Supporting Information

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SI Materials

Chemical reagents used include cyclosporine A, okadaic acid, KN-93, chelerythrine, brefeldin A, cycloheximide (Calbiochem), RP-8-bromo-cAMP, primaquine (Sigma), DL-APV, picrotoxin, strychnine (Tocris), TBB (a kind gift from Dr. D. Shugar, Institute of Biochemistry and Biophysics, Warsaw, Poland), and protease inhibitor tablets (Roche). Immunologic reagents used include rabbit anti-GIRK1 and anti-GIRK2 N-terminal antibodies (1), rabbit anti-GIRK2 antibody (Alomone Labs), rabbit anti-NR1 antibody, rabbit anti-GluR1-pS831 and C-terminal antibodies (a kind gift from Dr. R. Huganir, Johns Hopkins University, Baltimore, MD), mouse anti-HA monoclonal antibody (Covance), rat anti-HA-monoclonal antibody (Roche), mouse antisynaptophysin antibody (Chemicon), rabbit anti-DARPP32, anti-DARPP32-pThr34, anti-PP1α, and anti-PP1α-pThr320 antibody (Cell Signaling), all of the Alexa fluorophore-conjugated streptavidin and secondary antibodies (Amersham Life Science), and Cy2, Cy5, and biotin-conjugated secondary antibodies (The Jackson Laboratory).

DNA Constructs. Plasmid pcDNA3-Kir3.1 with extracellular HA tag (HA-GIRK1) (2) and pCMV- Kir3.2 with extracellular HA tag (HA-GIRK2, a kind gift from Dr. S. Nakajima, University of Illinois at Chicago) (3) have been previously described. HA-GIRK1 from pcDNA3-Kir3.1 and HA-GIRK2 from pCMV-Kir3.2 were subcloned into pEGFPN1 vector. Mutations in the internalization motifs of GIRK2 (V13A/L14A, S9A, and S9D) were generated by the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and verified by sequencing the entire construct. The following synthetic oligonucleotides were used: V13A/L14A (sense $^{5'}$CTGAATCCATGACTAATGCCGCGGAGGGGGATTCCATGGAC$^{3'}$, antisense $^{5'}$GTCCATGGAATCCCCCTCCGCGGCATTAGTCATGGATTCAG$^{3'}$); S9A (sense $^{5'}$GGCCAAGTTAACTGAAGCCATGACTAATGTCCTGGAG$^{3'}$, antisense $^{5'}$ CTCCAGGACATTAGTCATGGCTTCAGTTAACTTGGCC$^{3'}$); S9D (sense $^{5'}$ CAATGGCCAAGTTAACTGAAGACATGACTAATGTCCTGGAGG$^{3'}$, antisense $^{5'}$CCTCCAGGACATTAGTCATGTCTTCAGTTAACTTGGCCATTG$^{3'}$). All of the deletion mutations of HA-GIRK2 were made using standard PCR mutagenesis.

Primary Cultures and Neuronal Transfection. Primary hippocampal neurons (10^5) were plated onto 12-mm coverslips (Warner Instruments) pretreated with nitric acid and precoated with polyL-lysine (0.1 mg/mL; Sigma-Aldrich). For biochemistry, 6×10^5 cells or 1.4×10^6 cells were plated onto 30-mm and 60-mm culture dishes (Corning), respectively, and precoated with polyL-lysine (0.1 mg/mL; Sigma-Aldrich). Neurons were maintained in neurobasal media containing B27 extract, 2 mM glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen). For surface immunostaining, cultured-dissociated hippocampal neurons at 7 DIV were transfected with pEGFPN1-HA-GIRK1 and pcDNA3-GIRK2A wild type or various mutants to express heterotetrameric GIRK channels, or with pEGFPN1-HA-GIRK2 wild type or various mutants to express homomeric GIRK2 channels using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). The transfected neurons were maintained in the above media until 10–14 DIV.

