Supporting Information

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SI Materials and Methods

Electrophysiology. Parasagittal cerebellar slices (200-μm thick) were prepared from WT, *δ2 glutamate receptor* (*GluD2*) -null, or *megakaryocyte protein tyrosine phosphatase (PTPMEG)*-null mice on postnatal day (P) 21–35 as described previously (1). Whole-cell patch-clamp recordings were made from visually identified Purkinje cells using a 60× water-immersion objective attached to an upright microscope (BX51WI; Olympus) at room temperature. The resistance of patch pipettes was 3–5 MΩ when filled with an intracellular solution of the following composition: 110 mM K-gluconate, 50 mM Hepes, 10 mM KCl, 4 mM $MgCl_2$, 4 mM Na_2ATP, 1 mM Na_2GTP, and 5 mM sucrose (pH 7.23, 298 mOsm/kg) for slow excitatory postsynaptic current ($_{slow}$EPSC) recordings and 65 mM Cs-methanesulfonate, 65 mM K-gluconate, 20 mM Hepes, 10 mM KCl, 1 mM $MgCl_2$, 4 mM Na_2ATP, 1 mM Na_2GTP, 5 mM sucrose, and 0.4 mM EGTA (pH 7.25, 295 mOsm/kg) for long-term depression (LTD) recordings. Unphosphorylated (pep-3Y; YKEGYNVYG), phosphorylated (pep-3pY; pY-KEGpYNVpYG), or unphosphorable (pep-3A; AKEGANVAG) peptide was added to the patch pipette solution at the concentration of 500 μM for the decoy–peptide experiments. The peptides were provided by Operon Biotechnologies. A 4-amino-1-*tert*-butyl-3-(1′-naphthyl)pyrazolo[3,4-d]pyrimidine (PP1 analog; 10 μM; Calbiochem/Merck) was also applied through the pipette. The extracellular solution [artificial cerebrospinal fluid (ACSF)] used for slice storage and recording consisted of 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM D-glucose bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Picrotoxin (100 μM; Sigma) was always added to ACSF to block inhibitory synaptic transmission. Parallel fiber (PF) -EPSCs were elicited by application of square pulses to a stimulating electrode placed on the molecular layer, and the selective PF stimulation was confirmed by paired-pulse facilitation of EPSCs with a 50-ms interstimulus interval.

$_{slow}$EPSCs were elicited from Purkinje cells voltage-clamped at −80 mV by the application of tetanic stimulation (2–10 times at 100 Hz) to PFs in the presence of 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (100 μM). Before the $_{slow}$EPSC recordings, AMPA receptor-mediated PF-EPSCs were recorded in the absence of NBQX to normalize $_{slow}$EPSCs amplitudes and transferred charges. For the experiments of the LTD session, PF-EPSCs were recorded successively at a frequency of 0.1 Hz from Purkinje cells voltage-clamped at −80 mV. After stable PF-EPSCs were obtained at least for 10 min, a conjunctive stimulation [30 × (PF stimulus together with 500-ms depolarizing pulses from −60 to +20 mV) at 1 Hz] was applied. Access resistance was monitored every 10 s by measuring the peak currents evoked by 2-mV, 50-ms hyperpolarizing steps throughout the experiments. The measurements were discarded if access resistance changed by more than 20% of its original value. Current responses were recorded using an Axopatch 200B amplifier (Molecular Devices), and the pCLAMP system (version 9.2; Molecular Devices) was used for data acquisition and analysis. The signals were filtered at 1 kHz and digitized at 4 kHz.

Virus Vector Constructs and in Vivo Microinjection. For transduction of mutant transgenes into cerebellar Purkinje cells, we used a modified Sindbis virus vector (Invitrogen), which contained an additional subgenomic promoter and GFP (2, 3). When we transduced GluA2, we used GluA2, which carried glutamine (Q) in its Q/R RNA editing site to increase cell surface expression.

Surgery for microinjection into the mouse cerebellum was described previously. Briefly, mice were anesthetized by an i.p. injection of ketamine (80 mg/kg) and xylazine (20 mg/kg; Sigma). A glass pipette was inserted into the cerebellum 500–800 μm from the surface, and 0.5–2 μL virus solution were injected using a Nanoliter (World Precision Instruments). The mice were subjected to additional experimental manipulations 15–36 h after the virus injection. All procedures relating to the care and treatment of animals were performed in accordance with National Institutes of Health guidelines and permitted by Keio University Experimental Animal Committee.

