SUPPLEMENTARY METHODS

Cultured hippocampal neurons

Hippocampal neurons were cultured from postnatal day 1-2 Sprague-Dawley rats as previously described [Brewer et al., 1997]. Hippocampi were dissected in ice cold dissecting media (1x Hank’s buffer supplemented with 50 mM HEPES, pH 7.4; Gibco) and cut into 1-2 mm pieces. Hippocampal pieces were digested with 2 mg/mL papain (Worthington) in the dissecting media for 20 min at 37°C and triturated using a sterile polished glass pipette (0.8-1 mm pore diameter). Dissociated hippocampal cells were plated on 6-well plates (35 mm well diameter), precoated with 1 mg/mL poly-L-lysine (Sigma), in plating media (Neurobasal A media supplemented with 2% B-27 and 2 mM GlutaMAX™-I supplement; Gibco) at a density of 4 x 10⁵ cells per well. 3-4 hours after the initial plating, the media was replaced with fresh pre-warmed plating media. After 4 days in vitro (DIV), 2.5 M cytosine-β-D-arabinofuranoside was added to the plating media. Cultures were fed twice a week by replacing half of the media with fresh plating media and used for experiments at 13-15 DIV.

GISP (glycine-induced synaptic potentiation)

We used GISP paradigm [Lu et al., 2001] to induce synaptic plasticity in cultured hippocampal neurons and to assay GluR1 Ser831 phosphorylation in endogenous GluR1 homomers and GluR1/R2 heteromers (Fig. 2). Prior to GISP, the cultures were incubated for 30 min in the extracellular control solution (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 33 d-glucose, 0.02 d-APV (NMDA-R blocker), 0.003 strychnine (inhibitory glycine receptor blocker), 0.02 bicuculline (GABA A receptor blocker), 0.0005 TTX (to block action potentials), and 25 HEPES, pH 7.3. GISP was induced by treating cultures for 10 min with the same extracellular solution as above but with glycine (100-200 M) and without TTX, Mg²⁺ and d-APV. After glycine treatment, cultures were returned to the control solution. GISP was associated with long-lasting changes (1-3 hours) in frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) (Supplementary Fig. 1) [Lu et al., 2001]. This plasticity shared many key properties with hippocampal CA1 LTP as listed below.
Common properties between GISP and CA1 LTP

1. Induction requires NMDA-Rs (blocked by APV) [2]
2. Induction is Ca$^{2+}$ dependent [3]
3. Expression is largely due to AMPA-Rs [2]
4. Results in increased frequency and amplitude of mEPSCs [2, 3], Suppl. Fig. 1
5. Results in CaMKII-dependent phosphorylation of GluR1 at Ser831 Fig. 2a-b
6. Results in AMPA-R but not NMDA-R membrane insertion [2]
7. “Silent synapses” are converted to functional synapses [3]
8. Induction requires CaMK activation. [blocked by 3 M KN-93]
9. Results in CaMKII autophosphorylation at Thr286 Fig. 2a,c

Immunoprecipitation and immunoblotting

For biochemical analysis of endogenous AMPA-Rs, cultured hippocampal neurons were stimulated by GISP, followed by immunoblotting with the Ser831 phospho-specific antibody (UBI). Cells were scraped in cold homogenization buffer: 1% Triton X-100 with (in mM): 50 NaCl, 10 EDTA, 10 EGTA, 1 Na$_3$VO$_4$, 50 NaF, 25 NaPPi, 1 β-glycerophosphate, 1 PMSF, 0.001 microcystine, and 1 protease inhibitor cocktail tablet per 50 mL (Roche), and 50 HEPES, pH 7.5. 300 L of the buffer was used per well, and samples from 2 wells were combined for each condition. Scraped cells were sonicated for 20 min in ice-cold water bath and spun at 10,000g for 20 min. GluR1/R2 heteromers in the supernatant were immunoprecipitated by incubating with 4 g of anti-GluR2 antibody (Santa Cruz) for 1 hour on ice, followed by 3 hour incubation with 40 l of 50% Protein G Sepharose 4 Fast Flow (Amersham) on a rocker at 4°C. After the initial spin, the supernatant was saved and the precipitated pellet was rinsed four times with the homogenization buffer. This resulted in complete immunodepletion of GluR2 from the supernatant (Fig. 2a), suggesting that AMPA-Rs remaining in the supernatant were GluR2-lacking. Preliminary blots were run to normalize for differences in GluR1 amount between precipitate and supernatant (data not shown). Normalized volumes of samples were resolved in 10% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted using infra-red dye-coupled secondary antibodies (anti-rabbit IgG Alexa Fluor 680, Molecular Probes; anti-mouse IgG IRdye800, Rockland). Image acquisition and data quantitation were performed on the Odyssey Infrared Imaging System (Li-Cor).
Primary antibodies used were: anti-phospho-Ser831-GluR1 (UBI), anti-GluR1 (UBI), anti-GluR2 (Santa Cruz), anti-phospho-Thr286-CaM-KII (ABR), and anti-CaM-KII (ABR).

