Supporting Information

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SI Materials and Methods

Dissociated Neuronal Cultures. In brief, hippocampi and cortices from E18 Wistar rats were dissected, and the neurons were dissociated by enzymatic digestion with trypsin for 15 min followed by mechanical dissociation. Cells were plated at a density of 1,000,000 per 60-mm dish (cortical) or 100,000 (hippocampal) onto 22-mm glass coverslips coated with poly-L-lysine (Sigma-Aldrich). The culture medium was Neurobasal medium (Gibco) supplemented with horse serum (10%), B27 (Gibco), and 2 mM glutamine. On the second day, the media was changed for Neurobasal medium supplemented with B27, and neurons were fed each week with this glutamine-free medium until use (16–21 d in vitro).

Sindbis Viruses. YFP-myc-GluK2a (the variant comprising the full-length C terminus, including the SUMOylation motif) was constructed by inserting YFP at the 5′-ClaI site of the myc tag of pcDNA3-myc-GluK2. All mutations were introduced in pcDNA3-YFP-myc-GluK2 by site-directed mutagenesis using the QuickChangeXL Kit (Stratagene). Attenuated Sindbis virus-expressing YFP-myc-GluK2 was prepared and used as described previously (1). Neurons were transduced at at 17–20 d in vitro (DIV) and then returned to the incubator for an additional 18–24 h before use.

Quantitative Western Blots. YFP-myc-tagged GluK2 expressed in COS-7 cells was detected with sheep anti-myc antibody produced in-house. Monoclonal anti-GluK2 (NL9; Millipore) was used for detection of purified GluK2 C-termini in in vitro assays, and rabbit anti-GluK2 (Millipore) was used for endogenous GluK2 in neurons. For biotinylation experiments, monoclonal anti–β-actin (Sigma-Aldrich) was used to determine the specificity of labeling. Western blots were developed using ECL reagents, and analyzed using ImageJ. To quantify the level of GluK2 SUMOylation, the ratio of SUMOylated to non-SUMOylated GluK2 was determined. Because of the relatively low amount of SUMO-conjugated GluK2, blots were initially exposed to obtain a nonsaturated value for unconjugated GluK2 and then reexposed for a longer period to quantify the less intense higher molecular weight SUMOylated GluK2 band.

Phosphorylation Analysis. Cultured cortical neurons (14–18 DIV) were infected with YFP-myc-GluK2 for 20 h and stimulated with 20 μM KA for 5 min. Cells were then lysed in lysis buffer [1% Triton X-100, 0.1% SDS, 150 mM NaCl, 20 mM Hepes (pH 7.5), 1 mM EGTA, 50 mM sodium fluoride, and 10 mM sodium pyrophosphate], sonicated, and incubated on ice for 20 min. Lysates were cleared by centrifugation, and the resulting supernatant was incubated with 2 μg of rabbit anti-myc (Santa Cruz Biotechnology) antibody for 1 h at 4 °C. After incubation with protein-A Sepharose beads for another 1 h, the beads were washed three times in lysis buffer, and proteins were eluted with SDS/PAGE sample buffer. Samples were resolved using SDS/PAGE, and immunoblotted with anti-phosphoserine/threonine (BD Biosciences) and mouse anti-myc antibodies (9e10; Santa Cruz Biotechnology).

Immunofluorescence and Confocal Imaging. Hippocampal neurons were incubated for 30 min at 37 °C with kainate (KA) (10 μM), PMA (1 μm), KA/chelerythrine (10 μM/1 μm), or chelerythrine (1 μM) or without any added drug. For NMDA treatment, neurons were stimulated at 37 °C with NMDA (30 μM) for 3 min. Neurons were fixed for 20 min with 2% paraformaldehyde in PBS with 2.5% sucrose, permeabilized with digitonin (D141; Sigma-Aldrich) for 10 min, incubated with 10% horse serum for 20 min, and incubated with anti-GluK2 (rabbit polyclonal; Millipore; 1:100) and anti–SUMO-1 (mouse monoclonal 21C7; Developmental Studies Hybridoma Bank; 1:100) for 60 min at room temperature. Neurons were labeled with Cy2 anti-rabbit and Cy3 anti-mouse antibodies. Neurons transduced with Sindbis virus expressing YFP-myc-GluK2 were treated with 20 μM KA, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich), and stained with anti–SUMO-1 followed by anti-mouse Cy3. Confocal images were acquired with a Zeiss LSM 510 confocal microscope and quantified using the Manders colocalization coefficient (i.e., percentage of GluK2 colocalizing with SUMO-1) in ImageJ. At least 10 cells for each condition from three independent experiments were analyzed using identical confocal acquisition parameters. Data are expressed as mean ± SEM, and significance was determined using unpaired *t* tests.