Activation of Synaptic NMDARs. For synaptic NMDAR activation by removal of APV, neurons (11–14 DIV) were first treated with 200 μM DL-APV in neurobasal medium for 3 to 4 days and incubated with media containing 100 μM picrotoxin or ACSF containing picrotoxin, strychnine, and 100 μM glycine but not $MgCl_2$ for 15 min at 37 °C. Control neurons were transferred to media containing DL-APV and picrotoxin, or ACSF containing DL-APV, picrotoxin, and strychnine. For the experiment to test the requirement of Ca^{2+} influx, removal of APV was performed with the neuronal media containing EGTA and $MgCl_{2+}$ to the final concentration of 2.5 mM and 1.3 mM so that the media contained <0.001 μM Ca^{2+}, as calculated by Webmax C Stanford program, and the ACSF solution contained 1 mM EGTA and 2 mM $MgCl_2$ but no Ca^{2+}. To test whether various inhibitors block NMDAR-induced surface expression of GIRK channels and dephosphorylation of Ser-9-phosphorylation, neurons were incubated with phosphatase inhibitors (20 μM cyclosporine A, 5 μM FK520, 20 nM okadaic acid, or 1 μM okadaic acid), kinase inhibitors (5 μM chelerythrine, 10 μM RP-8-bromo-cAMP, 10 μM KN93, or 20 μM TBB), cycloheximide (5.5 μg/mL), brefeldin A (10 μg/mL), or primaquine (60 μM) for 1 h before and during activation of NMDA receptors.

Immunoblot Analysis and Quantification. Proteins were separated by SDS-PAGE and transferred to PVDF membrane (Immobilon-P membrane; Millipore). The membrane was blocked with blocking buffer [5% milk/0.1% Tween-20 in Tris-buffered saline (TBS; 50 mM Tris [pH 7.5], 150 mM NaCl)] for 1 h followed by 2-h incubation of primary antibodies with the following dilution: rabbit anti-GIRK2-pSer9 (1:200), rabbit anti-GIRK1 N terminus (1:200), rabbit anti-GIRK2 N terminus (1:200), rabbit anti-NR1 (1:500), and rat anti-HA (1:1,000). After several washes with washing buffer (0.5% milk/0.1% Tween-20 in TBS), the membrane was exposed to HRP-conjugated secondary antibodies (1:2,000) for 1 h. After extensive washes with washing buffer, the immunoblots were visualized by ECL development (Amersham Biosciences) and quantified on a ChemiImager Ready System (Alpha Innotech).

Surface Biotinylation. Hippocampal neuron cultures were cooled on ice, washed 2 times with ice-cold ACSF containing 1 mM $MgCl_2$, and then incubated with 1 mg/mL Sulfo-NHS-S-S-Biotin (Pierce) for 20 min on ice. Unreacted biotinylation reagent was washed 4 times with ice-cold ACSF containing 1 mM $MgCl_2$ and quenched by 2 washes in ice-cold TBS (pH 7.4; 50 mM Tris, 150 mM NaCl). Cultures were harvested in modified radioimmunoprecipitation assay (RIPA) buffer (pH 7.4; 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% SDS, 0.5% deoxycholic acid, and protease inhibitors). The homogenates were centrifuged at $20,000 \times g$ for 15 min at 4 °C. The resulting supernatant was incubated with 200 μL of 50% NeutraAvidin agarose (Pierce) for 3 h at 4 °C. After NeutraAvidin agarose was washed 5 times with RIPA buffer, bound proteins were eluted with SDS sample buffer by heating with DTT at 85 °C for 30 min. Total protein and isolated biotinylated proteins were analyzed by quantitative immunoblotting. To test whether all of the biotinylated surface GIRK1 and GIRK2 proteins were recovered by a single streptavidin precipitation, the supernatant remaining after the first streptavidin precipitation was reprecipitated and eluted as described above.

Degradation Assay Using Surface Biotinylation. For degradation assay of surface proteins, hippocampal neuron cultures (17 to 18 DIV) were first labeled with 1 mg/mL Sulfo-NHS-LC-Biotin biotin on ice, and followed by basal endocytosis and degradation at 37 °C for up to 22 h. Neurons were then harvested at various

time points without stripping. The surface proteins were recovered with streptavidin and visualized by immunoblotting.

Regulation of Ser-9 Phosphorylation of GIRK2, Thr-34/35 Phosphorylation of DARPP32/I1, Thr-320 Phosphorylation of PP1α, and Ser-831 Phosphorylation of GluR1 in Hippocampal Neurons. Characterization of anti-GIRK2-pSer9 antibody was performed on the HEK293T cells expressing HA-GIRK2 wild type or S9A, in which Ser-9 is mutated to alanine. The pCMV-HA-GIRK2 or S9A or pCMV vector alone was transfected into HEK293T cells using Fugene transfection reagent (Roche). The HEK293T cell or cultured hippocampal neuronal lysate was prepared by homogenizing in modified RIPA buffer and centrifugation at 20,000 $\times$ *g* for 15 min at 4 °C. The supernatant was collected and mixed with SDS sample buffer. Rat hippocampal membranes were prepared as previously described (4) and resuspended in SDS sample buffer. These samples were subjected to SDS-PAGE, transferred to PVDF membrane, and analyzed by immunoblotting with anti-GIRK2-pSer9 and anti-GIRK2 N-terminal antibodies for analyzing Ser-9 phosphorylation of GIRK2, anti-DARPP32-pThr34 and anti-DARPP32 for analyzing Thr-34/Thr-35 phosphorylation of DARPP32/I1, anti-PP1α-pThr320 and anti-PP1α antibodies for analyzing Thr-320 phosphorylation of PP1α, and anti-GluR1-pSer831 and anti-GluR1 C-terminal antibodies for analyzing Ser-831 phosphorylation of GluR1. To test the specificity of anti-GIRK2-pSer9, the immunoblot membrane was pretreated with λ-phosphatase (1,200 U/mL, NEB) for 1 h at 30 °C before immunoblotting.

