicts that, although the $EC_{50}$ for channel activation

was not altered in GluK2(E738D)/K5 heteromers, an increased glutamate concentration would be necessary to produce macroscopic desensitization of these receptors. We therefore compared the effect of glutamate concentration on the peak to steady-state current ratio for mutant and wild-type heteromers. At wild-type GluK2/K5 receptors, the peak to steady-state current began to increase at glutamate concentrations above 3 $\mu$M. At GluK2 (E738D)/K5 receptors, desensitization occurred at substantially higher glutamate concentrations (>30 $\mu$M), reflecting the decrease in GluK2 agonist affinity (Fig. 2*C*). The concentration at which desensitization first appeared correlated well with the lowest concentration of glutamate able to elicit a response from the GluK2(E738D) homomers. These results suggest that low-affinity activation of GluK2 subunits terminates the current through desensitization.

The above experiments were performed using whole-cell recordings. To ensure detection of peak currents, glutamate was rapidly applied to outside-out patches excised from cells expressing GluK2/K5 and GluK2(E738D)/K5 receptors (Fig. 2*D*). Current rise times (10–90%) in response to 2 s applications of 1 mM glutamate were 830 ± 50 $\mu$s ($n = 5$) for wild-type receptors and 690 ± 20 $\mu$s ($n = 6$) for mutant receptors, in good agreement with previous reports (Barberis et al., 2008). Current rise time slowed considerably at lower glutamate concentrations [at 30 $\mu$M glutamate GluK2/K5, 12 ± 2 ms, $n = 6$; GluK2(E738D)/K5, 26 ± 4 ms, $n = 7$]. In these experiments, the rise times (10–90%) of the junction potential at the open tip of the electrode were consistently faster than 400 $\mu$s, making it likely that the rise time of the glutamate-gated current was limited by the intrinsic binding and gating properties of the receptors. Results from analysis of currents at wild-type and mutant heteromeric receptors in excised patches were similar to those from whole-cell recordings (Fig. 2*D*). High glutamate (1 mM) produced strongly desensitizing currents at both receptors [GluK2/K5 $\tau_{desens} = 6 \pm 1.0$ ms, $n = 5$; GluK2(E738D)/K5 $\tau_{desens} = 85 \pm 22$ ms, $n = 6$]. A tail current after glutamate removal from the mutant receptor was also observed in these patch recordings. A lower glutamate concentration (30 $\mu$M) produced strong and complete desensitization at wild-type receptors (GluK2/K5 $\tau_{desens} = 49 \pm 3$ ms, $n = 6$), whereas at mutant receptors, little desensitization was apparent [GluK2(E738D)/K5 $\tau_{desens} = 249 \pm 74$ ms, $n = 7$], suggesting that the GluK2 subunit was minimally activated under these conditions. The rate of desensitization onset for wild-type heteromeric GluK2/K5 receptors at both low and high glutamate concentrations was similar to previously reported values for homomeric GluK2 receptors (Heckmann et al., 1996; Perrais et al., 2009), consistent with the suggestion that desensitization is controlled primarily by the GluK2 subunit.

### Kynurenate is a competitive antagonist at GluK2 homomeric receptors

The above experiments rely on the use of a mutation that could influence functional properties of the receptor other than glutamate binding. Therefore, we also tested our hypothesis through pharmacological manipulation of the wild-type receptor. Kynurenate is a well-characterized and commonly used competitive antagonist of ionotropic glutamate receptors. It has been reported to inhibit GluK2 homomers but not GluK2/K5 heteromers (Alt et al., 2004). As a competitive antagonist, kynurenate essentially reduces the sensitivity of the receptor to glutamate and therefore could be used as a pharmacological correlate of the point mutation. Before testing the effect of Kynurenate on heteromeric receptors, we first characterized its effect on GluK2 homomeric receptors.

Kynurenate coapplication reduced the current at homomeric GluK2 receptors and the E738D mutation did not appear to alter the effect of kynurenate (Fig. 3*A*). The effectiveness of the inhibition was dependent on the agonist concentration, as expected with a competitive antagonist. Thus, in 100 $\mu$M glutamate, the $IC_{50}$ for kynurenate at the wild-type receptor was 341 ± 16 $\mu$M ($n = 3$), whereas in 1 mM glutamate, it increased to 3577 ± 580 $\mu$M ($n = 5$; Fig. 3*B*). Kynurenate produced a concentration-dependent shift in the glutamate $EC_{50}$ at both wild-type (glutamate $EC_{50} = 294 \pm 36$ $\mu$M, $n = 6$; plus 300 $\mu$M kynurenate, glutamate $EC_{50} = 544 \pm 63$ $\mu$M, $n = 4$; plus 3000 $\mu$M kynurenate, glutamate $EC_{50} = 1796 \pm 201$ $\mu$M, $n = 5$) and E738D (glutamate $EC_{50} = 86 \pm 32$ mM, $n = 6$; plus 300 $\mu$M kynurenate, glutamate $EC_{50} = 206 \pm 64$ mM, $n = 4$) mutant receptors. Comparison of the effect of 300 $\mu$M kynurenate on wild-type and E738D mutant receptors revealed that it caused a similar shift in glutamate sensitivity at both receptors [GluK2, 2.2-fold shift; GluK2(E738D), 2.6-fold shift; Fig. 3*C*]. Together, these data are consistent with the action of kynurenate as a competitive antagonist and suggest that the E738D mutation does not alter the ability of kynurenate to inhibit the GluK2 subunit.