GluA2 Phosphorylation Levels in the Cerebellum. To study baseline phosphorylation of GluA2–tyrosine 876 (Y876) and -serine 880 (S880), the synaptosomal fraction was extracted from WT, *GluD2*-null, or *PTPMEG*-null cerebella. The whole cerebellum from a P21–P30 mouse was quickly dissected out and homogenized in 0.32 M sucrose in buffer A composed of 5 mM Hepes (pH 7.3) and 0.1 mM EDTA supplemented with protease inhibitors (Calbiochem), serine/threonine phosphatase inhibitors (Sigma), and 1 mM Na vanadate. After 1,000 × *g* centrifugation for 5 min at 4 °C, the supernatant was collected and centrifuged at 12,000 × *g* for 20 min. The resulting pellet was suspended with 0.32 M sucrose/buffer A overlaid on a gradient of 0.8 and 1.2 M sucrose/buffer A and ultracentrifuged at 105,000 × *g* for 2 h at 4 °C. The synaptosomal fraction was suspended in the 5× volume of 0.32 M sucrose/buffer A and centrifuged at 12,000 × *g* for 10 min. We added 200 μL 2× SDS/PAGE buffer to the pellet and boiled it for 5 min. Protein concentration was estimated by BCA protein assay kit (Thermo Scientific); 10–15 μg synaptosomal fraction protein were electrophoresed on the 5–20% gradient SDS polyacrylamide gel and transferred to the PVDF membrane (Immobilon). The antibody against Y876-phosphorylated GluA2 was donated by R. Huganir (Johns Hopkins University School of Medicine, Baltimore, MD) or purchased from Cell Signaling Technology. To detect S880 phosphorylation, we generated the polyclonal antibody against S880-phosphorylated GluA2 using a phosphorylated synthetic peptide of the most C-terminal 11 aa of GluA2 (YNVYGIEpSVKI) as an antigen. The specificity of the antibody was confirmed by in vitro phosphorylation assay using the C terminus of GluA2 (Fig. S8). Immunoreactive materials were detected with Immobilon Western (MilliPore), and its chemiluminescence signals were obtained by LAS-3000 (Fujifilm).

GluA2 Phosphorylation in Chemical LTD. Sagittal cerebellar slices from the P21–P35 mice were prepared as described above. After 1 h incubation in ACSF containing 100 μM picrotoxin, the slices were transferred to a humidified and oxygenated treatment chamber and recovered for additional 30 min. To induce chemical LTD, cerebellar slices were exposed to the K-glu solution (ACSF supplemented with 50 mM KCl and 10 μM L-glutamate) for 5 min. After 10 min washing, the slices were immediately frozen with liquid nitrogen. To obtain the lysates, we added the lysis buffer containing 150 mM NaCl, 50 mM Tris·HCl (pH 7.5), 5 mM EDTA, 1 mM Na vanadate, and 1% SDS with protease inhibitors and serine/threonine phosphatase inhibitors and applied sonication at 4 °C. The samples were boiled and centrifuged at 12,000 × *g* for 15 min. An equal volume of 2× SDS/PAGE buffer was added to the supernatant; 10–15 μg protein from each lysate were subjected to SDS/PAGE and analyzed by Western blotting. To induce chemical LTD in the presence of a PP1 analog, we pretreated cerebellar slices with 50 μM PP1 analog for 30 min and included

the PP1 analog throughout the LTD experiment. Potential bias was ruled out by performing posthoc immunoblot analyses in a blinded manner without any knowledge of the genotypes.

GluA2 Dephosphorylation in HEK293 Cells. pTracer-GluA2 and pCAGGS-HA-PTPMEG or -HA-PTPMEG^{DA} were cotransfected to HEK293 cells by the calcium phosphate method (CellPhect; BD Biosciences). After overnight incubation, cells were collected and treated with 2× SDS/PAGE loading buffer. The sonicated samples were boiled for 5 min, and 10 μg extracted protein were used for Western blot analysis. Quantification of Western blot was performed with MultiGauge (Fujifilm). For quantitative comparison, immunoreactivity of phosphorylated GluA2 was normalized by immunoreactivity of GluA2, and the ratio to the control group was calculated.