Immunoprecipitation of Ser-9-Phosphorylated GIRK2 Proteins. Neurons were washed with ice-cold ACSF, solubilized in RIPA buffer containing protease inhibitor cocktails, and subjected to centrifugation at 100,000 $\times$ *g* for 15 min. The supernatant was precleared with Protein A agarose beads (Pierce) for 1 h, followed by incubation with 2 μg of purified anti-GIRK2-pSer9 antibodies for 2 to 3 h at 4 °C with the agarose beads. The immunoprecipitates were washed with RIPA buffer 5 times and eluted with SDS sample buffer at 85 °C for 30 min.

***In Vitro* Dephosphorylation of GIRK2.** The pCMV-HA-GIRK2 was transfected into HEK293T cells using Fugene transfection reagent (Roche). Cells were washed with ice-cold PBS, solubilized in modified RIPA buffer, and centrifuged at 20,000 $\times$ *g* for 15 min at 4 °C. The supernatant was precleared first by incubating with 50 μL protein L agarose gels (Pierce) for 1 h at 4 °C. The precleared lysate was then used for immunoprecipitation of GIRK2 subunits with 1 μg rat anti-HA antibodies (Roche) for 2 h at 4 °C, followed by incubation with 50 μL protein L agarose gels for 2 h at 4 °C. The immunoprecipitates were washed with the modified RIPA buffer 5 times and then twice with the reaction buffer of PP1 (NEB provided the reaction buffer), PP2A (pH 7.0; 20 mM Hepes, 1 mM DTT, and 1 mM $MnCl_2$), and PP2B (pH 7.5; 25 mM Tris, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 10 μg/mL calmodulin), and λ-phosphatase (NEB provided the reaction buffer). The dephosphorylation reaction was performed in 50 μL reaction buffer with 50 μM ATP and PP1 (NEB, 2.5 U), PP2A (Upstate, 0.1 U), PP2B (Calbiochem, 50 U), or λ-phosphatase (NEB, 400 U) for 30 min at 30 °C, and stopped by adding SDS sample buffer. *In vitro* dephosphorylation reaction was analyzed by immunoblotting with anti-GIRK2-pSer9 and anti-GIRK2 N terminus antibodies.

Immunocytochemistry. For permeabilized immunostaining, the neurons were fixed with 4% formaldehyde/4% sucrose in PBS for 20 min, washed 3 times with PBS, and permeabilized via 10-min incubation with 0.2% Triton X-100 in PBS. Neurons were blocked with 10% normal donkey serum (NDS) in PBS for 1 h and then incubated with rabbit anti-GIRK2 antibody (1:200, Alomone) and mouse antisynaptophysin antibodies (1:500, Chemicon), or mouse anti-HA antibody (1:500, Covance) in 3% NDS in PBS overnight at 4 °C. After extensive washing with PBS, neurons were incubated with Cy5-conjugated antirabbit IgG and Cy2-conjugated antimouse IgG antibodies. After extensive washing with PBS, neurons were mounted on Superfrost microslides (VWR) using Prolong Antifade Kit mounting solution (Molecular Probes). For surface immunostaining, transfected neurons (10–14 DIV) with pEGFPN1-HA-GIRK1 and pcDNA3-GIRK2A or pEGFPN1-HA-GIRK2 were fixed with 4% formaldehyde/4% sucrose in PBS for 8 min, gently washed 3 times with PBS, blocked with 10% NDS in PBS for 1 h, and incubated with mouse anti-HA antibodies (1:300, Covance) in 3% NDS in PBS overnight at 4 °C. After extensive washing with PBS, neurons were incubated with biotin-conjugated antimouse IgG antibodies in 3% NDS/PBS for 2 h, washed 5 times with PBS, and incubated with Alexa 660-conjugated streptavidin (Amersham Life Science) in 3% NGS in PBS for 2 h. After extensive washing with PBS, neurons were mounted as described above. The total GIRK proteins were visualized by GFP fluorescence.