### Kynurenate acts as a weak competitive antagonist at GluK2/K5 receptors at low, non-desensitizing glutamate concentrations

Kynurenate had very different effects at GluK2/K5 heteromeric receptors depending on the glutamate concentration. At glutamate levels that produced little desensitization, kynurenate inhibited the response (Fig. 4). When coapplied with 1 $\mu$M glutamate, the $IC_{50}$ of kynurenate was very similar for both wild-type GluK2/K5 ($IC_{50} = 943 \pm 74$ $\mu$M, $n = 4$) and GluK2(E738D)/K5 ($IC_{50} = 778 \pm 78$ $\mu$M, $n = 4$) receptors (Fig. 4*A*,*B*) and much higher than for the homomeric receptors (Fig. 3). The similarity in the kynurenate $IC_{50}$ at the wild-type GluK2/K5 and mutant GluK2(E738D)/K5 receptor is consistent with our view that the GluK2 subunit does not contribute to the current at this low level of glutamate. As shown above, the GluK2 subunit has much higher sensitivity to kynurenate inhibition. At 1 $\mu$M glutamate, the mutated GluK2 would be much less likely to be bound by agonist and contribute to the current response. Thus, any contribution by GluK2 in the wild-type heteromer should have been evident as an increased kynurenate sensitivity compared with receptors with the mutant GluK2. Unlike the wild-type receptor, the GluK2(E738D)/K5 receptor does not desensitize in response to 10 $\mu$M glutamate. Kynurenate also inhibited the current in response to 10 $\mu$M glutamate, although with lower potency (1 $\mu$M glutamate, $IC_{50} = 778 \pm 78$ $\mu$M, $n = 4$; 10 $\mu$M glutamate, $IC_{50} = 4258 \pm 245$ $\mu$M, $n = 9$), as expected for a competitive antagonist (Fig. 4*C*). These data are consistent with kynurenate acting as a competitive antagonist at GluK5 subunits with a much lower affinity at GluK5 than at GluK2.

### Kynurenate reduces desensitization at GluK2/K5 and GluK2(E738D)/K5 receptors

At glutamate levels that caused desensitization, kynurenate potentiated the response of GluK2/K5 receptors by reducing desensitization (Fig. 5*A*). In wild-type receptors, kynurenate reduced the ratio of peak to steady-state current in a concentration-dependent manner, with millimolar concentrations eliminating desensitization without substantially reducing the peak current amplitude. The same result was obtained in excised patch config-

uration (Fig. 5*B*). At GluK2/K5 receptors, kynurenate (3 mM) slowed the onset of desensitization to current evoked by rapid application of 100 μM glutamate (glutamate alone, $\tau_{desens} = 21 \pm 4$ ms; plus kynurenate, $\tau_{desens} = 259 \pm 28$ ms; $n = 5$). In whole-cell recording, kynurenate was less effective as the glutamate concentration increased (Fig. 5*A*,*D*). Thus, kynurenate reduced desensitization to 10 μM glutamate with an $IC_{50}$ of $77 \pm 22$ μM ($n = 6$), whereas it reduced desensitization to 100 μM glutamate with an $IC_{50}$ of $393 \pm 130$ μM ($n = 4$). According to our hypothesis, this behavior can be explained if kynurenate is acting primarily at the GluK2 subunit, reducing its activation and subsequent desensitization. Thus, kynurenate appears to potentiate the current because it removes the inhibitory "brake" normally provided by GluK2. In support of this interpretation, the $IC_{50}$ for kynurenate to inhibit desensitization induced by 100 μM glutamate at the GluK2/K5 receptor (393 μM; Fig. 5*D*) is similar to its $IC_{50}$ for inhibiting the response of GluK2 homomers to 100 μM glutamate (341 μM; Fig. 3*B*). The effect of kynurenate on the GluK2(E738D)/K5 receptor also supports this mechanism (Fig. 5*C*,*E*). Although kynurenate inhibited the response to 1 and 10 μM glutamate (both non-desensitizing concentrations; Fig. 4*A*,*C*) at this mutant receptor, it enhanced the response to 100 μM glutamate. The $IC_{50}$ for this potentiation ($IC_{50} = 54 \pm 3$ μM, $n = 5$) was substantially lower than for the wild-type receptor ($393 \pm 130$ μM, $n = 4$) at the same glutamate concentration (Fig. 5*E*). This would be expected if kynurenate were acting preferentially at the GluK2 subunit, because 100 μM glutamate represents a much lower effective concentration at the mutated subunit compared with the wild type and therefore would be less able to compete with the antagonist for the binding site.