In Vitro Phosphorylation/SUMOylation Assays. The GST-tagged C-terminus of GluK2 (GST-CT-GluK2) was mutated using site-directed mutagenesis (QuikChange; Agilent) to create non-SUMOylatable (K886R), phosphomimetic (S846D and S868D), and phospho-null (S846A and S868A) mutants. Proteins were purified from BL21(DE3) *Escherichia coli* using glutathione Sepharose 4B beads (Pharmacia), as described previously (2), diluted in PBS, and stored at −80 °C until use. Purified protein was phosphorylated in 20 mM Hepes (pH 7.4), 1.67 mM $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 200 mM ATP, and 25 ng of purified PKC (Promega) at 30 °C for 30 min (3). The reaction mix was then used as a substrate for in vitro SUMOylation, performed according to the manufacturer's instructions (Enzo Life Sciences). GST-CT-GluK2 WT and mutants were also subjected to the in vitro SUMOylation procedure without previous phosphorylation. The reactions were stopped by adding sample buffer and resolved by SDS/PAGE.

KA Stimulation and Surface Biotinylation. Neurons were preincubated in 2 μM TTX for 1 h, washed with Earle's buffer (25 mM Hepes, Tris buffered to pH 7.4, 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM glucose) and incubated with 20 μM KA for 20 min in Earle's buffer. After KA stimulation, neuronal cultures were chilled on ice, washed twice with ice-cold PBS, and incubated with 0.15 mg/mL of sulfo-NHS-SS-biotin (Pierce) in PBS for 10 min on ice. Cells were then washed two times with cold PBS, incubated for 5 min in 50 mM NH_4Cl in PBS, washed three times with PBS, and lysed in lysis buffer [25 mM Hepes (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS]. After centrifugation, lysate was incubated with streptavidin-agarose beads for 3 h at 4 °C and washed four times in lysis buffer, after which bound proteins were detected by Western blot analysis. Surface biotinylation in COS-7 cells expressing YFP-myc-GluK2 was performed identically, but omitting incubation in Earle's buffer and KA stimulation.

Pulldown of SUMOylated Virally Expressed YFP-myc-GluK2. Neurons were infected with Sindbis virus expressing YFP-myc-GluK2 WT, S868A, or K886R 20 h before the experiments. Cells were stimulated with KA and lysed in lysis buffer as described above, but with the addition of 20 mM *N*-ethylmaleimide. YFP-myc-GluK2 was then pulled down using GFP-Trap A beads (ChromoTek) overnight at 4 °C. After three washes in lysis buffer, the bound proteins were detected by Western blot analysis with a sheep anti–SUMO-1 antibody. The membranes were then stripped and reprobed with rabbit polyclonal anti-GluK2 antibody (Millipore) to determine the total amount of YFP-myc-GluK2 pulled down. Corresponding input fractions were probed with the sheep anti-myc antibody.