Channel Endocytosis Assay. COS7 cells were cultured on 12-mm coverslips (Warner Instruments) precoated with poly L-lysine (0.1 mg/mL; Sigma-Aldrich) and transfected with pCMV-HA-GIRK2 wild type, VL/AA, S9A, or S9D for expression of GIRK2 homotetramers or pEGFPN1-HA-GIRK1 with pcDNA3-GIRK2 wild type, S9A, or S9D for expression of GIRK1/2 heterotetramers using Fugene transfection reagent (Roche) according to the manufacturer's instructions. Forty-eight hours later, the cells were washed 2 times with ice-cold PBS and incubated with blocking solution (5% normal goat serum in PBS) for 15 min, followed by incubation with Alexa 594-conjugated mouse anti-HA antibodies (Covance) in the blocking solution for 2 h at 4 °C. The cells were extensively washed 3 times with ice-cold PBS for 5 min. To quantify the surface HA-GIRK2 proteins, the cells were fixed with 4% formaldehyde/4% sucrose in PBS for 20 min at this time. To examine the colocalization of the internalized HA-GIRK2 proteins with endosomal markers, the cells were incubated with the fresh media for 80 min at 37 °C in the presence or absence of 60 μM primaquine (Sigma-Aldrich) and then fixed, permeabilized, and incubated with antibodies for EEA1 and Rab11 (BD Bioscience) and Lysosensor Blue (Invitrogen). EEA1 and Rab11 were visualized by Alexa 488 and Alexa 660-conjugated secondary antibodies. To quantify the extent of internalization, COS7 cells were transfected with pEGFPN1-HA-GIRK2 DNA wild type, VL/AA, S9A, or S9D and processed for the channel endocytosis assay using mouse anti-HA antibody. After inducing endocytosis, the cells were cooled to 4 °C and incubated with excess antimouse IgG for 2 h. The cells were then fixed, permeabilized, and incubated with Alexa 633-conjugated antimouse IgG antibodies to label the internalized GIRK2 proteins. The mean fluorescence intensity of internalized GIRK2 proteins at 80 min internalization was divided by that of surface proteins at 0 min internalization to obtain relative internalization. All cells were mounted on Superfrost microslides (VWR) using Prolong Antifade Kit mounting solution (Molecular Probes).

Electrophysiologic Recordings of GIRK Channels. Patch-clamp recording and analysis of single-channel currents of GIRK2 channels were performed as previously described (3). Single-channel currents were recorded from HEK293T cells at room temperature (22 °C-25 °C) from cell-attached patches, clamped at −100 mV with an Axopatch 200B patch-clamp amplifier (Axon Instruments), and low-pass filtered (3 dB, 1 kHz) with an 8-pole Bessel filter (Frequency Devices). The pipette solution contained 100 mM KCl (pH 7.2). All recordings were done on the HEK293T cells 2 days after transfection with pEGFPN1-GIRK2 wild type, S9A, or S9D (0.1 μg per 12-mm coverslip).

1. Liao YJ, Jan YN, Jan LY (1996) Heteromultimerization of G-protein-gated inwardly rectifying K+ channel proteins GIRK1 and GIRK2 and their altered expression in weaver brain. *J Neurosci* 16:7137–7150.
2. Ma D, *et al.* (2002) Diverse trafficking patterns due to multiple traffic motifs in G protein-activated inwardly rectifying potassium channels from brain and heart. *Neuron* 33:715–729.
3. Chen L, *et al.* (2002) A glutamate residue at the C terminus regulates activity of inward rectifier K+ channels: Implication for Andersen's syndrome. *Proc Natl Acad Sci USA* 99:8430–8435.
4. Luo J, Wang Y, Yasuda RP, Dunah AW, Wolfe BB (1997) The majority of N-methyl-D-aspartate receptor complexes in adult rat cerebral cortex contain at least three different subunits (NR1/NR2A/NR2B). *Mol Pharmacol* 51:79–86.