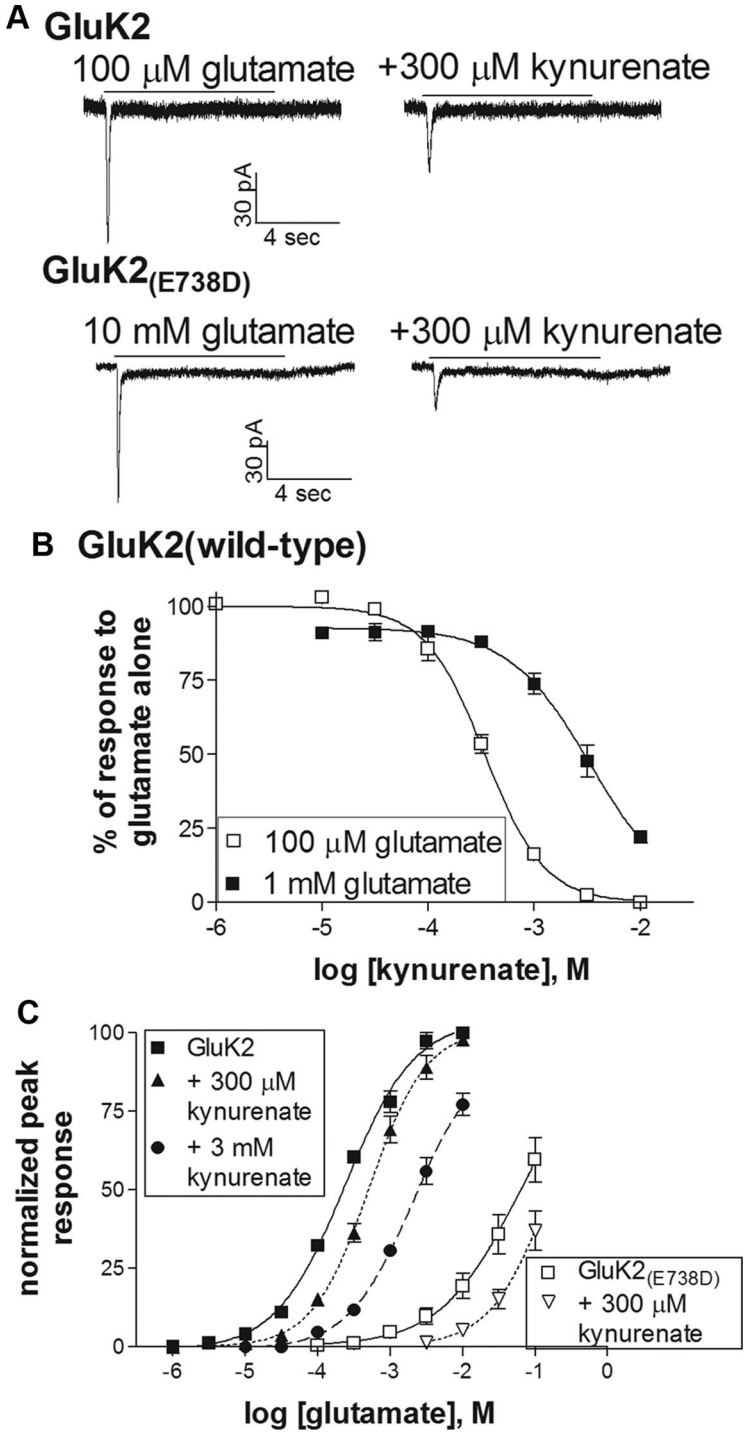


**Figure 3.** Response of GluK2 homomers to kynurenate. ***A***, Representative traces at GluK2 and GluK2(E738D) receptors in response to the indicated concentration of glutamate in the absence or presence of 300 μM kynurenate. ***B***, Concentration–response curve for kynurenate at GluK2 receptors activated by 100 μM glutamate (open squares; $n = 3$) or 1 mM glutamate (filled squares; $n = 5$). ***C***, Concentration–response curves to glutamate at GluK2 (filled symbols) and GluK2(E738D) (open symbols) receptors in the absence and presence of the indicated concentration of kynurenate.