In Vitro Phosphorylation of GST-GluA2 C-Terminal Cytoplasmic Region. GST-fusion protein of the GluA2 C-terminal cytoplasmic region (GST-GluA2-CT) was prepared. After autophosphorylation reaction of Src (Invitrogen) in the buffer containing 20 mM Hepes (pH 7.35), 3 mM $MnCl_2$, and 0.1 mM ATP at 30 °C for 15 min, 0.5–1 μg GST-GluA2-CT were added to the reaction solution and allowed for phosphorylation reaction by Src for 2 h. Then, the reaction solution was boiled to inactivate Src. $CaCl_2$, $MgCl_2$, DTT, and ATP (1.67, 10, 1, and 0.05 mM in final concentration, respectively) were added to the solution for the second phosphorylation reaction by PKC (Promega) at 30 °C for 2 h. The 2× SDS/PAGE buffer was added, and the samples were boiled.

Substrate Trap Assay. We prepared the catalytic domain of PTPMEG and its D820A mutant fused with the GST (GST-PTP^{WT} and GST-PTP^{DA}, respectively) and quantified their protein content by BCA protein assay kit (Thermo Scientific); 18 h after transfection of pCAGGS-GluA2 with Lipofectamine 2000 (Invitrogen), HEK293 cells were treated with the pervanadate solution composed of 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes (pH 7.35), 1 mM H_2O_2, and 0.1 mM Na vanadate for 30 min at 37 °C. Cells were washed with PBS two times and solubilized by the buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Hepes (pH 7.35), 50 mM NaF, and 1% TritonX-100 supplemented with the protease inhibitor mixture (Calbiochem) at 4 °C for 1 h. GST-fusion protein (4 μg) was added to the lysate and rotated at 4 °C for 1 h. Then, the samples were incubated with glutathione Sepharose beads (GE Healthcare) at 4 °C for an additional 1 h with rotation to pull-down GST-protein. After four times of washing with the solubilization buffer, 2× SDS/PAGE buffer was applied and boiled for 5 min. Pulled-down proteins were subjected to Western blot analysis using the antibodies against GluA2 (MilliPore), valosin-containing protein, *N*-ethylmaleimide–sensitive factor (BD Biosciences), and GST (GE Healthcare).

Mass Spectrometric Analysis. A synthetic phosphorylated GluA2 C-terminal peptide, pep-3pY (*SI Materials and Methods*, *Electrophysiology*), was used as a substrate. GST-PTP^{WT}, its mutants (D820A and C852S), and GST were prebound to glutathione Sepharose beads (GE Healthcare). Dephosphorylation reaction of pep-3pY (20 pmol) was performed for 30 min at 30 °C in the buffer containing 25 mM Hepes (pH 7.35), 5 mM EDTA, and 10 mM DTT by adding 2 μg each GST-proteins. The reaction solution was boiled and centrifuged to remove the GST-proteins. GluA2 peptides in the supernatant were purified and concentrated using StageTips (Empore extraction disk; 3M) and labeled with four iTRAQ reagents (Applied Biosystems/MDS Sciex) for 60 min at room temperature according to the manufacturer's instructions. iTRAQ-labeled GluA2 peptides were mixed and concentrated by vacuum evaporation, and they were subjected to mass spectrometric analysis using a 4800 MALDI-TOF-TOF Analyzer (Applied Biosystems). Mass spectral data were analyzed using the MASCOT program (Matrix Science), and the peaks derived from synthetic GluA2 peptides were identified. Peak areas for each iTRAQ signature were obtained by using ProteinPilot. For quantitative comparison, the data were normalized by the peak area of GST-treated sample.

Biotinylation Assay. HEK293 cells transfected with pCAGGS-GluA2, -GluA2^{Y876F}, or -GluA2^{Y869F,Y873F} were surface-biotinylated using an ECL protein biotinylation kit (GE Healthcare). After stopping the biotinylation reaction, the cells were collected and solubilized with the buffer containing 150 mM NaCl, 50 mM Tris·HCl (pH 7.5), 5 mM EDTA, 1% Nonidet P-40, and the protease inhibitors (Calbiochem/Merck) for 30 min at 4 °C. The samples were centrifuged at 12,000 × *g* for 15 min at 4 °C. Streptavidin beads (Neutravidin agarose; Thermo Scientific) were added to the supernatant and incubated for 1 h at 4 °C with rotation. Beads were washed four times with the solubilization buffer, boiled for 5 min, and subjected to the immunoblot analyses.

