Supporting information

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SI Materials and Methods

Antibodies and Plasmids. Rabbit polyclonal anti-GST (#06-332), mouse monoclonal anti-Src (clone GD11, #05-184), and rabbit monoclonal anti-GluK2/3 (clone NL9, #04-921) and anti-JNK3 (clone C05T, #04-893) antibodies were purchased from Millipore Biotechnology. Goat polyclonal anti-GluK2 (sc-7618), rabbit polyclonal anti-Actin (sc-10731) and anti-p-c-Jun (sc-16312), and mouse monoclonal anti-p-JNKs (clone G-7, sc-6254) antibodies were obtained from Santa Cruz Biotechnology. Rabbit monoclonal anti-c-Jun (clone 60A8, #9165) antibody was purchased from Cell Signaling Biotechnology. Mouse monoclonal anti-phosphotyrosine (PT-66, P3300) antibody, alkaline phosphatase-conjugated secondary antibodies, and nonspecific mouse IgG and goat IgG were obtained from Sigma Biotechnology.

The vector pcDNA3.1 (+) (Invitrogen) was used to express Src, GluK2, and various mutant constructs. The cDNA of full-length wild-type GluK2 was amplified by RT-PCR. The constructs of GluK2 and single tyrosine mutants (Y587F, Y590F, Y844F, and Y590E) were cloned by QuikChange Lightning Site-Directed Mutagenesis (Agilent Technologies). The constitutively active (SrcY529F) and inactive (SrcK297R) forms of Src were described previously (1). Vectors expressing HaloTag (pHTC) and NanoLuc luciferase (pNLF1-N or pNLF1-C) were purchased from Promega. All constructs were verified by DNA sequencing.

Brain Ischemia and Drug Administration. Adult male Sprague-Dawley rats weighing 220–260 g were kept on a 12-h light/dark cycle with free access to food and water before surgery. All experiments were performed following the guidelines of the local Animal Care and Use Committee. Transient brain ischemia was induced by the four-vessel occlusion method as described previously (1). Briefly, vertebral arteries were electrocauterized under anesthesia with chloral hydrate (300–350 mg/kg, i.p. injection). Electrocauterized rats were allowed to recover for 24 h. After an overnight fast, ischemia was induced by occluding carotid arteries with aneurysm clips; blood flow was restored by releasing the clips. A sham operation was performed using the same surgical procedures except occlusion of carotid arteries. PP2, PP3, SU6656 (Calbiochem/Millipore), or the same dose of the vehicle, DMSO, was delivered by microinjection into the right cerebral ventricles (anteroposterior, 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm from bregma) 30 min before inducing ischemia. After the indicated times of ischemia or reperfusion, the hippocampal CA1 regions were isolated rapidly and frozen in liquid nitrogen.

Primary Culture of Hippocampal and Cortical Neurons. Primary hippocampal and cortical neurons from 18-d-old embryonic Sprague-Dawley rats were plated onto poly-D-lysine–coated culture dishes as previously described (1, 2). The culture medium (Neurobasal serum-free medium supplemented with 2% (vol/vol) B27 and 0.5 mmol/L glutamine) was replaced every 3 d. After 14 d in vitro, the neuronal cultures were subjected to whole-cell patch-clamp recording or excitotoxicity induced by kainate (100 μmol/L) or NMDA (100 μmol/L) for 5, 10, or 30 min.

Surface Biotinylation. HEK293 cells were maintained in DMEM (Gibco/Invitrogen) supplemented with 10% (vol/vol) FBS and transfected with the indicated expression plasmids using polyethylenimine. After 24 h, HEK293 cultures were surface biotinylated on ice using EZ-link Sulfo-NHS-SS-Biotin (Pierce/Thermo). The cells were lysed in TNE buffer [50 mmol/L Tris·HCl (pH 8.0), 50 mmol/L NaF, 1% Nonidet P-40, 20 mmol/L EDTA, and 0.1% SDS] and then incubated with immobilized NeutrAvidin agarose (Pierce) to precipitate biotinylated surface proteins. Surface proteins and total cell lysates were analyzed by immunoblot.

