Supporting Information

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SI Materials and Methods

Pharmacological Treatments. Control neurons were incubated with 5 μM glycine for 30 min. Glutamate-treated neurons were incubated with 50 μM glutamate/5 μM glycine for 30 min. NMDA-treated neurons were incubated for 3 min with 75 μM NMDA (Tocris)/5 μM glycine. The inhibitors D-(-)-2-amino-5-phosphonopentanoic acid (APV, 100 μM for 2 h; Tocris), dynasore (80 μM for 15 min; Sigma), ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA-AM, 100 μM for 10 min; Molecular Probes), KN-93 (10 μM for 10 min; Tocris) and KN-92 (10 μM for 10 min; Calbiochem) were dissolved in DMSO. The final DMSO concentration never exceeded 1% (vol/vol). No significant difference was observed between NMDA and NMDA+DMSO treatment. Preincubation of neurons with DMSO 1% (vol/vol) for 2 h did not significantly affect the NMDA-mediated reduction in surface $GABA_{B1b}$ levels (control: 100% ± 13%; NMDA: 49.7 ± 7.4%, $P < 0.001$ compared with control; NMDA+DMSO: 58.6 ± 3.7%, $P < 0.01$ compared with control; mean ± SEM, $n = 10$). Inhibitors were applied to neurons before glutamate or NMDA treatment and kept in the medium during the treatment. Neurons were returned to conditioned medium for 27 min before analysis.

Quantification of Surface Receptors. After pharmacological treatment, neurons were fixed for 20 min with 4% paraformaldehyde in PBS containing 120 mM sucrose and then blocked for 2 h in 10% normal goat serum (GIBCO) in PBS (NGS/PBS). Neurons were incubated with primary mouse anti-HA (1:500 in 10% NGS/PBS; Covance) and anti-Myc antibodies (1:500; Roche) for 2 h to visualize surface $GABA_{B1b}$ receptors. Subsequently, neurons were permeabilized with 0.25% Triton-X-100 in PBS for 10 min. The total amount of $GABA_{B1b}$ receptors was detected by staining with rabbit anti-GFP antibodies (1:500 in 10% NGS/PBS; Molecular Probes). Secondary antibodies (Alexa Fluor 568 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, 1:500; Molecular Probes) were incubated for 1 h in 10% NGS/PBS. Immunolabeled neuronal cultures were mounted in FluorSave Reagent (Calbiochem) and viewed on a Leica TCS SPE confocal microscope. Digital pictures were captured with Leica Software (LAS AF) and identically processed with ImageJ software (1). The fluorescence intensity of labeled surface HA-GB1b-eGFP proteins was measured on the soma of transfected neurons and visualized in single optical planes using ImageJ software. For quantification of surface HA-GB1b-eGFP protein levels, the fluorescence intensity of surface HA-GB1b-eGFP (red) was normalized to the total HA-GB1b-eGFP (green) fluorescence intensity. Total protein levels were determined by using rabbit anti-GFP antibodies (1:500, Abcam). Statistical analysis was performed with GraphPad Prism 5.0. For each statistical analysis independent sample groups were analyzed.

Immunocytochemistry of Endocytosed Receptors. Anti-HA antibodies (1:50; Immunology Consultants Laboratory) and anti-Myc antibodies (1:50; Roche) were used to label the extracellular HA- and Myc-tag of surface HA-GB1b-eGFP and HA-GB1b-eGFP protein, respectively. Live neurons were then incubated at 37 °C/5% CO_2 for 30 min to allow internalization in the presence or absence of glutamatergic agonists and antagonists. After removal of residual anti-HA/anti-Myc antibodies from the cell surface by acid wash (2-min wash in Neurobasal medium pH 2.0 on ice, followed by intensive washing with Neurobasal medium pH 7.4 on ice), internalized anti-HA/anti-Myc antibodies were visualized with fluorescence-labeled secondary antibodies. Following fixation and permeabilization of neurons, the total amount of HA-GB1b-eGFP/Myc-GB1a-eGFP protein was determined by staining with mouse anti-GFP antibodies (1:500 in 10% NGS/PBS; Molecular Probes). Secondary antibodies (Alexa Fluor 568 goat antirabbit and Alexa Fluor 488 goat anti-mouse, 1:500; Molecular Probes) were incubated for 1 h in 10% NGS/PBS. To control for complete removal of anti-HA antibodies from the cell surface upon acid wash, nonpermeabilized cultures were stained with Alexa Fluor 568 goat anti-rabbit antibodies. For colocalization experiments, we used Rab11-eGFP (2).