To analyze Ser831 phosphorylation of recombinant GluR1/R2 heteromers expressed in HEK293 cells, we followed the same immunoprecipitation protocol described above using the anti-GluR2 antibody. For GluR1 homomers, supernatant from cells transfected with GluR1 subunit alone was used. Phosphorylation of Ser831 was analyzed for each receptor type with and without co-expressing constitutively active CaMKII. Relative phosphorylation for each receptor composition and condition was calculated by normalizing the phospho-Ser831 readings to the total GluR1 protein (Supplementary Fig. 2).

Electrophysiology

AMPA-R mEPSCs were recorded in the extracellular control solution indicated above. Cs\(^{2+}\)-based intracellular solution was used to block majority of K\(^+\) conductance (in mM): 100 Cs-methanosulfonate, 25 CsCl\(_2\), 2 MgCl\(_2\), 0.4 EGTA, 4 ATP, 0.4 GTP, 10 phospho-creatine, and 10 HEPES, pH 7.3. Input and serial resistances (<10-15 M\(\Omega\), 70-80% compensated) were monitored throughout the experiments, and cells with deviations >15% were discarded. Individual mEPSCs were detected using the template algorithm (AxoGraph 4.0, Axon Instruments), and their representative amplitudes and kinetic properties were determined based on averaged mEPSC (200-300 currents averaged) and from corresponding cumulative distributions (Supplementary Fig. 1). These measurements were made every 5 min of spontaneous activity to monitor changes in activated synapses.

HEK293 cells were transfected 24-36 hours after plating. Currents from all types of receptors were recorded either in whole-cell (cells lifted from the bottom) or outside-out patch-clamp configurations. Glutamate (10 mM, 100 ms pulses, 5 s interval) was delivered to receptors either by a piezo-driven application system or puffing, as described previously [Derkach et al., 1999]. Homomeric GluR1 and GluR2 receptors (flip isoforms) were recorded by expressing these subunits alone. Because edited GluR2 homomers produced very small currents (Supplementary Fig. 3d) [Greger et al., 2002], we increased the concentration of GluR2 cDNA during transfection by four fold. To obtain
GluR1/R2 heteromers, GluR1 and GluR2 cDNAs were co-transfected at a ratio of 1:1 (0.8 g of total cDNA per 35 mm well) using Lipofectamine 2000 transfection kit (GibcoBRL). We used the following criteria to ensure that tested receptors represent GluR1/R2 heteromers, with no or negligible contribution from GluR1 or GluR2 homomers: 1) absence of rectification (no contribution from GluR1 homomers) (Supplementary Fig. 3a-b); 2) no sensitivity to joro-toxin, the inhibitor of GluR1 homomers (Supplementary Fig. 3c); 3) difference in kinetics of GluR1 and GluR2 homomers from the GluR1/R2 heteromers (Supplementary Fig. 3d); and 4) small amplitude of current from edited GluR2 homomers (2-3 order of magnitude smaller at 0.4 g of cDNA per well) compared to the GluR1/R2 heteromers (Supplementary Fig. 3d) [Greger et al., 2002]. Extracellular solution for HEK293 cell recordings contained (in mM): 155 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 10 d-glucose, and 5 HEPES, pH 7.3. Intracellular solution was same as above. Currents were recorded at 2 kHz bandwidth and digitized at 20 kHz.

NSFA (non-stationary fluctuation analysis)

Because single-channel conductance of GluR1/R2 heteromers and GluR2 homomers was very low (Fig. 3) [Swanson et al., 1997], a reliable single-channel or silence analyses were not feasible [Derkach, 2003], and therefore, we used NSFA as a principle approach to measure channel properties throughout all experimental conditions in HEK293 cells and for all types of receptors tested. NSFA was performed essentially as described [Derkach et al., 1999; Derkach, 2003]. The quality of individual registrations and fitting were tested as described [Benke et al., 2000], and 31-78 currents were selected for each measurement. All statistics were evaluated by Student’s two-tailed t-test.

References