**Kynurenate reduces desensitization at GluK1/K5 and GluK3/K5 receptors**

In addition to GluK2, GluK5 also assembles with GluK1 and GluK3 to form functional receptors. We tested kynurenate on these heteromeric receptors to determine whether the role played by GluK5 subunits was unique to the GluK2/K5 receptor. Kynurenate (3 mM) blocked current produced by 100 μM glutamate at GluK1 homomers ($95 \pm 2\%$ blockade, $n = 4$), as expected (Alt et al., 2004). However, at GluK1/K5 receptors, kynurenate (3 mM) potentiated the steady-state response to 3 and 10 μM glutamate and reduced the peak to steady-state current ratio (Fig. 6). At GluK1/K5 heteromers, low concentrations of glutamate (3 and 10 μM) produced more complete desensitization than we observed at GluK2/GluK5 receptors, consistent with the reported higher glutamate sensitivity of the GluK1 subunit (Heckmann et al., 1996; Fletcher and Lodge, 1996). Furthermore, compared with GluK2/K5 receptors, kynurenate (3 mM) was less effective in slowing the onset of desensitization of GluK1/K5 heteromers, completely eliminating desensitization at only the lowest glutamate concentration (3 μM) tested. Because GluK1 has a higher affinity for glutamate

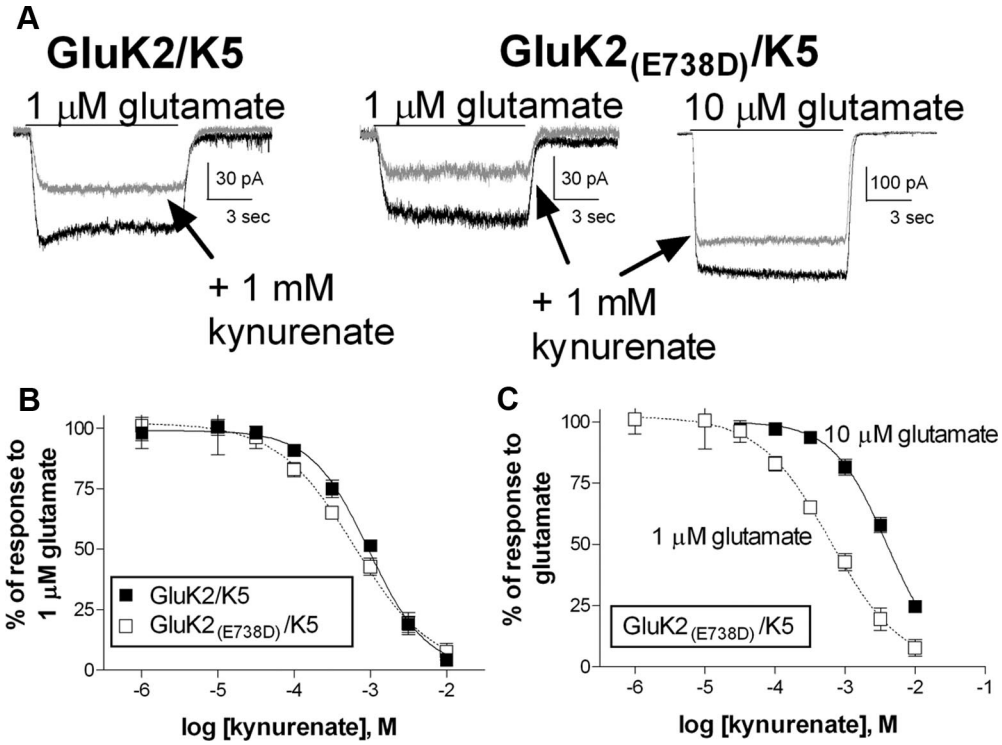


**Figure 4.** Inhibition of GluK2/K5 receptors by kynurenate at low glutamate concentrations. ***A***, Representative traces at GluK2/K5 and GluK2(E738D)/K5 receptors in response to the indicated concentration of glutamate in the absence or presence of 1 mM kynurenate. ***B***, Concentration–response curve for kynurenate at GluK2/K5 (filled squares; $n = 4$) and GluK2(E738D)/K5 (open squares; $n = 4$) receptors. ***C***, Concentration–response curve for kynurenate at GluK2(E738D)/K5 receptors activated by 1 $\mu$M glutamate (open squares; $n = 4$) or 10 $\mu$M glutamate (filled squares; $n = 9$).

than GluK2, this behavior can be explained by the reduced ability of kynurenate to effectively compete with the higher-affinity glutamate site of the GluK1 subunit and to subsequently block its activation and desensitization.

