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Physiological significance of high and low affinity agonist binding to neuronal and recombinant AMPA receptors

Markus Kessler, Erika Suzuki, Kyle Montgomery, and Amy C. Arai

Department of Pharmacology, Southern Illinois University School of Medicine, Springfield IL 62702, USA

Abstract

Radioligand binding studies have shown that AMPA receptors exist in two variants that differ about twentyfold in their binding affinities, with brain receptors being mainly of the low-affinity type and recombinantly expressed receptors having almost exclusively high affinity. However, the physiological correlate of high and low-affinity binding is not yet known. In this study we examined if physiological experiments similarly reveal evidence for two distinct receptor variants. We therefore measured equilibrium desensitization by glutamate and determined IC₅₀ values for neuronal receptors and for the homomeric receptors GluR1 to GluR4 expressed in HEK293 cells. Contrary to the prediction that these IC₅₀ values exhibit large differences commensurate with those of high and low-affinity binding, values for homomeric receptors (1–18 μ M) were on average not different from those of neuronal receptors (3–10 μ M). Moreover, simulations with kinetic receptor models suggest that the IC₅₀ values for neuronal and recombinant receptors correspond to the binding affinity of the low-affinity receptor variant. These findings indicate that the high-affinity binding measured in heterologous expression systems represents an immature receptor variant that does not contribute to the currents recorded from these cells, and that the functional low-affinity receptors are present in such small number that they are effectively masked in binding assays by the high-affinity receptors. Thus, in order to compare experimentally determined saturation binding profiles with those predicted by kinetic receptor models and with dose-response curves from physiological studies, it will be imperative to develop methods for isolating first the low-affinity receptors.

Keywords

GluR2; GluR3; steady-state desensitization; excised patch recordings; deactivation time constants; stargazin

Binding techniques have made important contributions to characterize the properties of AMPA receptors. They have been instrumental in establishing pharmacological profiles, in assessing regional variations in receptor density, and in searching for changes in receptor expression in various disorders. However, binding studies have also uncovered numerous experimental conditions that change the apparent affinity for agonists. For example, [³H]AMPA binding to brain membranes is altered by treatment with phospholipases (Massicotte et al., 1990; Menard et al., 2005), phosphatidylserine (Baudry et al., 1991), mercurials (Terramani et al., 1988) and

Corresponding author: Markus Kessler, SIU School of Medicine, 801 N. Rutledge St., POB 19629, Springfield IL 62794-9629, (217) 545-2204, (217) 545-0145 FAX, mkessler@siu.edu.

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sialidase (Hoffman et al., 1997). Agonist binding is also influenced by positive and negative modulators (Arai et al., 1996, 2000, 2002; Kessler et al., 1996; Kessler and Arai, 2006), thiocyanate (Hall et al., 1993) and purinergic antagonists like suramin and PPNDs (Suzuki et al., 2004). These changes in binding affinity may reflect modifications in diverse aspects of receptor kinetics. For example, binding modulation by positive allosteric modulators is to some extent correlated with changes in response deactivation (Kessler and Arai, 2006), and thiocyanate increases binding affinity probably by enhancing receptor desensitization (Bowie and Smart, 1993; Arai et al., 1995).

Further explorations of how receptor function is altered by biochemical processes and pharmacological agents will depend in part on molecular engineering and expression in heterologous cells. This, however, raises the problem that binding to recombinant receptors differs drastically from binding to neuronal AMPA receptors. For example, most brain receptors have binding affinities for AMPA of 200–1000 nM (Olsen et al. 1987; Honore and Drejer, 1988; Hall et al., 1992, Kessler and Arai, 2006) while the homomeric receptors GluR1–4, as well as heteromeric combinations of them, exhibit affinities of 5–50 nM (Keinanen et al., 1990; Andersen et al., 1996; Hennegriff et al., 1997; Nielsen et al., 1998; Nishimura et al., 2000). It should be noted, though, that brain AMPA receptors also exhibit a minor component of ‘high affinity binding’ with a K_D similar to that of recombinant receptors. Another important observation in this context has been that high- and low-affinity components in brain membranes can be partially segregated and thus represent separate receptor subpopulations. Moreover, high-affinity sites are enriched in intracellular membranes and low-affinity sites in synapses, suggesting that neuronal receptors at some point undergo a conversion to the low affinity state (Hall et al., 1992; Standley et al., 1994). However, where this conversion occurs is not yet understood.

The observation that recombinantly expressed receptors resemble high-affinity brain receptors thus suggests that the recombinant receptors detected in binding assays did not convert to the ‘mature’ state which is characteristic of synaptic receptors. There are two alternative hypotheses to interpret these findings. One is that recombinant receptors in heterologous cells generally fail to convert to low affinity, perhaps because of the absence of specific neuronal factors, and hence that *all receptors* in these cells are of the *high-affinity* variant. If this explanation is correct then we expect that recombinant and neuronal receptors also exhibit differences in at least some of their physiological properties that are as large as those in binding assays. The second possibility is that recombinant receptors do in fact undergo conversion to a state of low binding affinity, like in neurons, but that low-affinity receptors remain insignificant in number and hidden underneath high-affinity binding. Because receptors sampled in physiological recordings presumably would be of this low-affinity type, we would expect that their physiological properties do *not* differ to any major degree from those of neuronal receptors, at least not in a way that would reflect the difference between high and low-affinity binding. In order to distinguish between these two hypotheses we compared the physiological properties of the homomeric receptors GluR1–4 with those of AMPA receptors in patches from hippocampal neurons. Moreover, because binding is measured in an equilibrium state, we surmised that differences between recombinant and neuronal receptors would be most salient for physiological parameters related to steady-state currents, and we therefore focused on the IC_{50} constant for equilibrium desensitization.

EXPERIMENTAL PROCEDURES

Cell culture

GluR1o (flop), GluR2o, GluR2i (flip), GluR3o, GluR3i, and GluR4i receptors were stably expressed in HEK293 cell lines (Hennegriff et al., 1997; cell lines for GluR3i and GluR4i were provided by Drs. Choi and Turetsky, St. Louis). Cells expressing GluR1i, GluR2i(Q), and

GluR2o(Q) were generated by transient transfection using lipofectamine (Invitrogen) and a mixture of the plasmids for the receptor and for GFP (2 μ g: 0.2 μ g per 35 mm culture dish). Cells were cultured in MEM (Invitrogen) plus 10% new born calf serum and 100 μ M DNQX. One day before the experiment, the HEK293 cells were transferred to a 12 mm glass coverslip coated with poly-D-lysine (0.1 mg/ml). Recordings were performed 48–72 hr after transfection. For binding, HEK293 cells were transfected in a 10 cm culture dish using 7–15 μ g DNA (GluR1i, GluR4o) and harvested after 50–70 hours.