Immunoprecipitation. Hippocampal samples were homogenized, and cell cultures were lysed in ice-cold buffer [in mmol/L: Mops (pH 7.4) 50, sucrose 320, KCl 100, $MgCl_2$ 0.5, and inhibitors of proteases and phosphatases (β-glycerophosphate 20, sodium pyrophosphate 20, NaF 50, 1 mmol/L each of EDTA, EGTA, phenylmethylsulfonyl fluoride, benzamidine, sodium orthovanadate, and p-nitrophenyl phosphate, and 5 μg/mL each of aprotinin, leupeptin, and pepstatin A]. After centrifugation at 800 × *g*, the supernatant was collected, and protein concentration was determined by the Lowry method. Samples were stored at −80 °C until use. Hippocampal samples or cell lysates were incubated overnight at 4 °C with appropriate antibodies diluted in immunoprecipitation buffer [in mmol/L: Hepes 50 (pH 7.4), NaCl 150, glycerol 10% (vol/vol), $ZnCl_2$ 1, $MgCl_2$ 1.5, Triton X-100 1%, Nonidet P-40 0.5%, and the phosphatase and protease inhibitors mentioned above]. The mixture was then incubated with protein A-Sepharose CL-4B at 4 °C for an additional 2 h. Samples were then washed three times with immunoprecipitation buffer and eluted by boiling for 5 min in 4× Laemmli sample buffer.

Immunoblot. Sample proteins solubilized in 4× Laemmli sample buffer were separated by SDS/PAGE and then electrotransferred onto a nitrocellulose membrane (pore size, 0.45 μm). After blocking in 3% (wt/vol) BSA, the membrane was incubated with the indicated primary antibody. Detection was carried out by appropriate alkaline phosphatase-conjugated IgG and developed with the NBT/BCIP assay kit (Promega). Membrane bands were scanned and analyzed with Quantity One 1-D Analysis Software (Bio-Rad). Results are expressed as mean ± SD.

Electrophysiology. Whole-cell patch-clamp recording was performed in voltage-clamp mode using an Axon-700B amplifier (membrane voltage, −60 mV). The data were collected with Clampex 9.2 software and analyzed with Clampfit 9.2 software. The resistance of pipette was 3–4 MΩ when filled with the intracellular solution [in mmol/L: Hepes 10 (pH 7.2), CsCl 120, triethylamine 20, $MgCl_2$ 1, EGTA 10, and ATP 2]; the osmolarity was adjusted to 290–310 mOsm/L with sucrose. The standard extracellular solution contains (in mmol/L): Hepes 10 (pH 7.4), NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, and glucose 10; osmolarity was 310–330 mOsm/L. The whole-cell currents were stimulated by kainate (300 μmol/L, with GYKI-52466 100 μmol/L) in neuronal cultures or glutamate (1 mmol/L) in GluK2-expressing HEK293 cells through a Y-tube perfusion system. All results are expressed as mean ± SEM.

GST Pull-Down Assay. The full-length cDNA encoding wild-type Src was subcloned into the pGEX-4T-1 vector. Recombinant GST-Src and GST proteins were individually expressed in BL21(DE3) cells and purified using a GST protein interaction pull-down kit (Pierce). GluK2-overexpressing HEK293 cells were lysed in TNE buffer and incubated with immobilized GST-tagged proteins. The bound protein was eluted and analyzed by immunoblot.

Bioluminescence Resonance Energy Transfer Assay. HEK293 cells cultured in six-well plates were transfected with plasmids expressing GluK2-C-HT and Src-C-NLuc (optimal ratio, 20:1). After 24 h, cells (5×10^4) were resuspended in phenol red-free

medium and seeded in 96-well white plates (Corning) in the absence (control) or presence of the fluorescent HaloTag TMR ligand (0.5 μmol/L; Promega). After incubation for 4–18 h, the NanoLuc furimazine substrate (2 μmol/L; Promega) was added to both control and experimental groups. After 5 min, emission at 495 nm (donor) and 600 nm (acceptor) was measured using the GloMax Discover Detection System (Promega). The NanoBRET (BRET, bioluminescence resonance energy transfer) ratio is defined as the corrected ratio of acceptor/donor emission × 1,000 (3). Experiments are independently repeated at least three times, and results are expressed as mean ± SEM from a single experiment performed in triplicate.