Immunohistochemisty. For immunohistochemical analysis, mice were fixed under deep anesthesia by cardiac perfusion with 0.1 M sodium PB, pH 7.4, containing 4% paraformaldehyde; the cerebellum was then removed and soaked in 4% paraformaldehyde/PB for 4 h. After rinsing the specimens with PBS, parasagittal slices (100 μm) were prepared using a microslicer (DTK-2000; Dosaka) and permeabilized with 0.2% Triton X-100 in PBS with 2% normal goat serum and 2% BSA for 6 h at 4 °C. Immunohistochemical staining was performed using antimetabotropic glutamate receptor 1 (mGluR1; 1:1,000; provided by M. Watanabe, Hokkaido University, Sapporo, Japan), antitransient receptor potential channel 1 (anti-TRPC1; 1:200; Chemiccon) or anti-TRPC3 (1:200; Alomone), and anti-calbindin (1:10,000; Sigma) antibodies followed by incubation with Alexa546- and Alexa488-conjugated secondary antibodies (1:1,000; Invitrogen), respectively. The stained slices were viewed using a confocal laser-scanning microscope (Fluoview; Olympus).

1. Kakegawa W, et al. (2011) D-serine regulates cerebellar LTD and motor coordination through the δ2 glutamate receptor. *Nat Neurosci* 14(5):603–611.
2. Kohda K, et al. (2007) The extreme C-terminus of GluRdelta2 is essential for induction of long-term depression in cerebellar slices. *Eur J Neurosci* 25(5):1357–1362.
3. Kakegawa W, Kohda K, Yuzaki M (2007) The delta2 'ionotropic' glutamate receptor functions as a non-ionotropic receptor to control cerebellar synaptic plasticity. *J Physiol* 584(Pt 1):89–96.

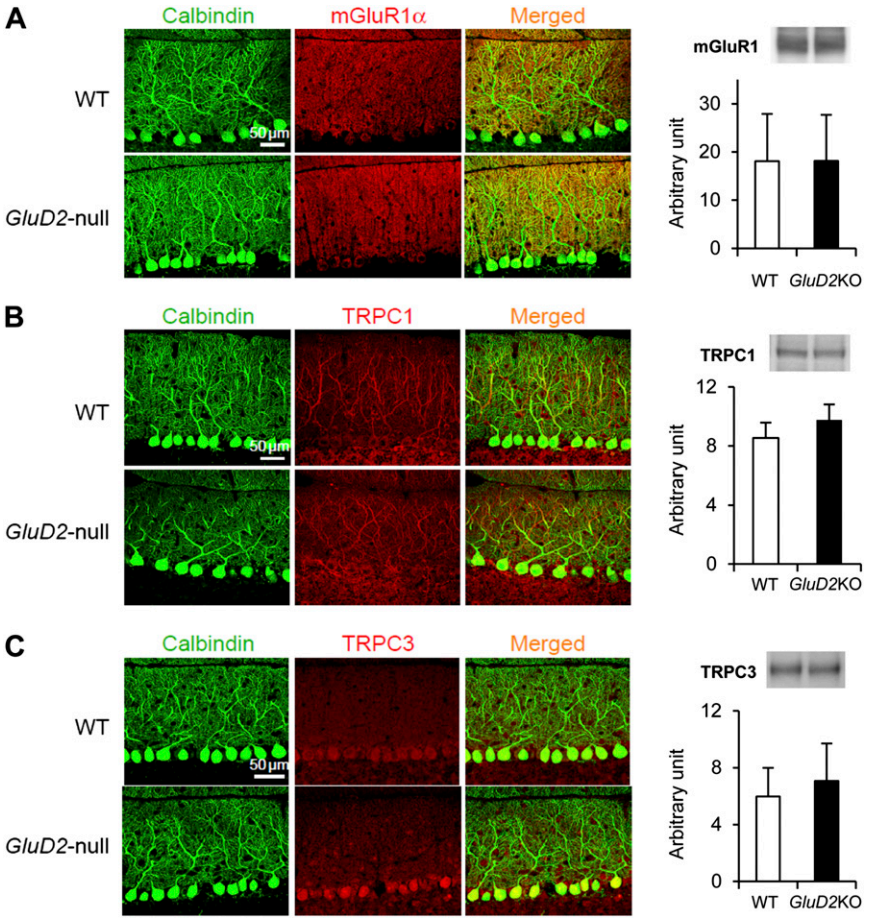

Fig. S1. Expression patterns of molecules involved in $_{slow}$EPSCs do not change in *GluD2*-null cerebellum. (*A*–*C*) Cerebellar slices from WT or *GluD2*-null mice were immunostained for mGluR1α (*A*), TRPC1 (*B*), and TRPC3 (*C*). Purkinje cells were coimmunostained for its marker calbindin (green). Representative immunoblot images of cerebellar synaptosomal fractions are shown in *Right*. The bar represents mean and SEM. There were no significant differences in band intensities for mGluR1α (paired *t* test, $P = 0.78$, $n = 8$), TRPC1 ($P = 0.16$, $n = 5$), and TRPC3 ($P = 0.12$, $n = 9$) between WT and *GluD2*-null cerebellum.