Electrophysiology

Electrophysiological recordings were carried out in HEK293 cells with whole-cell configuration (GluR3o) or in excised outside-out patches (all other subunits). Recordings were made with glass pipettes (2–5 MOhm) containing (in mM): CsF 130, EGTA-K 10, ATP-Mg 2 and HEPES 10 (pH 7.3). The external solution contained (in mM): NaCl 140, KCl 3, CaCl₂ 2, MgCl₂ 1, glucose 5 and HEPES 10 (pH 7.3). Glutamate was applied with an ultrafast solution application system using a piezo-translator, as described in more detail elsewhere (Arai et al., 1996). In this system, two solution streams flow continuously from a double-barrel pipette with a theta (θ) shaped cross-section, one carrying the background medium and the second one containing 10 mM L-glutamate. After a patch is excised, it is positioned in the background stream; glutamate pulses of 1 or 100 ms duration are then applied by moving the double-barrel pipette such that the patch becomes transiently exposed to the 10 mM glutamate stream. Based on measurement of open tip junction current, the solution exchange time (20–80%) with this system is 0.15 ms. For whole-cell recording, cells were lifted from the support glass after establishing whole-cell recording and glutamate was applied as described. Occasional experiments with one-millisecond pulses showing a 10–90% rise time slower than 1 ms and a peak amplitude smaller than that induced by a 100 ms-pulse were excluded from analysis. All recordings were carried out at room temperature (24 $\pm$ 1°C). The holding potential was –70 mV unless mentioned otherwise. For equilibrium desensitization experiments, various concentrations of glutamate (0.1–300 μ M) were included in the background stream, and the peak amplitude in the presence and absence of background ligand were compared. Measurements with a given patch were alternated repeatedly between control and various test conditions. Recordings were made with an Axopatch 1D amplifier (Axon Instruments) at a filter frequency of 5 kHz and digitized at 10 kHz with Digidata 1322A/pClamp 8 (Axon Instruments). Typically five responses with each condition were collected and averaged. For recordings from neuronal receptors, membrane patches were excised from the stratum pyramidale of field CA1 of hippocampal slice cultures. These slice cultures were prepared from PND14 Sprague-Dawley rats as described elsewhere (Arai et al., 2000). The animals were anesthetized before decapitation according to an institutionally approved animal use protocol. The slice cultures were maintained on Millicell inserts for several weeks. Recordings were made with a borosilicate pipette (3–8 MOhm) filled with a solution containing (in mM): CsF 65, CsCl 65, EGTA 10, MgCl₂ 2, ATP 2, and HEPES 10 (pH 7.3). The extracellular recording solution contained 130 mM NaCl, 3.5 mM KCl, 20 mM HEPES, 10 μ M MK-801 and 50 μ M D-AP5. Experiments were carried out at 24°C; holding potential –50 mV.

Binding to GluR1-4

HEK293 cells were collected into Tris-buffered saline (150 mM NaCl, 10 mM Tris/HCl, pH 7.4), spun down (2,000g for 10 min) and washed at least four times; after the first centrifugation, 0.1% saponin was added to permeabilize the membranes. For binding, the cells were suspended in the ‘external solution’ described above under ‘Electrophysiology’ or in HEPES-buffered saline (HBS; 150 mM NaCl, 20 mM HEPES, pH 7.4). Binding assays were mostly conducted with the filtration method. Aliquots of the cell suspension (5–50 μ g protein in 50 μ l) were placed in polystyrene tubes and incubated with radioligand and additions for 5–8 min at 24°C, or for 20–45 min at 0°C. In assays with [³H]AMPA, 50 mM potassium thiocyanate was

usually added to the incubation. Samples were then filtered through GF/A or GF/C filters after dilution in 5 ml of ice-cold wash buffer (100 mM NaCl, 10 mM Tris, 50 mM KSCN, pH 7.4), and the filters were rinsed with 10 ml wash buffer. Non-specific binding determined with 5 mM glutamate was subtracted from total binding and was usually between 3 and 30% of total binding. Protein was determined with the Bradford assay using bovine serum albumin as standard. Binding data were fitted with linear and non-linear regression using Graphpad Prism.

Binding to rat brain receptors

Brain membranes (lyzed and washed P2 fraction) were prepared from cortex and hippocampus of 6–10 week old Sprague-Dawley rats using conventional methods (Kessler and Arai, 2006) and suspended in HBS or the 'external patch solution' for the binding assays. Binding tests were usually conducted with the centrifugation method. Aliquots of the membrane suspension (20–60 µg protein) were incubated for 60 min at 0–4°C or 24°C with radioligand and additions and then spun for 15 min at 25,000g. The supernatant was then aspirated and the pellet quickly rinsed with ice-cold wash buffer. The pellets were dissolved in 20 µl tissue solubilizer BTS-450 (Beckman, Fullerton CA) before addition of scintillation fluid. To prepare solubilized receptors, brain membranes were treated with 3% octylglucoside, centrifuged, and dialyzed for 30 hours with three changes against HBS plus 1% octylglucoside. Binding assays were done with filtration through GF/B glass fiber filters soaked in 0.05% polyethylene-imine.

Materials

[³H]Fluorowillardiine (36 Ci/mmol) was obtained from Tocris Cookson (St. Louis, MO), [³H]S-AMPA (56 Ci/mmol; discontinued), [³H]RS-AMPA (46 Ci/mmol), and [³H]CNQX (26 Ci/mmol; discontinued) were from NEN/Perkin Elmer, Boston, MA). Unlabeled ligands were obtained from Tocris Cookson (St. Louis, MO, USA). Other reagents were obtained from Sigma (St. Louis, MO, USA) or Fisher (Pittsburgh, PA, USA).