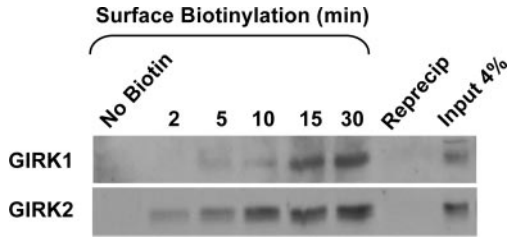

Fig. S1. Surface biotinylation of cultured dissociated hippocampal neurons. Surface biotinylation of GIRK channels in live hippocampal neurons in culture (17 DIV) was performed at 4 °C for 2–30 min to determine the time at which biotinylation of surface GIRK proteins was complete. Neurons were first incubated with membrane-impermeable sulfo-NHS-SS-biotin at 4 °C. After removal of the excess biotin, biotinylated surface proteins were isolated by streptavidin precipitation and visualized by immunoblot analysis. Supernatant remaining after streptavidin precipitation following the 30-min biotinylation reaction was reprecipitated (Reprecip). The biotinylation reaction was complete within 15 min, and all of the biotinylated surface GIRK1 and GIRK2 proteins were recovered by a single streptavidin precipitation because the second streptavidin precipitation yielded no further recovery.

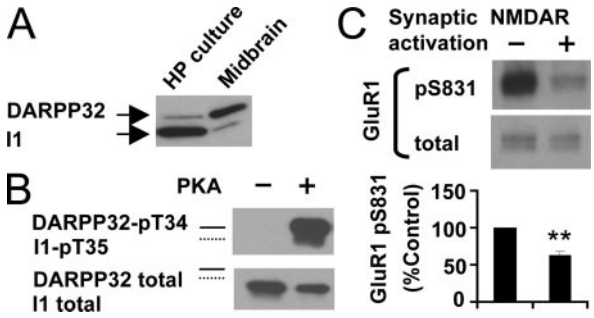

Fig. S2. DARPP32 and I1 proteins recognized by anti-DARPP32 and anti-DARPP32-pT34 antibodies and NMDAR-induced AMPA receptor dephosphorylation. (*A*) Anti-DARPP32 antibody recognized 2 proteins, DARPP32 and I1, which were differentially expressed in cultured rat hippocampal neurons and rat midbrain. (*B*) Phosphorylation site-specific anti-DARPP32-pT34 antibody recognized phosphorylated DARPP32/I1 proteins at Thr-34/Thr-35 in the mouse brain incubated with purified active protein kinase A (PKA). (*C*) Synaptic NMDAR activation induces dephosphorylation of GluR1 at Ser-831 ($n = 4$), a known PP1 substrate, as determined by immunoblot analysis with phosphorylation site-specific anti-GluR1-pS831 and phosphorylation-independent anti-GluR1 antibodies. $^{**}P < 0.01$.

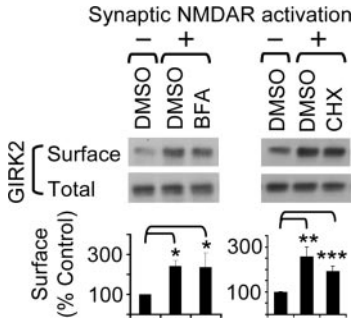

Fig. S3. NMDAR-induced GIRK surface expression does not require protein synthesis or endoplasmic reticulum to Golgi trafficking. NMDAR-induced surface expression of endogenous GIRK2 persisted in the presence of brefeldin A (BFA; 10 μg/mL, $n = 4$), which blocks endoplasmic reticulum to Golgi trafficking, or cycloheximide, which blocks protein synthesis (CHX; 5.5 μg/mL, $n = 15$).

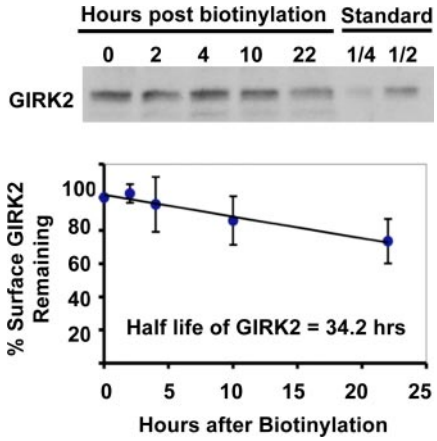

Fig. S4. Half-life of surface GIRK2 proteins in hippocampal neurons. Cultured hippocampal neurons (DIV 17) were biotinylated at 4 °C with NHS-LC-biotin and then incubated with the media at 37 °C up to 22 h to induce degradation of biotinylated surface proteins. The remaining biotinylated proteins were isolated by streptavidin precipitation and analyzed by immunoblotting. To quantify the amount of remaining biotinylated proteins as percentage surface protein remaining, the total intensity of biotinylated proteins at 0 h was taken as 100% surface protein, and the total intensity of biotinylated proteins remaining at different time points was normalized to the total intensity of biotinylated proteins at 0 h. The half-life of biotinylated surface GIRK2 proteins is 34.2 h in hippocampal neurons, which corresponds to a very slow degradation rate (≈0.025%/min, $n = 3$).