Calcium Imaging. Intracellular calcium ($[Ca^{2+}]_i$) was measured using a Ca^{2+}-sensitive fluorescent dye Fura-2/AM (Sigma). Fura-2 is an UV-excited Ca^{2+} indicator that allows ratiometric measurement. For ratiometric measurements, excitation at 340 and 380 nm is usually preferred. When the dye is excited at 340 nm, Ca^{2+} binding produces an increase in fluorescence, and a decrease in fluorescence is observed when the dye is excited at 380 nm. Fura-2 fluorescence was excited alternately at 340 and 380 nm using the Lambda DG-4 Ultra High Speed Wavelength Switcher, and the excitation light was focused on the cells via a ×20 objective. The emitted fluorescence was collected at 510 nm by a high-speed EMCCD camera, and the ratio of fluorescence at 340 nm to fluorescence at 380 nm was directly recorded with MetaFluor software, indicating $[Ca^{2+}]_i$ fluctuations. Results are expressed as mean ± SEM.

Statistical Analysis. Differences between the means of two groups were evaluated by Student *t* test. Multiple groups were compared by one-way ANOVA followed by Fisher's least significant difference test. $P < 0.05$ was considered significant.

1. Du CP, et al. (2009) Increased tyrosine phosphorylation of PSD-95 by Src family kinases after brain ischaemia. *Biochem J* 417(1):277–285.
2. Xu Y, Hou XY, Liu Y, Zong YY (2009) Different protection of K252a and N-acetyl-L-cysteine against amyloid-beta peptide-induced cortical neuron apoptosis involving inhibition of MLK3-MKK7-JNK3 signal cascades. *J Neurosci Res* 87(4):918–927.
3. Deplus R, et al. (2013) TET2 and TET3 regulate GlcNAcylation and H3K4 methylation through OGT and SET1/COMPASS. *EMBO J* 32(5):645–655.

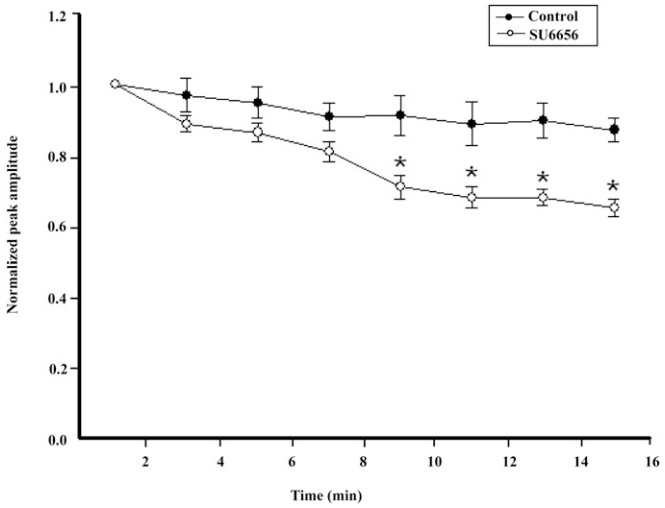

Fig. S1. Src family kinase inhibitor, SU6656, depresses kainate receptor-mediated currents in cultured hippocampal neurons. SU6656 (1 μmol/L, $n = 9$) or the vehicle control (DMSO, $n = 7$) was added to the patch pipette internal solution. After the whole-cell configuration was established, kainate receptor-mediated currents were elicited by kainate application (300 μmol/L kainate + 100 μmol/L GYKI-52466) at different time points. Kainate receptor-mediated currents are normalized to the peak current of the first recording. Results are expressed as mean ± SEM; $*P < 0.05$ compared with control at the corresponding time point.

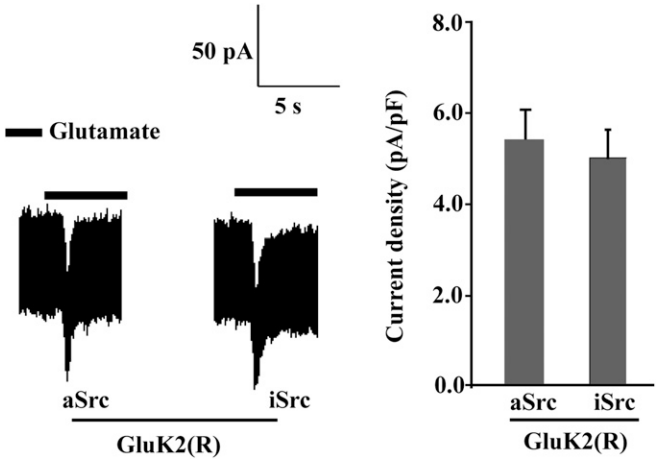

Fig. S2. Constitutively active Src kinases do not increase GluK2(R)-mediated currents induced by glutamate. Whole-cell recordings of GluK2(R)-mediated current from HEK293 cells cotransfected with wild-type GluK2(R) and either the constitutively active Src (aSrc) or inactive Src (iSrc). Results are expressed as current density (mean ± SEM; $n = 12$).