At GluK3/K5 receptors kynurenate strongly potentiated both the peak and steady-state currents to 10 and 100 $\mu$M glutamate in a concentration-dependent manner. GluK3 homomeric receptors have a low sensitivity to glutamate, desensitize rapidly, and are activated only by fast transients of high glutamate concentration (Perrais et al., 2009). Accordingly, we suggest that the strong potentiation of GluK3/K5 receptors by kynurenate reflected the removal of this desensitization by blockade of the GluK3 subunit, revealing the non-desensitizing current produced by activation of GluK5 subunits. A large tail current was observed after glutamate removal as would be expected with a large difference in glutamate sensitivity between the GluK3 and GluK5 subunits. The similar behavior of each of these heteromeric receptors in response to glutamate and kynurenate suggests that analogous roles are played by GluK5 and GluK1–GluK3 subunits in each receptor.

## Discussion

This study shows that the assembly of GluK5 and GluK1–GluK3 subunits creates a heteromeric receptor with two types of agonist binding sites: a high apparent affinity, non-desensitizing site and a low apparent affinity, strongly desensitizing site. Agonist occupancy of the high-affinity site is sufficient for channel activation but does not produce desensitization, whereas binding to the lower-affinity site rapidly leads to closure of the open channel through desensitization. With the native agonist glutamate, the high and low apparent affinity sites can be localized to the GluK5 and GluK1–GluK3 subunits, respectively. These results support the hypothesis that individual subunits can be activated within the heteromeric receptor and that agonist action at GluK2/K5 receptors is dependent on the identity of the activated subunits. Our findings demonstrate that GluK2 and GluK5 subunits do not merely differ quantitatively in agonist binding properties but also serve qualitatively different roles in channel gating.

### Activation of heteromeric KARs by glutamate

The ability of GluK5 subunits to independently activate the channel is supported by several observations. For example, we found that the E738D mutation in GluK2, which dramatically lowered the sensitivity of this subunit for glutamate, did not affect the glutamate sensitivity of heteromeric KARs assembled from wild-type GluK5 and mutant GluK2. Furthermore, glutamate at concentrations too low to activate GluK2 homomers produced non-desensitizing currents at both GluK2/K5 and GluK2(E738D)/K5 heteromers. Kynurenate is a competitive antagonist with substantially higher affinity for GluK2 than GluK5 subunits. At a concentration that blocked current at GluK2 homomers, kynurenate produced little inhibition of peak current and removed desensitization from GluK2/K5 receptors. These results are consistent with our model for heteromeric KAR gating in which high-affinity binding of glutamate to GluK5 subunits is responsible for channel activation (Mott et al., 2010). Furthermore, under our conditions, glutamate acting at GluK5 appeared able to fully activate the GluK2/K5 receptor channel, consistent with the suggestion that GluK2 activation contributes minimally to the channel conductance. However, single-channel recordings are required to resolve this issue.

In the present study, we found that channel activation by GluK5 occurred regardless of the identity of the GluK1–GluK3 subunit partner. The ability of the GluK5 subunit to gate current agrees with results of Swanson et al. (2002) who reported a GluK5-mediated conductance in GluK1/K5 heteromers in re-

