

Supplementary information on methods:

Construction of GluR2 CT mutants: GluR2 $_{\Delta 869}$ and HA-GluR2 $_{\Delta 880}$ were made by PCR using a common primer (3'GGGAAACATGTAAAGGGTACCAT), which covers the region of GluR2 containing a unique KpnI site, and primers 5'GGGGTCGACCTAAATTTTAACTCACTCGATGCCATA and 5'GGGGTCGACCTATGCAAATTCTGGGAATTCTG, respectively. The plasmid HA-GluR2 was digested with KpnI and Sall and the PCR products digested with the same enzymes were then ligated into this plasmid. HA-GluR2 $_{\Delta 834-843}$ was made by inverse PCR using primers AAACGAATGAAGGTGGCAAAGAAT and AATCAAAGCCACCAGCATTGCCAA with HA-GluR2 as a template. The PCR product was phosphorylated using T4 polynucleotide kinase and religated. Similarly, plasmid HA-GluR2 $_{\Delta 844-853}$ was made by inverse PCR using primers AATATTAACCCATCTTCCTCGCAG and CGCCTCGGCCCTTGACTTGTAACA. PCR products were phosphorylated and religated. GluR2 $_{\Delta 854}$ was made by PCR using primers GGGAAACATGTAAAGGGTACCAT and GGGCTCGAGCCCGGGCTACTACTACTGTGGATTCTTTGC. The PCR products were digested with KpnI and XhoI and subcloned into the GluR2 plasmid (in pcDNA3) previously digested with the same enzymes. The HA-GluR2 $_{3Y-3A}$ plasmid was made using a Quick-Change Site Directed Mutagenesis Kit (Stratagene) using primers TTTGCAACTGCTAAGGAAGGTGCCAACGTAGCATGCATCGAGAGT and ACTCTCGATGCATGCTACGTTGGCACCTTCCTTAGCAGTTGCAA according to the manufacturer's instructions. To make the GST-GluR2 $_{3Y}$ plasmid, oligonucleotides AATTCTATAAGGAAGGTTACAACGTATATGGCATCTGAG and

TCGACTCAGATGCCATATACGTTGTAACCTTCCTTATAG were hybridized, phosphorylated on the 5' end using T4 polynucleotide kinase, and then ligated into the multiple cloning region of pGEX 4T-1 previously digested with EcoRI and SalI. Similarly, to make the GST-GluR2_{3A} plasmid, oligonucleotides AATTCGCTAAGGAAGGTGCCAACGTAGCTGGCATCTGAG and TCGACTCAGATGCCAGCTACGTTGGCACCTTCCTTAGCG were hybridized, phosphorylated, and cloned into pGEX4T-1 using the EcoRI and SalI restriction sites.

Colorimetric (Cell ELISA) assays: Colorimetric assays were performed essentially as previously reported (Man et al., 2000b). Briefly, HEK293 cells were transfected as described in Methods but with 10 or 15 µg of each plasmid in 10 cm or 15 cm dishes, respectively. Twenty-four hours after transfection, the cells from each large plate were detached and reseeded in a 12-well plate at a density of 2.5×10^5 cells/well. Forty-eight hours after transfection, cells were washed and treated with insulin as described in Materials and Methods. To minimize transfection efficiency-related variation, treatment and control studies were always performed on sets of 12-well plates derived from the same population of transfected cells. After treatment with insulin, cells were fixed using paraformaldehyde (2% in PBS for 3 min for non-permeant and 4% in PBS for 15 min for permeant conditions). Cells were then washed three times under either non-permeant (PBS alone) or permeant (PBS + 0.2% Triton X-100) conditions, blocked for 1 h at room temperature with 3% bovine serum albumin, and then incubated overnight at 4°C with an anti-HA antibody (1:2000) to detect HA-tagged receptors, or monoclonal antibody raised against an N-terminal sequence of GluR2/4 to detect wild type GluR2 (1:1000;