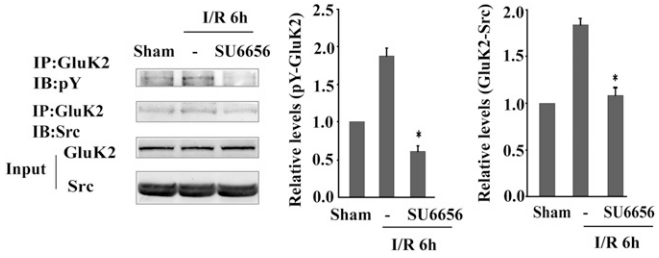

Fig. S3. Src family kinase inhibitor SU6656 prevents GluK2 tyrosine phosphorylation and GluK2-Src binding after 6-h reperfusion after 15-min ischemia (I/R6h) in the rat hippocampal CA1 region. SU6656 (10 nmol per rat in 5 μL DMSO) was administered by unilateral intracerebroventricular infusion 30 min before inducing ischemia. Tyrosine phosphorylation of GluK2 (pY-GluK2) and GluK2-Src binding were examined by immunoprecipitation (IP) followed by immunoblot (IB). Results are normalized to the sham control and expressed as mean ± SD ($n = 3$); *$P < 0.05$ vs. untreated rats that underwent I/R.