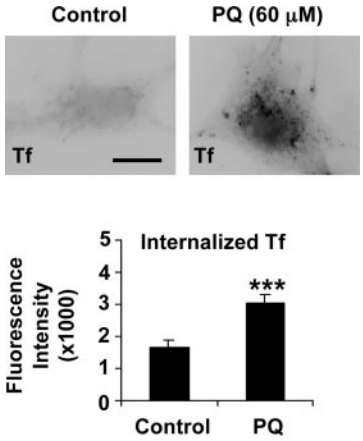

Fig. S5. Primaquine blocks recycling of endocytosed transferrin receptors. Live COS7 cells were incubated with Alexa 488-conjugated human transferrin at 4 °C to label surface transferrin receptors. After removing the excess unbound transferrin, cells were incubated with the media in the presence or absence of 60 μM primaquine (PQ) at 37 °C for 0–80 min to induce basal internalization. Quantification of the mean fluorescence intensity of endocytosed transferrin receptors (Tf) after 80 min of internalization showed that the endocytosed transferrin receptors were retained inside the cell to a greater extent by the primaquine treatment (Control $n = 14$; PQ $n = 16$; $^{***}P < 0.005$).

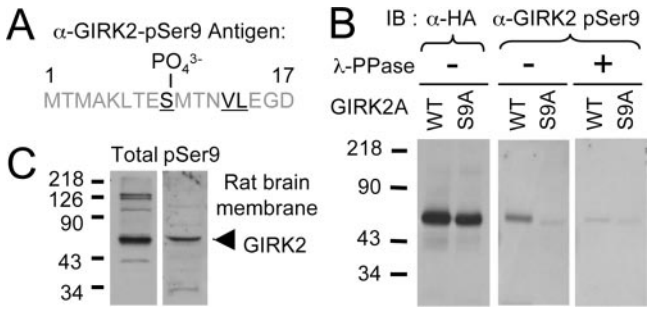

Fig. S6. Generation and characterization of phosphorylation site-specific antibody against GIRK2 Ser-9. (*A*) Anti-GIRK2-pSer9 antibody was raised against the synthetic peptide corresponding to amino acids 1–17 of GIRK2 with phosphoserine included at the Ser-9. (*B*) Immunoblot analysis with phosphorylation site-specific anti-GIRK2-pSer9 antibody and phosphorylation-independent anti-HA antibody. Anti-GIRK2-pSer9 specifically detected wild-type (WT) but not S9A mutant HA-GIRK2A in HEK293T cells. Phosphorylation-dependent recognition was eliminated by λ-phosphatase treatment before immunoblotting. (*C*) Immunoblot analysis with anti-GIRK2-pSer9 antibody (pSer9) and anti-GIRK2 N terminus antibody (Total) in rat hippocampal membranes.