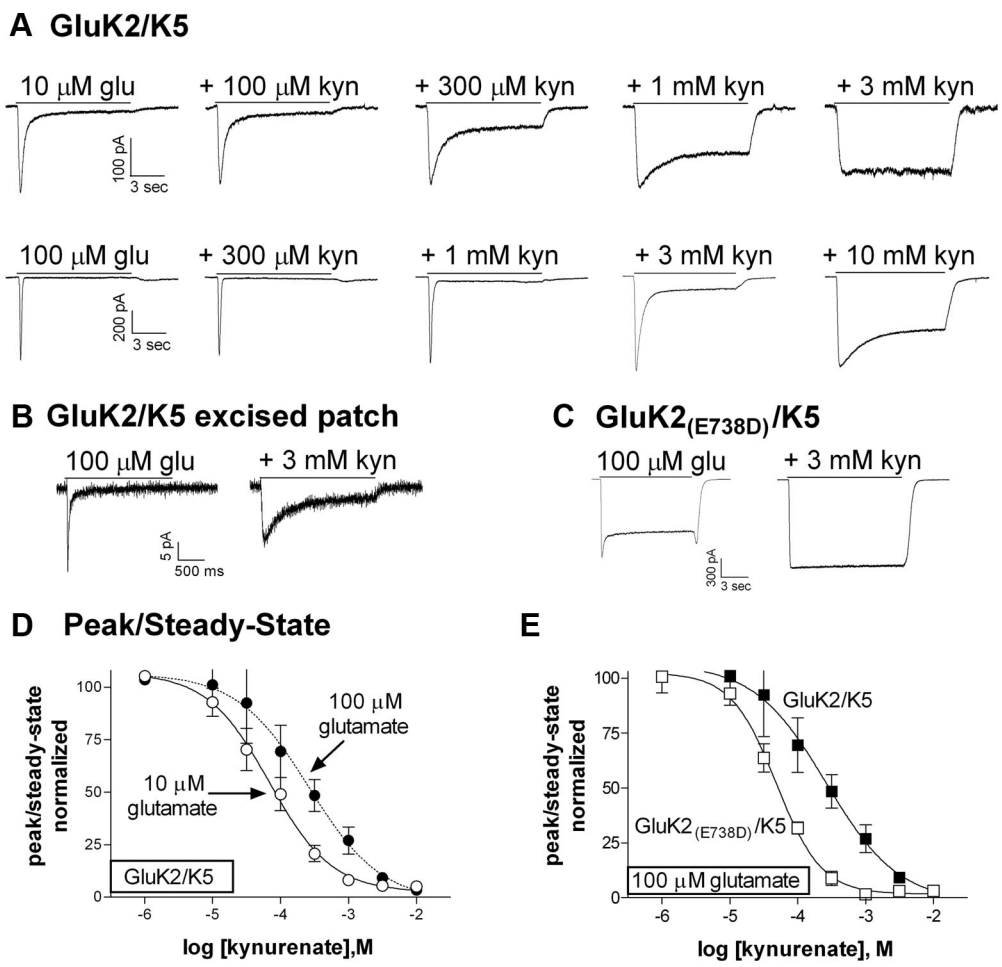


**Figure 5.** Potentiation of current at GluK2/K5 receptors by kynurenate at high glutamate concentrations. ***A***, Representative traces at GluK2/K5 receptors in response to 10 or 100 $\mu$M glutamate in the presence of the indicated concentration of kynurenate. ***B***, Representative current evoked by a 2 s application of 100 $\mu$M glutamate in the absence and presence of 3 mM kynurenate on outside-out patches expressing GluK2/K5 receptors. ***C***, Representative trace of GluK2(E738D)/K5 receptors in response to 100 $\mu$M glutamate in the absence and presence of 3 mM kynurenate. ***D***, Kynurenate concentration–response curve of peak to steady-state current ratio for GluK2/K5 receptors activated by 10 $\mu$M (open circles; $n = 6$) or 100 $\mu$M (filled circles; $n = 4$) glutamate. ***E***, Kynurenate concentration–response curve of peak to steady-state current ratio for GluK2/K5 (filled squares; $n = 4$) and GluK2(E738D)/K5 (open squares; $n = 5$) receptors activated by 100 $\mu$M glutamate.

sponse to CNQX, when dysiherbaine, a KAR agonist, was bound to GluK1 subunits. Similarly, we have reported previously a model of activation and desensitization behavior of GluK2/K4 receptors based on slow application of agonists to *Xenopus* oocytes (Mott et al., 2010). This model distinguished a high-affinity glutamate binding site on the GluK4 subunit responsible for channel activation and a lower-affinity binding site on the GluK2 subunit responsible for receptor desensitization. Together with the results of the present study, these findings suggest that the presence of high- and low-affinity binding sites responsible for channel activation and desensitization can be generalized to other heteromeric KARs, regardless of the identity of the GluK4–GluK5 or GluK1–GluK3 subunit.

**Desensitization of heteromeric KARs by glutamate**

Our data suggest that desensitization at GluK2/K5 receptors occurs only at glutamate concentrations sufficient to activate GluK2 subunits. Any alteration in glutamate sensitivity of the GluK2 subunit should therefore alter the onset of desensitization. Accordingly, we found that the E738D mutation in GluK2 produced a parallel rightward shift both in the concentration of glutamate necessary to activate the homomeric receptor and the concentration necessary to produce desensitization of the GluK2(E738D)/K5 receptors. In addition, blockade of GluK2 subunits with kynurenate removed desensitization from both GluK2/K5 and GluK2(E738D)/K5 receptors. These findings strongly support the idea that desensitization results from glutamate occupancy of the GluK2 agonist binding site.

At homomeric GluK2 receptors, occupancy of two subunits is sufficient for channel activation. However, homomeric KARs are desensitized by concentrations of glutamate below those required for activation, consistent with the idea that glutamate occupancy of a single subunit is sufficient for desensitization (Heckmann et al., 1996; Paternain et al., 1998). GluK5-containing receptors display strikingly different behavior. Because of the higher glutamate affinity of GluK5 than GluK2 subunits (Egebjerg et al., 1991; Herb et al., 1992), low concentrations of glutamate bind GluK5 subunits to activate the receptor without desensitization. Desensitization does not occur until lower-affinity GluK2 subunits are activated. These results suggest that, unlike GluK2 homomers, GluK2/K5 heteromers do not desensitize without activation.