Biotinylation Experiments. After treatment with pharmacological agents, rat cortical neurons were rinsed twice with PBS on ice and incubated with 1 mg/mL EZ-Link Sulfo-NHS-SS-Biotin (Pierce Chemicals) in PBS for 30 min. The biotinylation reagent was removed by washing with PBS and quenched for 5 min with 50 mM glycine in PBS. Neurons were rinsed with Tris-buffered saline, washed twice by resuspension in PBS, followed by centrifugation at 2,000 × *g*. Finally, neurons were lysed in RIPA buffer for 30 min. Lysates were cleared by centrifugation at 16,000 × *g* for 20 min and incubated with immobilized NeutrAvidin gel (Pierce Chemicals) overnight. Beads were washed five times with RIPA buffer. All steps were performed on ice or at 4 °C. Adsorbed proteins were separated by SDS/PAGE.

Whole-Brain Lysate and Brain Membrane Preparation. Whole brains from BALB/c mice were polytron-homogenized in Hepes buffer (4 mM Hepes pH 7.4, 320 mM sucrose, 1 mM EDTA, 1 mM EGTA) containing Complete Protease Inhibitor Mixture (Roche) and centrifuged for 10 min at 1,000 × *g* to remove debris and the crude nuclear fraction. Samples were kept at 4 °C throughout the procedure. The supernatant (S2) containing the cytosolic fraction of whole-brain lysates was removed and used for in vitro phosphorylation assays. The remaining pellet (P2) containing the membrane fraction was solubilized in RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate) and centrifuged for 30 min at 10,000 × *g* to remove insoluble material. Solubilized brain membranes were used for immunoprecipitation and Western blot analysis. Synaptic plasma membranes were purified from total mouse brain by combined flotation-sedimentation density gradient centrifugation (3).

Coimmunoprecipitation Experiments. Solubilized brain membranes were precleared for 3 h by incubation with protein-G agarose beads (Roche). Rabbit anti-$GABA_{B1}$ antibody 174.1 (4) or guinea pig anti-$GABA_{B2}$ antibody (Chemicon) were added to the precleared lysates for 1 h before overnight incubation with protein-G agarose beads. Rabbit and guinea pig preimmune serum were used as a control. Immunoprecipitated complexes were repeatedly washed with RIPA buffer, separated by SDS/PAGE and probed with an anti-CaMKIIα antibody (1:1,000, Santa Cruz).

Pull-Down Assays. To generate GST-GB1 and GST-GB2, the full-length C-termini of $GABA_{B1}$ (amino acids 857–960) and $GABA_{B2}$ (amino acid 745–940) were amplified by PCR from the rat cDNAs and inserted in-frame into the pGEX-4T-1 fusion vector (GE Healthcare). GST-fusion proteins were expressed in *E. coli* BL21 (DE3). Whole-brain lysates from mice were prepared by polytron-homogenization in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% Triton X-100) containing Complete Protease Inhibitor Mixture. Lysates were cleared by centrifugation at 15,700 × *g* for 30 min at 4 °C and incubated for 4 h with GST-fusion proteins immobilized on Sepharose beads. Following extensive washing in RIPA buffer, iso-

lated proteins were separated by SDS/PAGE and probed with an anti-CaMKII antibody (1:5,000; BD Biosciences).