RESULTS

[³H]AMPA binding to neuronal and recombinant AMPA receptors

To obtain favorable signal/background ratios, [³H]AMPA binding is usually measured at 0°C and with the chaotropic ion thiocyanate which enhances agonist affinity about ten-fold (Honore and Nielsen, 1985; Hall et al., 1993). Figure 1 shows binding saturation data for [³H]S-AMPA under these conditions. Binding to receptors in brain membranes exhibits a high degree of curvature in Scatchard plots and two-site fits provide affinity estimates of 10 and 380 nM, with >90% of binding being of the low affinity type. Similar results were obtained with [³H]fluorowillardiine as ligand (Hawkins et al., 1995; Kessler and Arai, 2006). In contrast, binding to recombinant receptors exhibits high affinity with binding constants in the low nanomolar range. Five of the subunits, including all flop variants, exhibited strict linearity in Scatchard plots with K_D 's between 4 and 30 nM (table 1). GluR2i and 4i, and to a minor extent GluR1i, showed concave curvature, indicating either negative cooperativity between subunits or else the presence of two receptor populations with high and low affinity. According to the latter interpretation, the sites would have K_D 's of 2.2 and 34 nM for GluR2i, and 7 and 115 nM for GluR4i. Similar results were obtained with [³H]fluorowillardiine (Kessler and Arai, 2006). Also, earlier studies showed that binding affinities of recombinant *heteromeric* receptors of various subunit combinations are in the same range as those of the homomeric receptors (Nielsen et al., 1998; Nishimura et al., 2000). Taken together, these data indicate that the affinity of the predominant component of brain receptors is 4–100 times lower than the affinities obtained for recombinant receptors.

It has recently become clear that AMPA receptors in the brain are constitutively associated with auxiliary proteins of the TARP family, such as stargazin (Tomita et al., 2005). These

proteins control the trafficking of the receptor from the ER to the synapse, but they also alter receptor kinetics, for instance by slowing rates of deactivation and desensitization. We therefore examined if coexpression with a TARP might convert recombinant receptors to low affinity. As shown in figure 1, this appears not to be the case as the binding affinities were not significantly altered by stargazin. Similar results were obtained with other TARPs ($\gamma 3$, $\gamma 4$, $\gamma 8$; not shown).

Similarly large disparities between brain and recombinant receptors were obtained in affinity estimates for glutamate (figure 2). Because binding constants for glutamate will be compared also with physiological equilibrium constants, the assays were done at 24°C and in the medium used for physiological recordings. Assays with radiolabeled glutamate are not feasible because affinity is low and thus K_D values were determined from the displacement of the higher-affinity ligands [^3H]CNQX and [^3H]FW. As shown in figure 2 (bottom), displacement of [^3H]CNQX binding by glutamate yielded K_D 's of 33 μM and 1.7 μM , the latter accounting for about 9% of binding and presumably representing high-affinity receptors. Indeed, binding to detergent solubilized brain receptors - which exhibit only high-affinity binding (Hall et al., 1992; Kessler and Arai, 2006) - gave a K_D of 0.8 μM which is similar to the high-affinity component in intact membranes. The low-affinity K_D is comparable to the value of 30 μM previously reported by Honore et al. (1989). For the recombinant receptors GluR1-4, K_D 's were between 0.5 and 2 μM (table 1). These values are again similar to the high-affinity component in the brain. [^3H]FW was used for these tests because [^3H]CNQX binding is unmeasurably low for most subunits, but additional experiments with GluR4i using [^3H]CNQX gave similar K_D 's (figure 2). Taken together, the affinities of GluR1-4 for glutamate are about an order of magnitude higher than those of brain receptors, as for AMPA and FW.

Physiological properties of recombinant versus neuronal AMPA receptors

Figure 3 and table 2 summarize the fast kinetic properties of the GluR1-4 receptors used for these studies. These properties were determined from responses to 1 ms or 100 ms pulses of 10 mM glutamate. In agreement with earlier reports (Erreger et al., 2004), the deactivation time constants were rather uniform (0.7–1.3 ms) while desensitization time constants varied between 1.1 and 5.8 ms and were generally slower for flip variants. GluR1 receptors often stood apart, for instance in having the slowest deactivation and in showing no consistent differences between splice variants. They also exhibited a slower recovery from desensitization (time constants 105–151 ms; figure 3D) than other subunit (16–43 ms), and had a two-fold lower EC_{50} for the peak current (1.1 mM; figure 3C) than GluR2-4 (1.7–2.4 mM).

Two physiological measures are commonly employed to characterize receptor properties under steady-state conditions, namely (i) the EC_{50} for the steady-state current (SS-EC_{50}) and (ii) the IC_{50} for equilibrium desensitization, which in this report is called EqD_{50} . To determine the latter, the excised patches are sequentially exposed to a range of concentrations of glutamate in the background stream. Once a steady-state is reached at each of these concentrations, a 1 ms test pulse of near-saturating (10 mM) glutamate is applied. The glutamate in the background stream causes a fraction of the receptors to become desensitized and hence unresponsive to the test glutamate pulse, leading to a reduction in the amplitude of the test pulse. By plotting the response amplitude against the background glutamate concentration and fitting a sigmoidal inhibition curve to the data, one obtains the equilibrium constant EqD_{50} . Several studies have shown that the steady-state EC_{50} and the EqD_{50} differ, the latter typically being lower (Robert and Howe, 2003). Because steady-state currents were about 1% of the peak current and often extremely small in excised patches, we focused in this study on the EqD_{50} for equilibrium desensitization. Inhibition curves were constructed for each subunit by exposing patches to background glutamate concentrations between 0.01 and 300 μM . As shown in figure 4 and table 1, equilibrium desensitization differed substantially across subunits. For GluR2 and 3,

the pattern showed some correlation with the desensitization time constants in that the flop variants ($\sim 4.0 \mu\text{M}$) had significantly smaller EqD50s than the flip variants ($14\text{--}18 \mu\text{M}$). GluR1 receptors stood again apart in that EqD50 values were similar for flip and flop ($\sim 1.0 \mu\text{M}$) and lower than for the other subunits ($4\text{--}18 \mu\text{M}$). The EqD50s shown here are reasonably close to published values ($0.56 \mu\text{M}$ for GluR1i and $7.5 \mu\text{M}$ for GluR4i, in Robert and Howe, 2003).