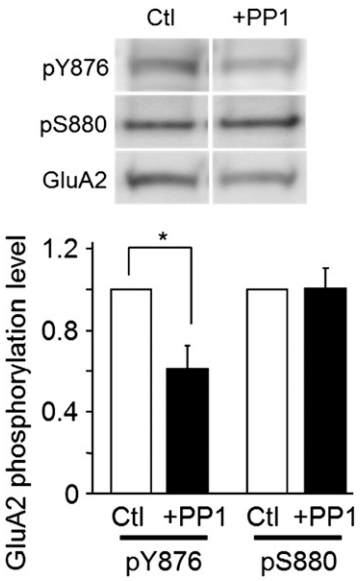

Fig. S2. Incubation with an Src family tyrosine kinase (SFK) inhibitor decreases basal-state Y876 phosphorylation levels without changing S880 phosphorylation levels in *GluD2*-null cerebellum. Cerebellar slices from *GluD2*-null mice were incubated with a PP1 analog for 15 min and subjected to immunoblot analyses using phosphorylation-specific antibodies against pY876 and pS880. Representative immunoblot images are shown in *Upper*. In *Lower*, intensities of pY876 and pS880 bands in PP1-treated slices were normalized to those band intensities in untreated slices. The bar represents mean and SEM. $^*P < 0.05$ ($n = 4$ each).

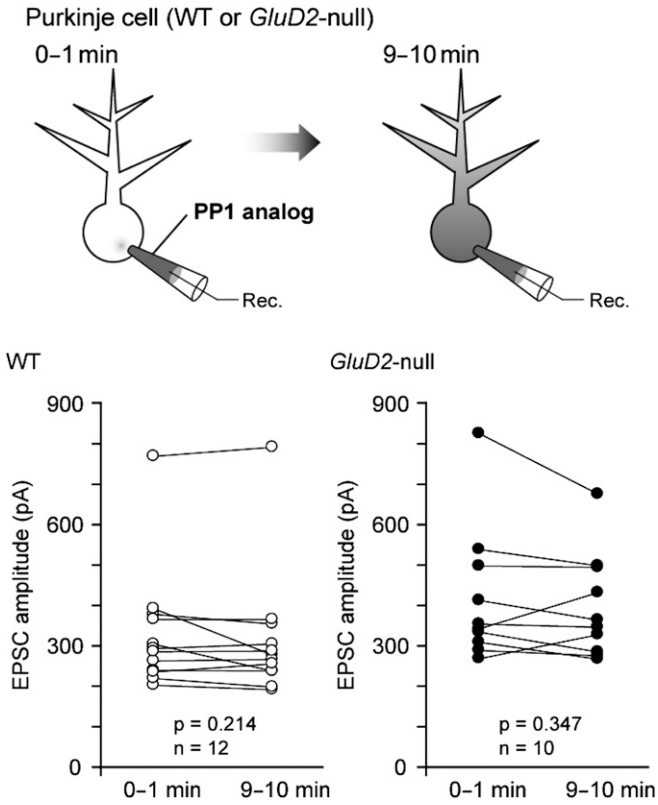

Fig. S3. Application of an SFK inhibitor does not change PF-EPSC amplitudes in WT and *GluD2*-null Purkinje cells. When a PP1 analog was included in the patch pipette, there were no changes in amplitudes of PF-EPSCs just after breaking into whole-cell mode and 10 min later.

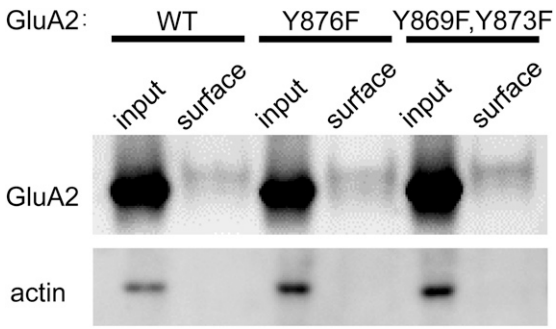

Fig. S4. Tyrosine phosphorylation does not affect cell surface trafficking of GluA2. Phosphorylation sites at Y869, Y873, and Y876 were replaced with phenylalanine to produce unphosphorable GluA2 mutants termed as GluA2^{Y876F} and GluA2^{Y869F,Y873F}. WT GluA2 (GluA2^{WT}), GluA2^{Y876F}, and GluA2^{Y869F,Y873F} were expressed in HEK293 cells and surface-biotinylated. Total GluA2 proteins (input) and GluA2 proteins on the cell surface (surface), which were precipitated by avidin-conjugated beads, were subjected to immunoblot analyses using anti-GluA2 antibody. Endogenous actin, an intracellular protein, was blotted as a control.