In Vitro Phosphorylation Assays. GST-fusion proteins immobilized on Sepharose beads were phosphorylated with recombinant CaMKII (New England Biolabs), recombinant PKC (Calbiochem) or the cytosolic S2 fraction of whole-brain lysates prepared from BALB/c mice. Recombinant kinases were activated according to the manufacturer's instructions. For whole-brain lysates, the following phosphorylation buffer was used (in mM): 20 Hepes, pH7.4, 1.7 $CaCl_2$, 100 DTT, 10 $MgCl_2$, 1.6 unlabeled ATP. A 20-μg quantity of GST fusion-protein was incubated with recombinant CaMKII (500 units), recombinant PKC or whole-brain lysates (50 μg) in phosphorylation buffer in the presence of 1 μL [γ-^{32}P]-ATP (3,000 Ci/mmol) for 30 min at 30 °C. Free [γ-^{32}P]-ATP was removed by extensive washing with ice-cold phosphorylation buffer. To inhibit CaMKII activity, whole-brain extracts were preincubated with KN-93 (10 μM for 20 min) at 4 °C. Phosphorylated GST-fusion proteins were either separated by SDS/PAGE and subjected to autoradiography or used for RP-HPLC and ESI-MS/MS analysis.

RP-HPLC. For HPLC analysis, 20 μg phosphorylated GST-fusion protein was digested with the endoproteinase Lys C (Wako Chemicals) followed by digestion with trypsin (Promega). Digestion was stopped by adding TFA (Applied Biosystems) to a final concentration of 0.1% (vol/vol). Insoluble material was removed by centrifugation (12,000 rpm, 5 min) and the supernatant subjected to RP-HPLC on Vydac C18 reverse-phase columns (218TP52, 2.1 × 250 mm; Grace Vydac) connected to a Hewlett Packard 1090 HPLC system. Bound peptides were eluted during 60 min at 150 μL/min using a linear gradient of 0.1% TFA/2% acetonitrile to 0.09% TFA/75% acetonitrile. The effluent was monitored at 214 nm. Fractions were collected at 1 min intervals and phosphopeptides identified by liquid scintillation counting.

ESI-MS/MS. Radioactively labeled peptides were analyzed by capillary liquid chromatography tandem MS (LC/MS/MS) using a set up of a trapping 300SB C-18 column (0.3 × 50 mm, Agilent Technologies) and a separating column (0.1 × 100 mm) packed with Magic 300 Å C18 reverse-phase material (5-μm particle size, Michrom Bioresources.). Columns were connected online to an Orbitrap FT hybrid instrument (Thermo Finnigan). A linear gradient from 2 to 80% solvent B (0.1% acetic acid and 80% acetonitrile in water) in solvent A (0.1% acetic acid and 2% acetonitrile in water) was delivered for 85 min with a Rheos 2200 pump (Flux Instruments) at a flow rate of 100 μL/min. A precolumn split was used to reduce the flow to 100 nL/min. Eluted peptides were ionized at 1.7 kV. The mass spectrometer was operated in a data-dependent fashion. The precursor scan was done in the Orbitrap set to 60,000 resolution, and the fragment ions were mass analyzed in the LTQ instrument. The five most intense precursors were selected for fragmentation. The MS/MS spectra were searched against the NCBI database using TurboSequest or Mascot software (5, 6).

Two-Photon Imaging. The slice was placed into a perfusion chamber and superfused with ACSF containing (in mM): 127 NaCl, 25 $NaHCO_3$, 25 d-glucose, 2.5 KCl, 4 $CaCl_2$, 1.25 NaH_2PO_4, 0.03 serine and 0.001 TTX (320 mosm, pH 7.4). In a subset of experiments, 0.02 mM dCPP was added to the ACSF. Experiments were performed at 28 °C. Stacks of images (256 × 256 pixels) from secondary dendritic branches were obtained from transfected CA1 pyramidal neurons (Z step: 0.5 μm). Green fluorescence (SEP-GB1b or SEP-GB1bS867A) and red fluorescence (RFP) was imaged at 5-min intervals before and after bath application of NMDA and quantified in regions of interest (ROIs) containing either a dendritic shaft or spines. Control ROIs in the image background were always included and did not show changes in fluorescence during the course of the experiment. To quantify NMDA-mediated changes, the green and red fluorescence intensities after NMDA application (seven values) were normalized to the intensities before NMDA application (two values). For the ratio images, we used a hue/saturation/brightness model, in which hue was determined by the G/R ratio (using a rainbow color table), and the intensity in the red channel was used to set the brightness.