HEK293 Cell Transfection and Electrophysiology. HEK293 cells were transfected on 22-mm coverslips in six-well dishes with 2 μg of DNA encoding YFP-myc-GluK2 WT, S868A, or S846A, using Lipofectamine 2000 (Invitrogen). Whole-cell patch clamp recordings were made 48 h after transfection using an Axopatch 200B amplifier and pClamp 10.2 software (Molecular Devices). Borosilicate patch electrodes had series resistances of 2–6 MΩ. The 305-mOsm extracellular solution contained 135 mM NaCl, 5 mM KCl, 30 mM glucose, 2 mM CaCl2, 1 mM MgCl2, and 10 mM Hepes (pH 7.3). The intracellular solution contained 117 mM CsMeSO4, 8 mM NaCl, 10 mM Hepes, 5 mM QX-314Cl, 4 mM MgATP, 0.3 mM NaGTP, 0.2 mM EGTA, 0.1 mM bestatin, and 0.1 mM leupeptin at pH 7.4 and 280 mOsm. Before whole-cell configurations were obtained, cells were lifted from the coverslip into a laminar stream of extracellular solution containing conconavalin A (0.3 mg/mL) to block receptor desensitization. 500-ms KA (100 μM) applications were then performed using a multibarrel fast perfusion system controlled by Clampex software. The first response amplitude was obtained within 30 s of achieving a whole-cell configuration. Analysis was performed off-line using Clampfit software. Data are expressed as mean ± SEM. The unpaired Student t test and one-way ANOVA were performed with a Newman–Keuls posttest for multiple-comparison datasets when required.

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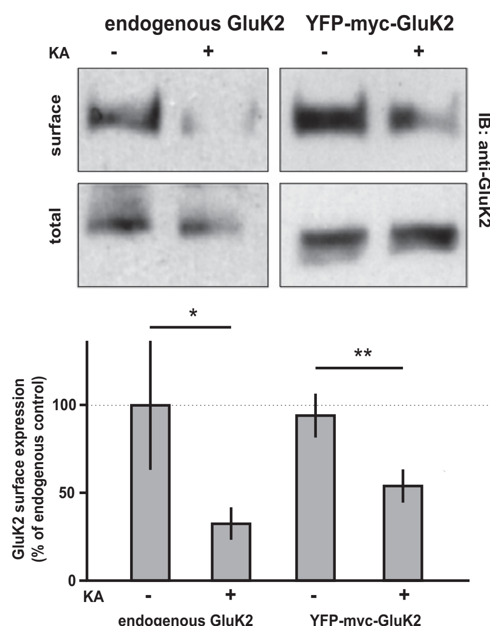

Fig. S1. Comparison of the surface expression of endogenous GluK2 and virally expressed YFP-myc-GluK2 in neurons. Total (12%) and surface-expressed biotinylated GluK2 was detected with rabbit polyclonal anti-GluK2 antibody. The graph shows normalized surface expression of GluK2 vs. YFP-myc-GluK2 in control and KA-stimulated neurons. *$P < 0.05$; $n = 5$.

Fig. S2. In vitro SUMOylation of PKC phosphomimetic/phospho-null mutants of GST-CT-GluK2 probed using anti-GluK2 antibody. The graph quantifies in vitro SUMOylation. For each lane, the intensity of the upper (SUMO-modified) band was divided by intensity of the lower (nonmodified) band, and results were normalized to the WT. $^{**}P < 0.01$; $n = 7$.

Fig. S3. Surface biotinylation of COS-7 cells expressing WT and serine mutants of YFP-myc-GluK2. Total (10%) and surface-expressed biotinylated GluK2 were detected with anti-myc antibody. The lower panels show the same blots reprobed for β-actin. The graph quantifies the surface expression of GluK2 WT and serine mutants, normalized to the WT. $^{*}P < 0.05$; $n = 10$.

Fig. S4. Surface biotinylation of COS-7 cells expressing WT, non-SUMOylatable mutant (K886R), single phosphomimetic mutants, and non-SUMOylatable phosphomimetic double mutants (K886R + S846D and K886R + S868D) of YFP-myc-GluK2. Total (20%) and surface-expressed biotinylated GluK2 were detected with anti-myc antibody. The lower panels show the same blots probed for β-actin. The graph quantifies the surface expression of GluK2 WT, K886R, phosphomimetic, and phosphomimetic/non-SUMOylatable mutants, normalized to the WT. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 3$.

Fig. S5. (*A*) SUMOylation of virally expressed YFP-myc-GluK2 in neurons. Immunoprecipitated YFP-myc-GluK2 was probed with SUMO-1 antibody. Note the lack of a SUMOylated band in the K886R mutant of GluK2, confirming the specificity of the pulldowns. (*B*) The increase in GluK2 SUMOylation after KA stimulation in neurons is abolished by mutation of S868 to a nonphosphorylatable alanine. The blots are representative images of at least three independent experiments.