Figure 5A shows the corresponding data obtained with patches excised from pyramidal cells in hippocampal area CA1; the inhibition curves for the recombinant receptors from figure 4 are included for comparison. The EqD50 value in hippocampal patches was $4.1 \pm 0.5 \mu\text{M}$, which is within the range of those for the recombinant receptors ($1.1\text{--}18.4 \mu\text{M}$). It is similar to the values for GluR2o and 3o and bracketed by those for GluR1, 2i and 3i. CA1 pyramidal neurons contain the subunits GluR1-3 at comparable densities (Gold et al., 1997), with flop variants probably more prevalent as judged by sensitivity to cyclothiazide (Arai and Lynch, 1996). Thus, the inhibition curve for the hippocampal receptors can reasonably be understood as being the ensemble average for the subunits present in this region. This is also in agreement with the data of Colquhoun et al. (1992) who found an EqD50 of $4.2 \mu\text{M}$ in CA1 and $9.6 \mu\text{M}$ in CA3 which contains more flip variants. EqD50 values for autaptic responses of hippocampal neurons were slightly larger ($10 \mu\text{M}$; data not shown) but still within the same range. This then implies that there are no major differences between recombinant and brain receptors. Similar conclusions were reached with respect to other physiological measures. The average EC_{50} for the peak current of glutamate responses was $1.1 \pm 0.3 \text{ mM}$ (figure 5B) which is close to the range of $1.1\text{--}2.4 \text{ mM}$ for recombinant receptors. Similarly, the time constant for resensitization in hippocampal patches was $74 \pm 8 \text{ ms}$ (figure 5C) which is in between those for the individual subunits ($16\text{--}151 \text{ ms}$). The only notable differences were seen in the time constants for deactivation and desensitization. In patches from cortical and hippocampal neurons, deactivation time constants are $2\text{--}3 \text{ ms}$ and desensitization time constants are mostly on the order of 10 ms (e.g. Colquhoun et al., 1992; Arai and Lynch, 1996; Andrasfalvy and Magee, 2001), and hence they are two to four times larger than the median time constants of recombinant receptors (table 2). Taken together, the physiological experiments shown here indicate that recombinant and neuronal receptors have similar properties. Decay time constants differed, but only by a modest factor when compared to the disparity between high and low affinity binding affinity. Most important for this study, no major disparity was detected in the equilibrium desensitization constants, which would be expected to be most closely related to binding affinity.

Relationships between binding constants and physiological steady-state constants in kinetic receptor models

The preceding sections have shown that brain AMPA receptors and recombinant receptors expressed in heterologous expression systems appear to differ drastically when binding affinities are measured, but not when physiological properties are examined. Any attempt to interpret these findings raises several additional questions, such as (i) whether there are predictable relationships in the first place between binding affinities and EqD50 constants, and (ii) whether the EqD50 constants measured in our tests are more likely to correspond to high-affinity or low-affinity binding sites. To examine these issues, we resorted to simulations with various kinetic receptor models described in the literature.

AMPA receptors are tetramers with four agonist binding sites, but the earliest models considered the binding of only one ligand (Patneau and Mayer, 1991; Ambros-Ingerson and Lynch, 1993). In all these one-site models, binding always exhibits simple dose-response relations with a Hill slope $n_H=1$, and Scatchard transforms are strictly linear, the K_D value being given by the inverse of the slope of the regression line (figure 6A). It should also be emphasized at this point that the term K_D that is commonly used in the binding literature to

denote binding affinity is in reality a complex function of *all* the independent rate constants (Ambros-Ingerson and Lynch, 1993) and that for most ligand-receptor interactions it does *not* represent an elementary dissociation constant of the form of k_{-1}/k_1 (Colquhoun, 1998). Figure 6A further indicates that the values for the EqD50 and the SS-EC₅₀ (marked by short lines; see also summary tables on the right) are virtually identical to the binding K_D and again have Hill coefficients near 1. The figures 6B-6D illustrate simulations with several more recently developed multi-ligand models. Most of these models assume two agonist sites which allows for some cooperativity yet keeps computations manageable, but one model has been constructed which incorporates all four agonist sites (Robert and Howe, 2003). An obvious downside of these models is that the choice of the rate constants is less constrained by experimental data and the models are accordingly more diverse. As evident from the sigmoidal plots of figure 6, concentration-effect relations for multi-ligand models may show significant deviation from basic saturation profiles with Hill slopes larger or smaller than one. For binding data, this is particularly easy to discern in the Scatchard transformations shown on the right. For instance, in both variants of the neuronal receptor model by Jonas et al. (1993), a marked positive cooperativity is apparent (figure 6B). In the models for GluR3o and R3i by Sekiguchi et al. (2002), some curvature is again apparent, but in opposite directions, with GluR3i showing negative and GluR3o positive cooperativity (figure 6C). In fact, in both of these two models the rather modest curvilinearity results from an imperfect balancing of a much more extreme negative cooperativity assumed to underlie agonist binding and a similarly extreme positive cooperativity underlying desensitization. Even more drastic deviations from 'linearity' are predicted by the four-subunit model of Robert and Howe (2003). According to their model for GluR1i (figure 6D), binding should be biphasic and exhibit an abrupt bend in Scatchard plots, with a more than 100 fold difference in the slope of the line fitted to the highest versus lowest four points (0.52 vs. 159 μ M). This binding heterogeneity results from the assumption underlying this model that only one subunit per receptor desensitizes with high affinity and that two of the remaining three subunits – despite being non-conducting and hence 'functionally desensitized' – retain the low-affinity of the non-desensitized receptor states. A similarly large disparity in affinities is seen for GluR4i, but the latter in addition exhibits some positive cooperativity in the high-affinity component (figure 6D). In summary, binding to multi-site receptors must be expected to show marked deviations from simple saturation kinetics, and some receptors are likely to exhibit positive cooperativity. It should also be noted, however, that the affinity of the lowest-affinity components predicted by the four-ligand models may in fact be so low that assays with [³H]AMPA or [³H]FW would be unable to detect this component. Binding to R1i might in this case appear to follow simple saturation kinetics and R4i should exhibit positive cooperativity.

A second conclusion from these simulations is that the steady-state EC₅₀, the EqD50 and the binding K_D no longer coincide in multi-ligand models (see tables in figure 6B–D). More importantly, however, inspection of the data in figure 6 also shows that there appear to be consistent relationships among the various equilibrium constants despite the great diversity in the design of these models. The most conspicuous relation was that the EqD50 always was *lower* than the binding K_D or the steady-state EC₅₀. That EqD50 constants are lower than the SS-EC₅₀ has indeed been shown for two subunits (Robert and Howe, 2003) and probably also holds true for receptors in hippocampal neurons as the SS-EC₅₀ of 19 μ M reported by Patneau and Mayer (1990) is higher than the EqD50 shown in figure 5 (4 μ M). Moreover, the K_D of ~30 μ M for low-affinity brain AMPA receptors - which have been shown to represent mainly mature synaptic receptors (Hall et al., 1992) - is larger than the EqD50 values, in agreement with the predictions made by our simulations.