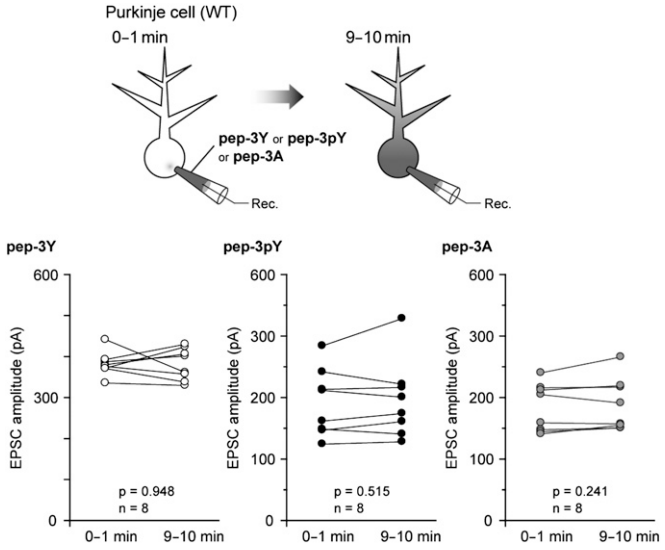

Fig. S5. Application of synthetic peptides mimicking the C terminus of GluA2 does not affect PF-EPSC amplitudes. Synthetic peptides corresponded to the GluA2 C terminus between positions 869 and 877. Alanine replaced three tyrosine residues in the original peptide (pep-3Y) to produce unphosphorable peptide (pep-3A). All tyrosine residues were phosphorylated in pep-3pY. When these peptides were included in the patch pipette, there were no changes in amplitudes of PF-EPSCs just after breaking into whole-cell mode and 10 min later.

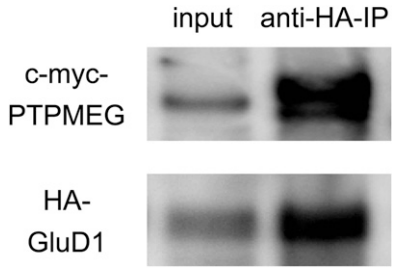

Fig. S6. PTPMEG was coimmunoprecipitated with GluD1. HA-GluD1 and c-myc-PTPMEG were cotransfected to HEK293 cells and immunoprecipitated with anti-HA antibody. Anti–c-myc and anti-HA antibody were used for immunoblotting.

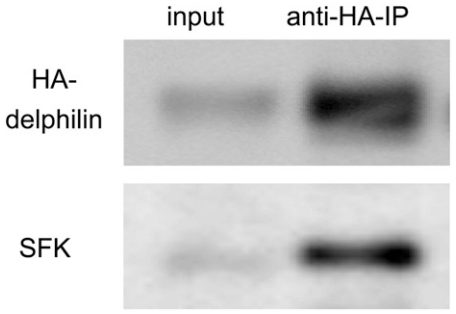

Fig. S7. Endogenous SFK was coimmunoprecipitated with delphilin. HA-delphilin was transfected to HEK293 cells and immunoprecipitated with anti-HA antibody. For immunoblotting of SFK, we used monoclonal anti-panSrc antibody (Cell Signaling).

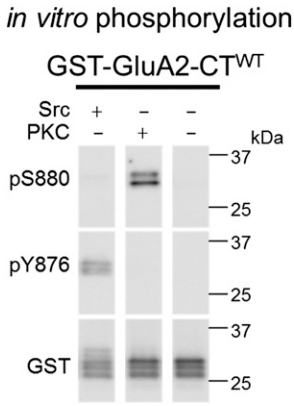

Fig. S8. Specificity of phosphorylation-specific antibodies. C-terminal region of GluA2 was conjugated with GST (GST-GluA2-CT^{WT}). GST-GluA2-CT^{WT} was incubated with Src or PKC) and subjected to immunoblot analyses using antibodies against pS880, pY876, or GST.