At least two models of channel behavior could explain these findings. First, GluK2 subunits could act independently to produce desensitization. This suggestion is supported by several lines of evidence showing similarity in desensitization properties of GluK2 homomers and GluK2/K5 heteromers. For example, the

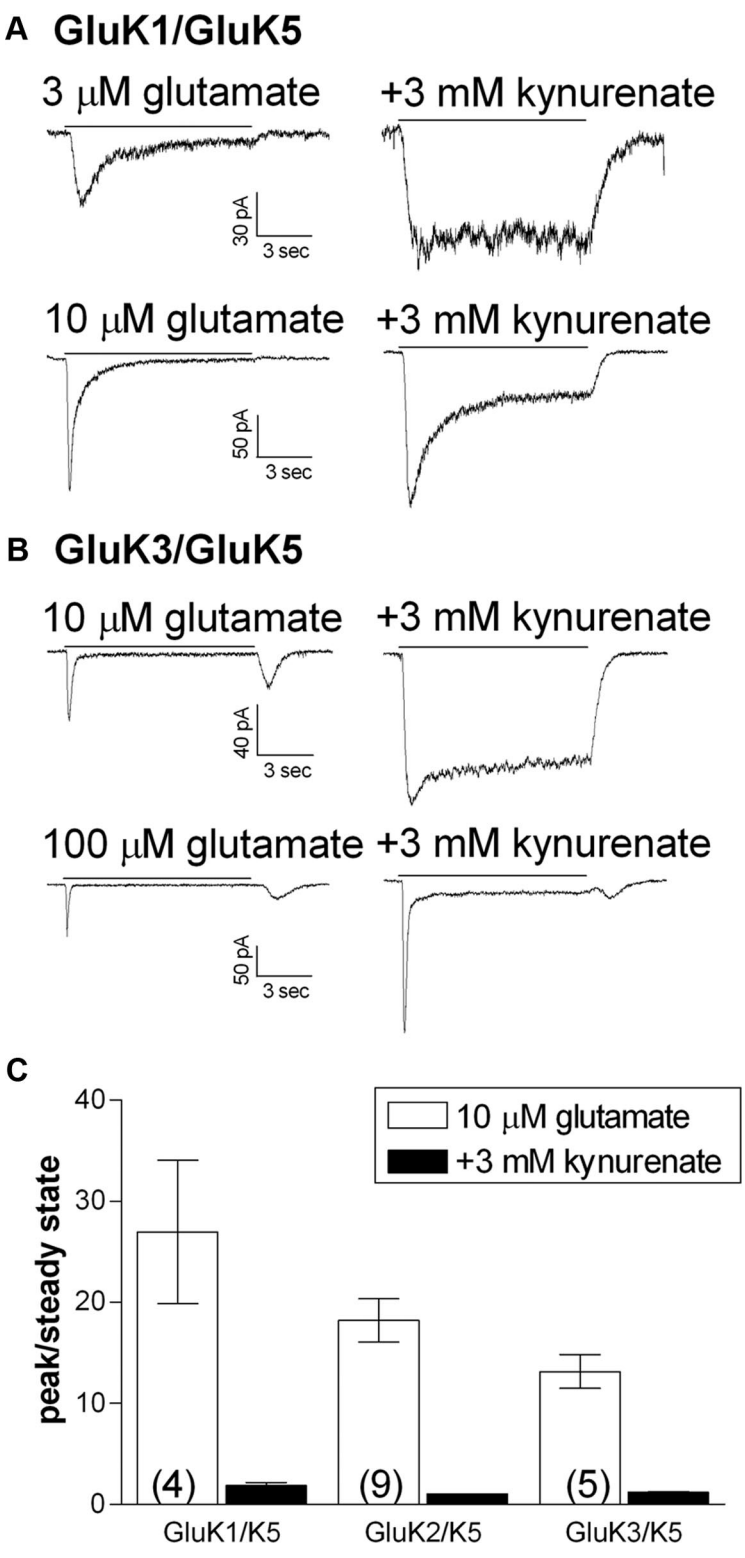


**Figure 6.** Potentiation of current at GluK1/K5 and GluK3/K5 receptors by kynurenate at high glutamate concentrations. ***A***, ***B***, Representative traces at GluK1/K5 (***A***) and GluK3/K5 (***B***) receptors in response to glutamate in the presence of the indicated concentration of kynurenate. ***C***, Average peak to steady-state current ratio for each indicated receptor in the absence (open bars) and presence (filled bars) of 3 mM kynurenate. At each receptor, kynurenate potentiated the steady-state current, thereby reducing the peak to steady-state current ratio. Currents were evoked by 10 $\mu$M glutamate. For each receptor, the number of cells tested is indicated in parentheses.