Electrophysiology in Cultured Hippocampal Neurons. Recordings were performed 1–2 wk after transfection at DIV14–21 at room temperature (23–24 °C). Neurons were continuously superfused with an extracellular solution (ECS) composed of (in mM): 145 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, 25 glucose; pH 7.3, 323 mOsm supplemented with 5 μM DNQX, 0.5 μM TTX, 0.3 μM strychnine, and 100 μM picrotoxin. Patch pipettes had 3- to 4-MΩ resistances when filled with the intracellular solution composed of (in mM): 107.5 potassium gluconate, 32.5 KCl, 10 Hepes, 0.1 EGTA, 4 MgATP, 0.6 NaGTP, 10 Tris phosphocreatine; pH 7.2, 297 mOsm. Series resistance (<5 MΩ) was compensated to 80%. $GABA_B$ responses were evoked by fast application of baclofen and recorded with an Axopatch 200B patch-clamp amplifier; filtering and sampling frequencies were set to 1 kHz and 5 kHz, respectively. Normalized K^+ current amplitudes were calculated as the maximal baclofen-induced K^+ current amplitudes recorded 30 min after pharmacological treatment relative to the maximal baclofen-induced K^+ current amplitudes recorded before pharmacological treatment of the same neuron.

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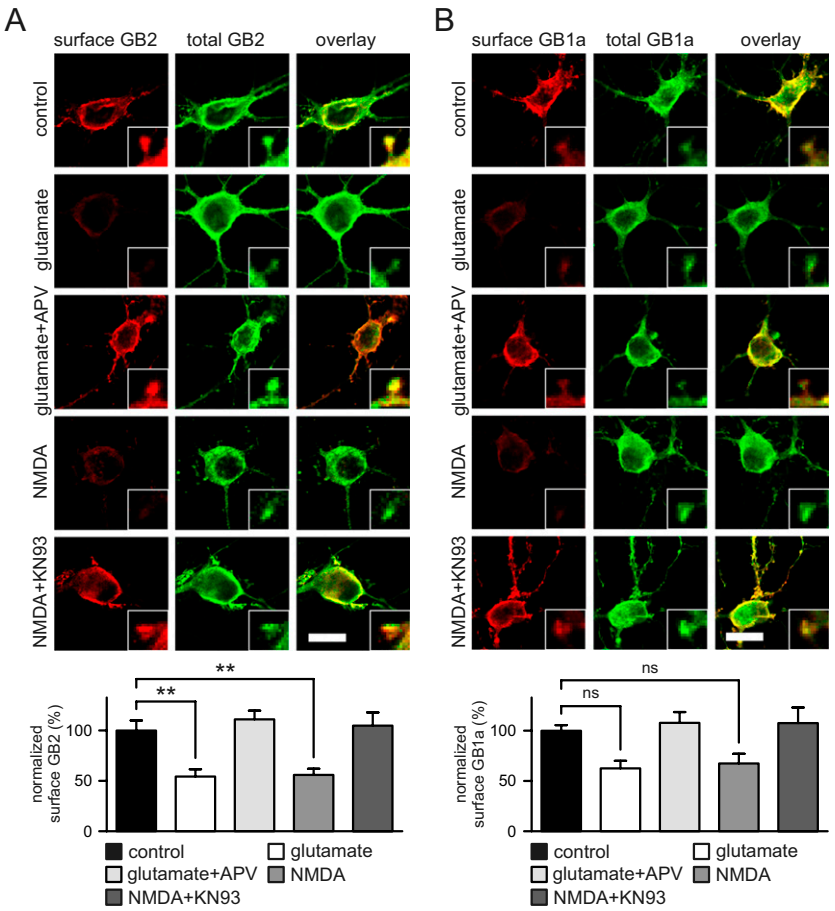