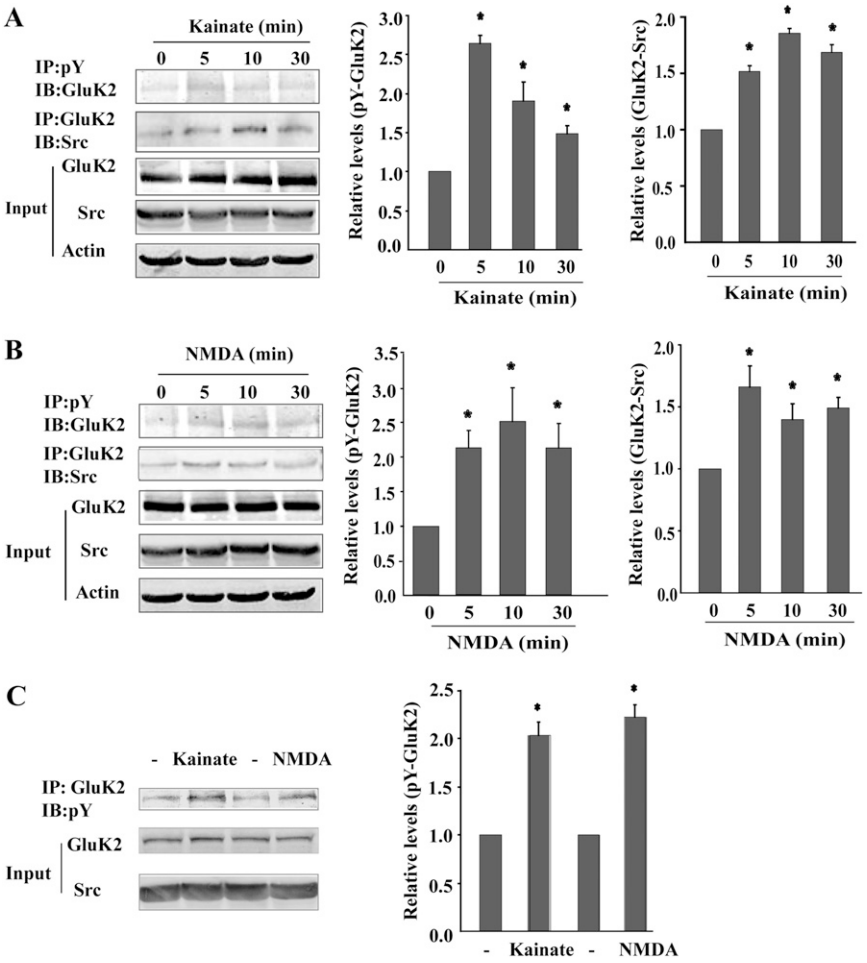

Fig. S4. Both kainate and NMDA receptors participate in the regulation of GluK2 tyrosine phosphorylation. Results are normalized to the untreated control and expressed as mean ± SD ($n = 3$); *$P < 0.05$ vs. untreated group. (*A* and *B*) GluK2 tyrosine phosphorylation and GluK2-Src binding after treating cultured cortical neurons with kainate (100 μmol/L) and NMDA (100 μmol/L) for the indicated times. GluK2 tyrosine phosphorylation (pY-GluK2) was examined by immunoprecipitation (IP) with an anti-phosphotyrosine (pY) antibody followed by immunoblot (IB) with an anti-GluK2 antibody. (*C*) Tyrosine phosphorylation of GluK2 increases after treating cultured cortical neurons with kainate or NMDA for 10 min. GluK2 tyrosine phosphorylation (pY-GluK2) was examined by immunoprecipitation (IP) with an anti-GluK2 antibody followed by immunoblot (IB) with an anti-phosphotyrosine (pY) antibody.

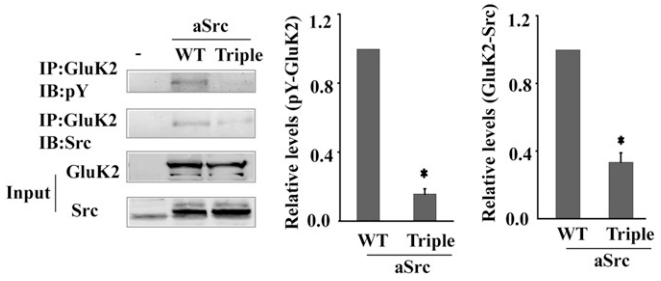

Fig. S5. The triple Y587/590/844F mutant nearly abolishes GluK2 tyrosine phosphorylation. Wild-type GluK2 (WT) or the Y587/590/844F triple mutant (Triple) was coexpressed with constructively active Src (aSrc) in HEK293 cells. Tyrosine phosphorylation of GluK2 (pY-GluK2) and GluK2-Src binding were examined by immunoprecipitation (IP) followed by immunoblot (IB). Results are normalized to the WT group and expressed as mean ± SD ($n = 3$); *$P < 0.05$ vs. WT.

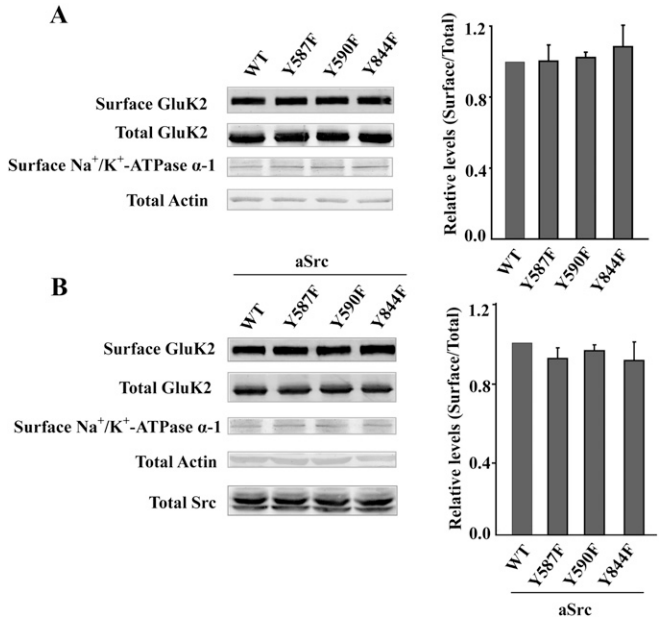

Fig. S6. A single tyrosine mutation at Y587F, Y590F, or Y844F does not change surface localization of GluK2. (*A*) Constructs encoding wild-type GluK2 (WT) or the single tyrosine mutants (Y587F, Y590F, or Y844F) were transfected into HEK293 cells. (*B*) Wild-type GluK2 (WT) or a single tyrosine mutant (Y587F, Y590F, or Y844F) was coexpressed with constitutively active Src (aSrc) in HEK293 cells. Surface localization was evaluated using the biotinylation assay. Surface biotinylated and total GluK2 were detected by immunoblotting using an anti-GluK2/3 antibody. Na^+/K^+ ATPase α-1 subunits served as an internal control to show that membrane protein levels were the same across samples, and Actin served as a loading control for total protein. The surface/total ratio of GluK2 is normalized to WT. Results are expressed as mean ± SD ($n = 3$); $^*P < 0.05$ vs. WT.