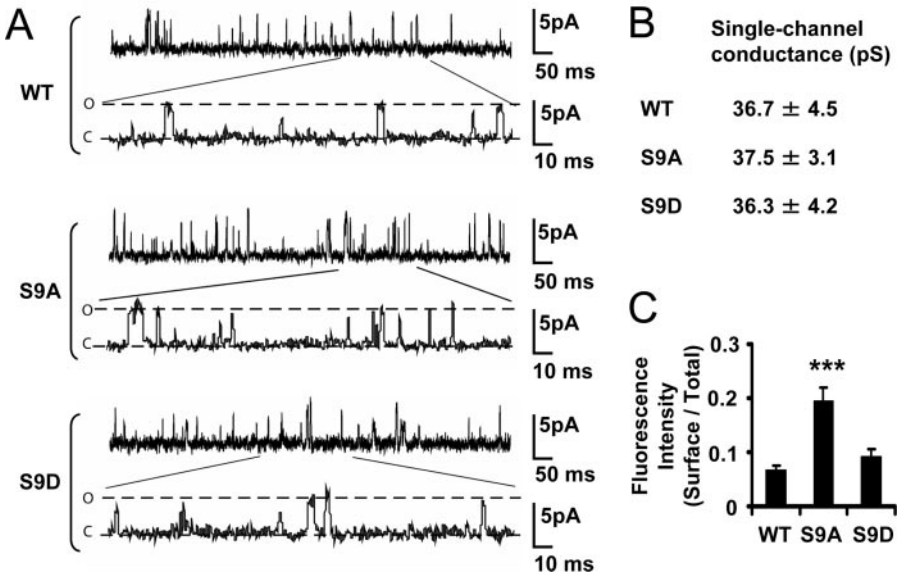

Fig. S7. Single-channel recording and surface expression of GIRK2 wild-type (WT) and Ser-9 mutant channels in HEK293T cells. (*A*) Representative recordings of HA-GIRK2 wild-type channels and mutant channels in which Ser-9 is mutated to alanine (S9A) or aspartate (S9D) obtained in the cell-attached configuration from HEK293T cells, at a holding potential of −100 mV. Mutation of Ser-9 to alanine (S9A) and aspartate (S9D) mimics dephosphorylated and phosphorylated Ser-9, respectively. Channel openings are upward deflections. Single-channel current amplitudes from 3 or more different patches recorded under the same conditions were subsequently averaged for the single-channel conductance calculation. (*B*) There was no difference in single-channel conductance between wild-type and mutant GIRK2 proteins ($n = 5$ each). (*C*) Quantitative measurement of surface HA-GIRK2 proteins in HEK293T cells in 12-well plates, showing that surface density of S9A is 3-fold higher than that of wild type or S9D ($n = 4$, each well). $***P < 0.001$.