DISCUSSION

Earlier studies have shown that binding affinities of recombinant receptors for AMPA and FW are at least an order of magnitude higher than those of the dominant binding component in the brain. Our binding experiments have confirmed this and they have shown that a similar disparity exists for the natural ligand glutamate. In contrast, physiological experiments did not reveal major differences between recombinant and brain receptors. Most importantly, the EqD50 for equilibrium desensitization of brain receptors was close to the median value of recombinant receptors. Some differences were seen in the decay time constants but they were modest and less likely to be related to equilibrium binding constants. Moreover, differences in decay time constants are explained by the influence of the TARPs which are present in neurons but not HEK293 cells and which slow desensitization and deactivation time constants up to 3 times (Tomita et al., 2005; Milstein et al., 2007). However, TARPs, which associate with receptors already early in the biosynthetic pathway (Cuadra et al., 2004; Vandenberghe et al., 2005), do not seem to account for the conversion from high to low affinity binding because they had no significant impact on binding (figure 1).

Taken together, these findings favor the second hypothesis outlined in the Introduction which predicted that recombinant and neuronal receptors would fail to exhibit major differences in their physiological properties. This hypothesis stated (i) that recombinant receptors, like those in the brain, undergo conversion to a state of low binding affinity while being trafficked to the surface, and (ii) that the low affinity population remains invisible in binding tests. A plausible explanation for the latter is that the number of mature receptors is simply too small to make a significant contribution to overall binding. Low-affinity components in mixed receptor populations are inherently difficult to detect unless their density is considerably larger than that of the high-affinity population. This is the case in the brain where low-affinity receptors accumulate in synapses, but perhaps not in heterologous expression systems. Hall et al. (1997) reported that only 25–40% of recombinant receptors were at the cell surface. In our preparations surface receptors often accounted for less than 10% of the receptors and even when co-expressed with TARPs rarely exceeded 30% (not shown). Thus, if we make the further assumption that the population of mature, low-affinity receptors is roughly identical to that of the surface receptors, then the mature receptors detected in physiological experiments may indeed have been virtually impossible to detect in binding assays.

Stated differently, we propose that the recombinant receptors responsible for generating currents in physiological recordings were not the same as those detected in binding tests, and hence that their kinetic properties should not be directly compared. This interpretation is supported further by comparisons between experimental data and receptor simulations. The latter suggested that EqD50 constants in multi-site models are likely to be *lower* than the corresponding binding K_D , and brain receptors indeed exhibited the predicted relationship. For recombinant receptors, however, the EqD50 for each subunit was *larger* than the corresponding K_D , sometimes up to ten-fold, and thus this relationship between the two constants was opposite to our expectations. The predictions would be fulfilled, however, if the ‘hidden’ *functional* receptors in our HEK293 cells have a binding K_D that is about an order of magnitude higher than the one actually measured.

This interpretation also implies that the binding data commonly reported for recombinant receptors pertain to receptors with unknown physiological characteristics, or perhaps even to receptors that lack the competence for conducting current. It further implies that it will be indispensable in the future to separate ‘low-affinity receptors’ from the dominant population of high-affinity receptors if the binding properties of functional receptors are to be investigated. Only then will it be meaningful to make quantitative comparisons between experimentally determined saturation curves on the one hand and those predicted by the various receptor

models such as in figure 6. Minimally we expect that mature GluR1-4 receptors will exhibit binding affinities for glutamate on the order of tens of micromolar, as in brain membranes (the K_D of which should be understood to be always an ensemble average of the individual subunits). But one could then also test whether Scatchard plots exhibit concave and/or convex curvature as predicted by the models shown in figure 6, or the prediction arising from model D that glutamate displacement of [3 H]CNQX binding to GluR1i and R4i should exhibit two components with drastically different affinities.

If these interpretations are correct then it also follows that conversion to low affinity occurs in heterologous expression systems and hence does not require specific neuronal or synaptic factors. The question then remains which mechanism is responsible for the conversion. Standley et al (1998) proposed that the affinity change may correlate with the transition from immature, high-mannose glycosylation to complex glycosylation. Other posttranslational modifications may occur, including palmitoylation (Hayashi et al., 2005), but they do not seem to be an obligatory property of surface receptors. A further possibility is that changes in binding affinity reflect stages in receptor assembly. Perhaps a substantial fraction of newly synthesized receptors are present in dimers or proto-tetramers that are not yet fully integrated into mature receptors. This might also explain another puzzling aspect of the binding data, namely that Scatchard plots for many subunits appear strictly linear. In the simulations of figure 6B–D, binding to multi-subunit receptors always exhibited a significant degree of convex or concave curvature due to subunit cooperativity, and thus the strict ‘linearity’ of most recombinant receptors is highly unusual. One explanation may be that subunits in early stages of assembly do not yet interact in ways that permit cooperativity and that upon agonist binding each subunit undergoes conformational transitions - including those leading to the desensitized conformation which is the state with the highest binding affinity - independent of the neighboring subunits. In this case, binding properties would be adequately represented by a one-site model as in figure 6A. It should also be noted that subunit monomers are unlikely to contribute significantly to binding because high-affinity agonist binding is modulated by positive allosteric modulators (Kessler and Arai, 2006) which bind at the interface between two subunits (Jin et al., 2005).

The latter explanation - i.e., that conversion to low affinity reflects changes in quaternary structure in the course of receptor assembly - can perhaps also be most readily reconciled with observations that brain receptors switch to high-affinity binding after exposure to radiation and some chemical agents. Honore and Nielsen (1985) made the seminal observation during radiation-inactivation experiments that AMPA receptors anomalously switch to high affinity for [3 H]AMPA (but not [3 H]CNQX) when exposed to modest radiation and they ascribed this to the destruction of a 113 kD protein. This could indicate that radiation destroyed one subunit, leading to disintegration of the receptor into fragments of dimers and monomers. A similar switch to high-affinity occurs after ultraviolet irradiation (Hall et al., 1996). Moreover, the observation that brain receptors convert to a pure high-affinity state after solubilization (Hall et al., 1992; Kessler and Arai, 2006) is perhaps explained by the finding that some detergents dissociate AMPA receptor tetramers (Nakagawa et al., 2006). Conversion to high affinity was also observed when treating brain membranes with phenylglyoxal, which derivatizes arginine residues (unpublished data), and with the mercurial pCMBS which reacts with cysteines (Terramani et al., 1988, Hall et al., 1996). If the interpretation outlined above is correct then these observations may help to identify amino acids that specifically mediate allosteric interactions in the mature tetrameric receptor.