Pharmingen). The cells were then incubated for 1 h at room temperature with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP) (1:800, Amersham Biosciences) followed by incubation with 1 volume of the chromogenic HRP substrate OPD (Sigma) for approximately 2 min. Reactions were stopped with 0.2 volumes of 3 N HCl, and the absorbance at 492 nm of 1 ml of supernatant was measured using a spectrophotometer. To control for the specificity of the receptor detection, the primary antibody was omitted from the color reactions in two wells of each of the individual plates, and the absorbance readings were typically less than 2% of the specific readings obtained with the primary antibody. The levels of AMPA receptors expressed on the cell surface were presented as the ratio of colorimetric readings under non-permeabilized conditions to those under permeabilized conditions.

Hippocampal neuron cultures, transfection, and fluorescence-based internalization assays:

Medium-density hippocampal neurons were prepared from E18/19 rat embryos and maintained in Neurobasal medium containing B-27 supplement (Invitrogen) as described (Passafaro et al., 2001). Neurons were transfected at 14 days in vitro (DIV) using a calcium phosphate method and used for internalization experiments at 5-8 days posttransfection (DIV19-22). ELISA-based cell-surface receptor assays were performed as described for HEK cells using an antibody raised against an N-terminal sequence of GluR2/4 to detect wild type GluR2 (1:1000; Pharmingen). The fluorescence-based internalization assay was performed as described (Lee et al., 2002; Lin et al., 2000) with minor modifications. Briefly, HA-tagged surface AMPA receptors were “live”-labeled

with mouse anti-HA monoclonal antibody by incubating neurons in conditioned medium containing the antibody (10 $\mu\text{g/ml}$) for 10 min at 37°C. After brief washing in pre-warmed DMEM, neurons were returned to conditioned medium (control) or medium containing either 100 μM AMPA or 0.5 μM insulin and incubated for a further 10 min at 37°C. Subsequently, neurons were fixed in 4% formaldehyde/4% sucrose in PBS for 8 min at room temperature and surface receptors were visualized with Alexa488-conjugated secondary antibody (Molecular Probes). Internalized receptors were detected with Cy-3-conjugated secondary antibody (Jackson Laboratories) after permeabilizing cells in methanol (-20°C) for 1 min. Images were acquired using z-serial section scanning mode on an LSM 510 confocal microscope (Carl Zeiss). The same confocal acquisition setting was applied to all samples from the same experiment. Collected z-section images were first converted to projection images and analyzed using the Metamorph image analysis program (Universal Imaging Corporation). After setting threshold levels for green and red channels (the same threshold applied for each experimental set of images), integrated fluorescence values (which includes both area and intensity) from each channel were quantified. The “internalization index” for transfected GluR2 was determined by dividing the computed red fluorescence (surface staining) by the green fluorescence (total staining).

Hippocampal slice preparation and electrophysiological recordings: Hippocampal slices (400 : m thickness) were prepared from Sprague-Dawley rats aged 16-26 postnatal days and perfused at room temperature with artificial cerebrospinal fluid containing (mM): 126 NaCl, 26 NaHCO₃, 10 glucose, 3 KCl, 1.2 KH₂PO₄, 1 MgCl₂, and 1 CaCl₂,

saturated with 95% O₂/5% CO₂ (Man et al., 2000b). The recording pipettes (4-5 MΩ) were filled with solution containing (mM): 135 CsCl, 10 HEPES, 5 QX-314, 4 Mg-ATP, 2 MgCl₂, 0.5 EGTA, 0.2 GTP and 0.1 CaCl₂, pH 7.4, 310 mOsm. Whole-cell recording of CA1 neurons and the induction of LFS-LTD were performed as previously described (Man et al., 2000b). Series and input resistance were monitored throughout each experiment by injecting a test pulse (10 ms) through recording pipettes prior to each stimulation and cells were excluded from data analysis if a greater than 15% change in the series or input resistance occurred during the course of the experiment.