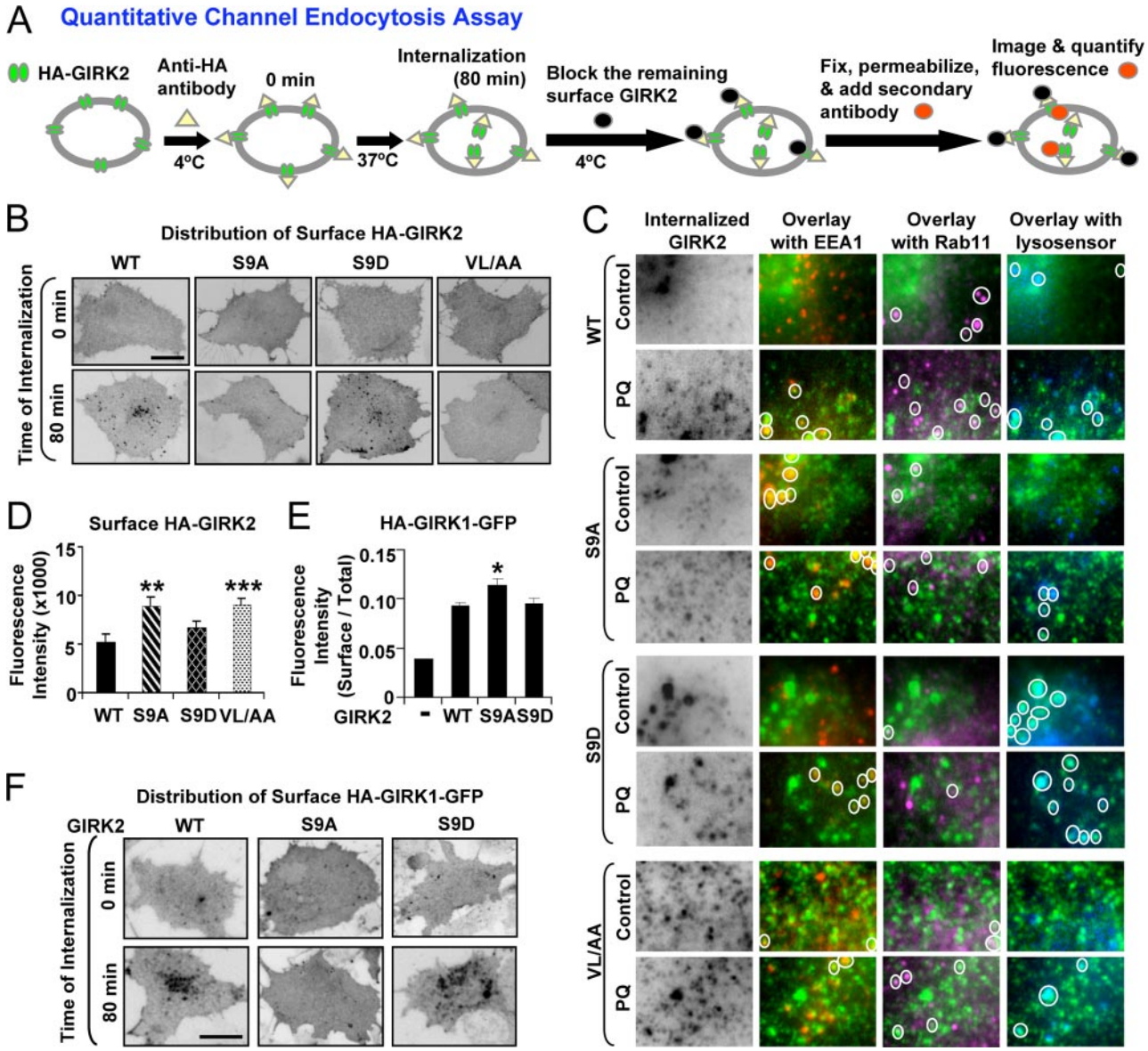

Fig. S8. Primaquine treatment increased colocalization of HA-GIRK2 wild-type (WT), S9A, and VL/AA mutant proteins with recycling endosomes. (*A*) Experimental design of quantitative immunoassay of channel endocytosis. HA-GIRK2 proteins are labeled at 4 °C with mouse anti-HA antibody in live COS7 cells. After inducing basal endocytosis at 37 °C, the cells are incubated with excess unlabeled antimouse IgG at 4 °C to block HA-GIRK2 proteins remaining on the plasma membrane. After fixation and permeabilization, the internalized HA-GIRK2 proteins are visualized by Alexa 633-conjugated antimouse secondary antibodies and imaged to quantify. (*B*) Channel endocytosis assay in COS7 cells transfected with HA-GIRK2 wild type or mutants (S9A, S9D, and VL/AA). After 80 min internalization, S9A mutation decreases redistribution of surface HA-GIRK1-GFP proteins to intracellular endosomes compared with wild type or S9D. (Scale bar, 15 μm.) (*C*) Localization of internalized HA-GIRK2 proteins in endosomes with or without primaquine (PQ), which blocks recycling. After surface labeling, endocytosis was induced in the media at 37 °C for 80 min with or without 60 μM PQ. The cells are then incubated with excess unlabeled antimouse IgG at 4 °C to block HA-GIRK2 proteins remaining on the plasma membrane. After fixation and permeabilization, the colocalization of internalized HA-GIRK2 proteins with endosomal markers including EEA1 (early endosomes), Rab11 (recycling endosomes), and LysoSensor (lysosomes) was examined. Primaquine increased colocalization of GIRK2 WT, S9A, and VL/AA mutants with Rab11, consistent with its ability to inhibit GIRK2 recycling. In contrast, the surface-labeled S9D mutant proteins were mostly localized to lysosomes regardless of primaquine treatment. Primaquine also increased colocalization of the wild-type and S9D mutant proteins with EEA1-containing early endosomes, possibly owing to a secondary consequence of inhibiting recycling. (*D*) Quantification of mean fluorescence intensity of surface-labeled HA-GIRK2 wild type ($n = 36$) and S9A ($n = 31$), S9D ($n = 36$), and VL/AA ($n = 38$) at 0 min internalization. After surface labeling, cells were fixed (0 min internalization) and visualized by Alexa 594-conjugated antimouse secondary antibodies. (*E*) Quantitative measurement of surface HA-GIRK1-GFP proteins in COS7 cells cotransfected with GIRK2 in 12-well plates, showing that S9A mutation of GIRK2 increases surface density of HA-GIRK1-GFP compared with wild-type or S9D mutation, or HA-GIRK1-GFP alone ($n = 3$, each well). *$P < 0.05$. (*F*) Channel endocytosis assay on COS7 cells transfected with HA-GIRK1-GFP and GIRK2 wild-type, S9A, and S9D. S9A mutation decreases redistribution of surface HA-GIRK1-GFP proteins to endosomes compared with wild type or S9D after 80 min internalization.

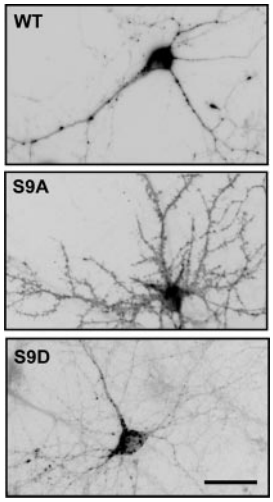

Fig. S9. S9A or S9D mutation affects subcellular localization of GIRK2 in hippocampal neurons. HA-GIRK2-GFP WT, S9A, or S9D in hippocampal neurons (14 DIV) was visualized by GFP fluorescence. Both wild-type GIRK2 and S9D mutant proteins showed punctate distribution in soma and dendrites, whereas S9A mutant proteins displayed more diffuse distribution in both proximal and distal dendrites, as well as spines. (Scale bar, 20 μm.)