Another noteworthy conclusion from our data is that binding K_D 's and physiological equilibrium constants are sufficiently close to allow for meaningful comparisons between them. This is in contrast to many earlier reports that alleged drastic discrepancies between these constants. For instance, Patneau and Mayer (1990) reported a >100-fold difference for neuronal

receptors by juxtaposing a binding K_D for glutamate of 0.16 μM with their observed steady-state EC_{50} of 19 μM . In many of those instances, however, glutamate K_D 's were determined at 0°C in assays containing thiocyanate - which increases affinity several-fold for all agonists with the exception of kainate (Hall et al., 1994) - and under conditions that favored binding to high-affinity sites. In contrast, our assays were done at 25°C using [^3H]CNQX (without thiocyanate) which labels mainly low-affinity sites in brain membranes. The K_D estimate for glutamate obtained under these conditions (~30 μM) has a similar order of magnitude as the physiological constants and exhibits the predicted relationship with the EqD_{50} .

To summarize, the most plausible interpretation of our data is that AMPA receptors, even when expressed in heterologous cells, undergo an obligate conversion from high binding affinity to a state of low binding affinity and that it is the latter variant of the receptor which is responsible for *all* physiological responses. In heterologous expression systems, however, binding to mature receptors is effectively masked (i) because high-affinity receptors are more abundant and (ii) because high-affinity receptors exhibit much higher binding site occupancy at any given radioligand concentration. This implies that the binding data on recombinant receptors reported in the literature are not representative of the properties of functional receptors, and that it will be essential to isolate membranes containing mature, low-affinity receptors if binding data are to be compared with physiological data and kinetic receptor models. The same caveat holds true also for efforts to examine receptor changes induced by the biochemical and pharmacological treatments mentioned in the Introduction, most of which were studied in brain AMPA receptors and most likely apply to the low-affinity receptor variant.

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Abbreviations

CNQX

6-cyano-7-nitro-quinoline-dione

EqD_{50}

concentration producing 50% inhibition in equilibrium desensitization experiment

FW

fluorowillardiine

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Figure 1. Binding affinities of brain AMPA receptors and GluR1-4 for $[^3\text{H}]$ AMPA

Left: Rat brain membranes were incubated for 30 min at 0°C with $[^3\text{H}]$ S-AMPA plus 50 mM potassium thiocyanate; representative experiment. AMPA concentrations were varied between 3.3 nM and 4 μM . Binding was measured in duplicate or triplicate and is shown as mean and s.e.m. The original binding data were fitted with a double-hyperbolic function and the best-fit curve was then transformed into Scatchard format, along with the data points. The Y-axis shows the ratio bound/free ligand, the units are in pmol/mg protein/ μM . K_D values for the high and low affinity components and percent contribution to total B_{max} are shown as insets. **Center:** Scatchard plots for $[^3\text{H}]$ S-AMPA binding to GluR1-4 expressed in HEK293 cell. AMPA concentrations were selected between 0.7 and 500 nM. The data points for most subunits were fitted with linear regression. For GluR2i and 4i, which consistently exhibited deviation from linearity, the original binding data were fitted with a double-hyperbolic function, as for brain receptors. All panels show representative experiments and their K_D constants; summary data are compiled in table 1. The data for GluR2o, 3o and 4i were also included in the left panel along with those for brain receptors (data for GluR2o and 4i were proportionally scaled down 2.5 and 5 fold while preserving the correct slopes). In Scatchard plots, binding affinity (K_D) is equal to $-1/\text{slope}$ and hence high-affinity binding is characterized by a steeper slope. These comparisons show that binding to brain membranes exhibits a dominant low-affinity component for which there is no equivalent component in homomeric receptors. **Right:** Binding of $[^3\text{H}]$ fluorowillardiine to GluR1i and GluR2o when expressed alone and together with stargazin (TARP $\gamma 2$) (cDNA ratio 1:1). K_D values from linear regression were essentially unchanged (7.4 nM vs 7.4 nM for GluR1i; 14 vs 11 nM for GluR2o).

Figure 2. Binding affinity of GluR1-4 and brain AMPA receptors for glutamate

Assays were done at $24 \pm 1^\circ\text{C}$ and in the buffer used for the patch recordings in figures 4 and 5. **Top:** Displacement of [^3H]fluorowillardiine (FW) binding to GluR1-4 by glutamate. In each experiment, binding (in duplicate) was normalized to that without glutamate (in quadruplicate). The data points show the mean and s.e.m. of the percentages from 3–7 experiments (most error bars are smaller than the symbol size). The data were fitted with sigmoidal functions with $n_H = -1$ (solid lines) or with variable Hill slope (dashed lines). IC_{50} values are shown as insets. [^3H]FW concentrations were less than half-saturating at 24°C (5 nM for GluR1-2, 100 nM for GluR3-4) and Cheng-Prusoff correction factors accordingly were between 0.53 and 0.9 (Cheng and Prusoff, 1973). K_D values for glutamate were obtained by multiplying the IC_{50} with this

factor and are summarized in table 1. The panel for GluR4i also shows displacement of [^3H] CNQX (40 nM) instead of [^3H]FW (n=6). The IC_{50} in this case was $4.9 \pm 0.3 \mu\text{M}$ ($n_{\text{H}} = -1.07$) and the K_{D} after Cheng-Prusoff correction is $4.0 \mu\text{M}$. **Bottom:** Displacement of [^3H]CNQX (20 nM) from brain AMPA receptors by glutamate (filled squares); mean and s.e.m. from 7 experiments. A statistically better fit ($F=23.1$, $p<0.001$) was obtained with the sum of two sigmoidal functions ($n_{\text{H}} = -1$ each; solid line) than with a single sigmoidal function (dashed line). Multiplying the IC_{50} 's with a Cheng-Prusoff factor of 0.95 gives K_{D} values of 1.7 and $33 \mu\text{M}$. For comparison, data and regression curves are also shown for the recombinant receptors (open circles, thin dashed lines). In addition, displacement curves were determined for solubilized brain AMPA receptors, which exhibit only high-affinity binding (Hall et al., 1992). Displacement of [^3H]S-AMPA (50 nM, no thiocyanate; n=2) gave an IC_{50} of $1.2 \mu\text{M}$ and a K_{D} for glutamate of $0.8 \mu\text{M}$.