rate of onset of desensitization for heteromeric GluK2/K5 receptors is similar to reported values for homomeric GluK2 receptors (Heckmann et al., 1996; Perrais et al., 2009), suggesting that GluK2 subunits desensitize at a similar rate regardless of their subunit partner. Likewise, the concentration dependence of desensitization for GluK2/K5 heteromers is similar to that reported for GluK2 homomers. Thus, increasing glutamate from 30 $\mu$M to 1 mM produces an approximately ninefold decrease in the time constant for desensitization at both GluK2/K5 heteromers (present study) and GluK2 homomers (Heckmann et al., 1996). Finally, recovery from glutamate-induced desensitization is similar for GluK2 (Heckmann et al., 1996) and GluK2/K5 (Barberis et al., 2008) receptors. These similarities in desensitization of GluK2 homomers and GluK2/K5 heteromers support the idea that GluK2 subunits behave independently with respect to agonist binding and receptor desensitization.

Alternately, GluK2 subunits could act in a concerted manner with GluK5 subunits to produce desensitization. Like AMPA receptors, KARs have been proposed to assemble as dimers of dimers, with each dimer containing two agonist binding sites (Das et al., 2010). Desensitization is thought to occur through conformational change at the dimer interface, which uncouples the ligand binding domain from the channel gate (Sun et al., 2002; Zhang et al., 2006). At AMPA receptors and KAR homomers, agonist binding to a single subunit in one of the two dimers is sufficient for desensitization (Heckmann et al., 1996; Robert and Howe, 2003). However, our data suggest that, at GluK2/K5 receptors, desensitization does not occur until agonist occupies both binding sites in at least one of the GluK2/K5 dimers. Thus, GluK5 occupancy can produce activation but is not sufficient for desensitization. This model gains support from the observation that glutamate binding to GluK2 subunits can desensitize current produced by agonist occupancy of GluK5 subunits, indicating a concerted conformational change of receptor subunits. The above listed similarities in desensitization kinetics of GluK2 homomers and GluK2/K5 heteromers could be explained if conformational change at the dimer interface was rate limited by the GluK2 subunit. Studies with dysiherbaine at GluK1/K5 receptors support this hypothesis. Unlike glutamate, dysiherbaine binds GluK1 subunits with much higher affinity than GluK5 subunits (Swanson et al., 2002). Removal of dysiherbaine from GluK1/K5 receptors reveals a long-lasting tail current attributed to persistent occupancy of GluK1 subunits. Subsequent application of glutamate binds to GluK5 subunits within the heteromer and strongly desensitizes this tail current. These observations support the hypothesis that

desensitization at GluK5-containing heteromers requires agonist occupancy of both binding sites in the dimer. Interestingly, dysiherbaine (binding at GluK1; Swanson et al., 2002) and glutamate (binding at GluK5; present study) produce steady-state current by acting at different subunits within the heteromer. Together, these observations suggest that, at GluK5-containing receptors, occupancy of a single binding site in the heteromeric dimer produces a non-desensitizing current, regardless of the identity of that subunit, and that desensitization does not occur until both binding sites in the dimer are occupied. Additional experiments using low-affinity GluK5 mutants and selective agonists are needed to distinguish these possibilities.

### Functional significance of heteromeric KARs

The slow decay of synaptic KARs contributes to synaptic integration at glutamatergic synapses (Frerking and Ohliger-Frerking, 2002). Recent studies have shown that GluK5 subunits are required for proper function of synaptic KARs and contribute to their slow kinetics (Contractor et al., 2003; Barberis et al., 2008; Fernandes et al., 2009). Our data are consistent with these findings and show that, rather than being modulatory, GluK5 subunits play a central role in gating current at heteromeric receptors. In addition, our data suggest that, at low glutamate concentrations, GluK2/K5 receptors could produce a tonic depolarizing current mediated by activation of the high-affinity, non-desensitizing GluK5 subunit. GluK5-containing KARs are present extrasynaptically and on axons (Contractor et al., 2003). These extrasynaptic KARs could produce GluK5-mediated steady-state current when exposed to low glutamate concentrations, as may occur during glutamate overspill. Ambient glutamate in the hippocampus has been estimated at 25 nM (Herman and Jahr, 2007), below the threshold concentration of glutamate required to activate heteromeric receptors (100–300 nM; Fig. 1). However, extracellular glutamate concentrations can rise by orders of magnitude during seizures and ischemic stroke (Timmerman and Westerink, 1997; Nyitrai et al., 2006). Emergent tonic current mediated by GluK5-containing KARs may contribute to hyperexcitability and excitotoxicity underlying these disorders (Benveniste et al., 2010). Receptor desensitization mediated by GluK2 subunits would be a critical regulator of this tonic depolarizing current.

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