Figure 3. Fast kinetic properties of GluR1-4

Outside-out patches excised from HEK293 cells expressing GluR1-4 flip or flop were exposed to 1 ms or 100 ms pulses of glutamate with the use of a piezo-driven liquid-filament switch system. The unedited version of GluR2 (GluR2(Q)) was used for these experiments. **A.** Representative recordings of currents mediated by GluR1 (R1i, R1o) and GluR2 (R2i, R2o) in response to a 100 ms pulse of 10 mM glutamate. The graph on the right shows the same traces at an expanded time scale. Summary data for desensitization time constants and steady-state/peak current ratios are shown in table 2. **B.** Representative responses to 1 ms pulses of 10 mM glutamate to determine response deactivation. **C.** The peak amplitude of responses to 1 ms pulses of glutamate was measured at concentrations of 0.01 to 10 mM and normalized to that at 10 mM glutamate. The data points show the mean and s.e.m. of 3 (R2o), 6 (R1o, R2i, R4i), 7 (R1i) and 8 (R3i, R3o) experiments and were fitted with a logistic equation; EC₅₀ values and Hill coefficients (n_H) are shown as insets and in table 2. Absence of an error bar indicates that the error was smaller than the symbol. **D.** Recovery from desensitization. Pairs of 1 ms glutamate pulses (10 mM) were applied at intervals of 20–600 ms. The amplitude of the second response was normalized to that of the first response and plotted against the pulse interval (mean and s.d. from 3–8 experiments). Recovery time constants (τ) were calculated by fitting with a single exponential function and the maximum desensitization was determined by extrapolating to $t=0$; summary data are shown in table 2. Note that GluR1 is plotted on a different time scale. Data for GluR3 were obtained with whole-cell recording.

Figure 4. EqD50 values of GluR1-4 for equilibrium desensitization by glutamate

Patches were equilibrated with the concentration of glutamate indicated on the x-axis. A 1 ms pulse of 10 mM glutamate was then applied and the peak amplitude was normalized to that in the absence of background glutamate. The data points represent the mean and s.e.m. of 3 (R1i), 7 (R3o), 8 (R2o), 11 (R4i), 12 (R3i, R2i), and 15 (R1o) experiments. The concentration producing 50% inhibition (EqD50) was calculated by fitting the data with a logistic equation and is shown as inset, together with the Hill slope n_H . Representative traces are from an experiment with GluR1i; the thick horizontal bar denotes the duration of the 10 mM glutamate pulse and the thin line indicates the continuous presence of glutamate in the background flow line.

Figure 5. Physiological properties of hippocampal AMPA receptors in comparison with GluR1-4 Patches were excised from stratum pyramidale of hippocampal field CA1. Recordings were made at 24°C in the presence of 50 μM D-AP5 and 10 μM MK-801; the holding potential was -50 mV. Data are presented as mean and s.e.m. and curves fitted to the data points are shown by thick lines. For comparison, the curves obtained with recombinant receptors in figures 3 and 4 are shown with thin lines. Values given in the insets are for hippocampal receptors. **A.** Equilibrium desensitization by 1–100 μM glutamate; mean and s.e.m. of 6 experiments. Shown on the right are representative responses to 10 mM glutamate in the presence of the indicated background concentration of glutamate. **B.** Concentration-response relation for the peak current of responses to glutamate. Data are averages from 4–12 experiments, normalized in each experiment to the peak current at 10 mM glutamate. **C.** Recovery from desensitization. Pairs of 1 ms pulses were given at different intervals as in figure 3D. The data are from 5–15 experiments and are fitted with a single exponential function.

Figure 6. Steady-state-currents, equilibrium desensitization, glutamate binding, and peak currents according to four receptor models

Simulations were carried out with four models described in the literature. The nature of the model is shown on the left, with R indicating closed receptor states, O open states, and D, D* and E desensitized states; the number following these letters indicates the number of agonists bound. Lines indicate transitions between states assumed to be significant. These transitions are governed by forward and reverse rate constants shown next to the line (left to right = above the line; right to left = below; downward = right side; upward = left side). Rate constants involving agonist association are in $\mu\text{Mol}^{-1} \cdot \text{sec}^{-1}$, all other rate constants in sec^{-1} . The center panels show concentration-effect curves for the steady-state current, equilibrium desensitization ('EqD50'), binding, and peak current. Steady-state current is plotted on the right y-axis, other measures on the left y-axis. Currents and binding were calculated for glutamate concentrations from 0.01 μM to 10 mM and fitted with four-point logistic equations. EC50 values are marked by a vertical line and summarized in the table to the right, with Hill slopes in brackets. The panels on the right show Scatchard transformations of the binding data. The models were as follows: **A.** Single-ligand model of Ambros-Ingerson and Lynch (1993), as modified to match physiological and binding data for GluR1o (Suzuki et al., 2004). **B.** Two-ligand model of Jonas et al. (1993) to simulate currents produced by neuronal receptors (CA3 pyramidal cells). D*2 represents an additional desensitized state directly accessed from the open state. **C.** Two-ligand models used by Sekiguchi et al. (2002) for GluR3i and GluR3o. **D.** Four-ligand model developed by Robert and Howe (2003) to emulate physiological characteristics of GluR1i and GluR4i and general molecular aspects of AMPA receptor function. This model introduces an additional E state (called D₂ in Robert and Howe, 2003) but the significance of D- and E-states is different from other models. 'D2' in models B and C indicates that both subunits are in a desensitized state, and no allowance is made for the

possibility that only one is desensitized. In model D, the D-states indicate a configuration in which one subunit is desensitized and binds with high affinity, while the other subunits retain low agonist affinity. Thus, D4 contains one subunit in a high-affinity state and three subunits which remain of low affinity. The authors postulate, however, that all D and E-states are non-conducting and hence *functionally* desensitized. When entering the E-states, a second subunit desensitizes and assumes increased affinity. The remaining two subunits do not enter into a high-affinity state; also, in GluR1i, the affinity gain in the E-state is small and thus only one out of four subunits binds with truly high affinity. This explains the enormous disparity between high- and low-affinity binding evident in the Scatchard plot. Similarly, binding in the center graph could only be adequately fitted with the sum of two sigmoidal functions (the single logistic fit is indicated by a dotted line). The summary table accordingly shows data for both one- and two-sigmoidal fits; for the latter, the K_D values for the high and the low-affinity components are shown and the percent of total binding represented by the low-affinity component. For GluR4i, D and E states are assumed to have similar affinity and thus about half of total binding has high affinity. The R1i Scatchard also shows linear regression lines fitted to the binding at the lowest four (0.01 to 0.3 μM) and highest four (500–10000 μM) glutamate concentrations. Binding in all graphs is shown as percent of total possible binding (1 ligand per receptor in A, 4 ligands per receptor in D, etc) and hence upper asymptotes are 100%. Current measures indicate the percentage of receptors being in the open state and thus upper asymptotes are lower than 100%.

Physiological equilibrium constants and binding affinities for rat GluR1-4 and brain AMPA receptors.

	physiological constants		binding affinities		N	S-AMPA		N	K _D high K _D low (% low)	
	EqD50	μM [n _H]	K _D , μM [n _H]	glutamate		K _D , nM	1-site fit		2-site fit	
R1i	1.1 ± 0.1 [1.0]		0.82 ± 0.12 [.88 ± .01]		4	11.4 ± 0.6*		6		
R1o	1.0 ± 0.1 [1.0]		0.47 ± 0.03 [.99 ± .03]		7	10.6 ± 0.6		4		
R2i	13.6 ± 0.9 [1.3]		1.94 ± 0.15 [.81 ± .04]		4	11.3 ± 2.6*		5	2.2 ± 0.3 34 ± 11 (72%)	
R2o	4.4 ± 0.5 [1.4]		0.98 ± 0.07 [.90 ± .04]		4	4.1 ± 0.6		7		
R3i	18.4 ± 2.1 [1.1]		1.83 ± 0.14 [.96 ± .06]		4	19 ± 2		5		
R3o	4.0 ± 0.3 [1.5]		1.98 ± 0.16 [.98 ± .05]		3	16 ± 2		4		
R4i	6.6 ± 0.9 [1.1]		1.65 ± 0.15 [.90 ± .05]		3	63 ± 4*		12	6.9 ± 1.1 115 ± 10 (86%)	
R4o	—		1.54 ± 0.15 [.92 ± .02]		3	31 ± 4		3		
Brain	4.1 ± 0.5 [0.92]		1.7 33 (91%)		7			2	11 ± 1 377 ± 8 (94%)	

The left column shows EqD50 values from the equilibrium desensitization experiments in figure 4, the other columns show binding affinities. Glutamate: Summary data for the experiments of figure 2, except that IC50 values and n_H were determined separately for each experiment by fitting a four-point logistic equation. The binding constants ('K_D') were then calculated by applying a Cheng-Prusoff correction, and K_D values and Hill slopes were averaged (N=number of experiments). AMPA: Binding was measured at 0°C in the presence of 50 mM KSCN. K_D values were determined in each experiment by fitting a regression line after transformation into the Scatchard format; values with an asterisk are approximate because binding exhibited nonlinearity. Data for GluR2i and 4i were in addition fitted with the sum of two hyperbolic functions to obtain high and low affinity affinities. EqD50 data for brain receptors (hippocampal area CA1) are taken from figure 5A. K_D values for glutamate for brain receptors (mostly from cortex and hippocampus) are from figure 2. AMPA K_D values for brain receptors are averages from 2 experiments, one of which is shown in figure 1A. High and low affinity sites in brain membranes should in any case be understood to represent ensemble averages of all the receptor subunits present in brain membranes.

Table 2
Response characteristics of GluR1-4 homomeric receptors and hippocampal AMPA receptors.

	10-90% rise time (ms)	desensitization time constant (ms)	steady-state/ peak (%)	deactivation time constant (ms)	resensitization time constant (ms)	maximum desensitization (%)	EC ₅₀ peak current (mM)
R1i	0.3 ± 0.02	2.5 ± 0.1 ⁽¹³⁾	1.3 ± 0.2	1.1 ± 0.1 ⁽¹³⁾	151 ± 19 ⁽³⁾	75 ± 4	1.1 ± 0.1 [1.1]
R1o	0.4 ± 0.03	3.1 ± 0.1 ⁽²⁷⁾	0.6 ± 0.1	1.3 ± 0.05 ⁽²⁷⁾	105 ± 13 ⁽⁷⁾	66 ± 4	1.1 ± 0.1 [1.2]
R2i	0.4 ± 0.02	5.8 ± 0.2 ⁽³⁰⁾	5.6 ± 1.0	0.8 ± 0.1 ⁽³⁰⁾	20.9 ± 2.5 ⁽⁶⁾	40 ± 3	2.1 ± 0.1 [0.8]
R2o	0.3 ± 0.03	1.1 ± 0.1 ⁽¹⁷⁾	0.4 ± 0.1	0.7 ± 0.05 ⁽¹⁷⁾	29.9 ± 3.4 ⁽⁶⁾	89 ± 5	1.7 ± 0.2 [0.9]
R3i	0.6 ± 0.03	4.6 ± 0.2 ⁽³³⁾	1.7 ± 0.3	0.8 ± 0.03 ⁽³³⁾	16.2 ± 3.2 ⁽⁵⁾	43 ± 3	2.4 ± 0.1 [1.2]
R3o	0.5 ± 0.03	1.1 ± 0.04 ⁽¹⁸⁾	0.4 ± 0.1	0.8 ± 0.1 ⁽¹⁸⁾	43.7 ± 3.0 ⁽⁵⁾	100	2.1 ± 0.1 [1.3]
R4i	0.4 ± 0.02	3.6 ± 0.1 ⁽³⁵⁾	2.4 ± 0.3	0.8 ± 0.1 ⁽³⁵⁾	24.0 ± 2.5 ⁽³⁾	58 ± 1	1.9 ± 0.2 [1.1]
Brain		11.1 ± 0.7 ⁽²⁷⁾ a	5.1 ± 0.6 ⁽¹²⁾ b	2.5 ± 0.2 ⁽⁹⁾ b	74 ± 8	54 ± 5	1.1 ± 0.3 [1.2]

GluR1-4 receptors were expressed in HEK293 cells. For GluR2, the unedited forms (GluR2(Q)) were employed. Data for GluR3o were obtained with whole-cell recording, those for all other subunits from excised patches. Currents were induced by applying pulses of 10 mM glutamate. Desensitization time constants and steady-state currents were determined by fitting the decay phase of the responses to a 100 ms pulse of glutamate with a single exponential function. The 10-90% rise time and deactivation time constants were measured in responses induced by a 1-ms pulse of glutamate. The results are expressed as the mean and s.e.m. of the number of experiments shown in parenthesis. Resensitization time constants and 'maximum desensitization' were obtained after fitting a curve to the averaged data, as described in figure 3D; error values were in this case given by the curve fitting program. For GluR3o, the maximum desensitization determined in this manner was >100% which agrees with observations that resensitization follows a sigmoidal (Hodgkin-Huxley type) time course at very short intervals (Robert and Howe, 2003). The EC₅₀ values for the peak current are taken from figure 3C; Hill slopes are shown in brackets. Data for brain receptors were obtained from patches excised from area CA1 of hippocampal slice cultures and are taken from figure 5, from Arai et al., 1995 ⁽⁴⁾, and from Arai and Lynch, 1996 ^(b).