Fig. S1. NMDA-dependent internalization of $GABA_B$ receptors. (*A*) Rat hippocampal neurons coexpressing exogenous HA-GB2 and $GABA_{B1b}$ were treated at DIV14 as indicated. Surface $GABA_{B2}$ (GB2) protein was fluorescence-labeled with anti-HA antibodies before permeabilization. Total GB2 protein was fluorescence-labeled with anti-GB2 antibodies after permeabilization. Single optical planes captured with a confocal microscope are shown. (Scale bar, 15 μm.) (*Insets*) Representative spines at higher magnification. Surface GB2 protein was quantified by the ratio of surface to total fluorescence intensity. Values were normalized to control values in the absence of any pharmacological treatment. Surface GB2 protein was significantly decreased following glutamate or NMDA treatment. No significant reduction was observed with glutamate and NMDA treatment after preincubation with APV and KN-93, respectively (glutamate: 54.4 ± 13.2% of control, $n = 10$, $^{**}P < 0.01$; glutamate + APV: 111 ± 8.7%, $n = 8$; NMDA: 55.9 ± 6.2%, $n = 10$, $^{**}P < 0.01$; NMDA + KN-93: 105 ± 13.2, $n = 10$). (*B*) Rat hippocampal neurons coexpressing exogenous Myc-GB1a-eGFP and $GABA_{B2}$ were treated at DIV14 as indicated. Surface $GABA_{B1a}$ (GB1a) protein was fluorescence-labeled with anti-Myc antibodies before permeabilization. Total GB1a protein was fluorescence-labeled with anti-eGFP antibodies after permeabilization. Single optical planes captured with a confocal microscope are shown. (Scale bar, 15 μm.) (*Insets*) Representative spines at higher magnification. Note that GB1a does not efficiently penetrate the spines. Surface GB1a protein was quantified by the ratio of surface to total fluorescence intensity. Values were normalized to control values in the absence of any pharmacological treatment. Surface GB1a protein was decreased following glutamate or NMDA treatment, but this decrease did not reach statistical significance. No reduction in surface GB1a protein was observed with glutamate and NMDA treatment after preincubation with APV and KN-93, respectively (glutamate: 62.5 ± 7.5% of control, $n = 10$; glutamate + APV: 108 ± 10.7%, $n = 9$; NMDA: 67.4 ± 9.6%, $n = 8$; NMDA + KN-93: 108 ± 15.5, $n = 9$). Quantification was from nonsaturated images. Data are presented as mean ± SEM. ns, Not significant.

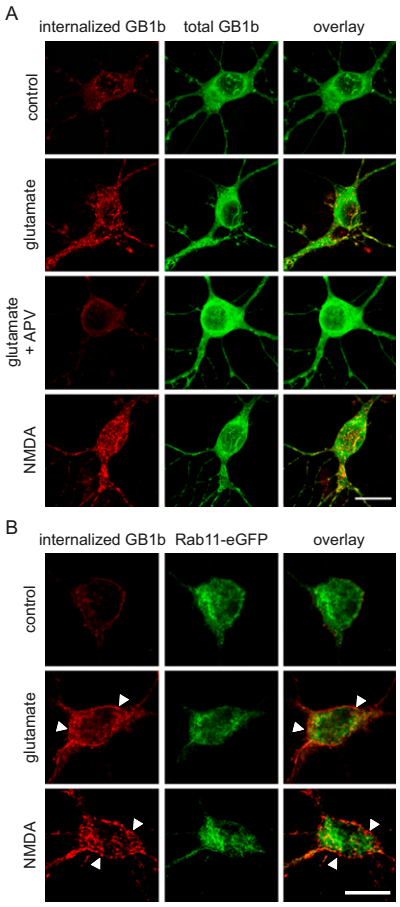

Fig. S2. NMDA-dependent internalization of $GABA_B$ receptors in neurons. (*A*) Rat hippocampal neuronal cultures were cotransfected with HA-GB1b-eGFP and $GABA_{B2}$ expression vectors and analyzed at DIV14. Constitutive internalization was observed under control conditions. After glutamate treatment, the rate of HA-GB1b-eGFP internalization was visibly increased. Preincubation with APV prevented glutamate-induced internalization of HA-GB1b-eGFP above basal levels. NMDA treatment was sufficient to increase internalization of HA-GB1b-eGFP. Maximum projections of representative neurons visualized by confocal microscopy are shown. (Scale bar, 15 μm.) (*B*) Rat hippocampal neurons were cotransfected with Myc-GB1b, $GABA_{B2}$, and Rab11-eGFP, which labels recycling endosomes. Under control conditions constitutively internalized GB1b appears to mostly colocalize with Rab11-eGFP. Following glutamate or NMDA treatment a fraction of internalized Myc-GB1b is observed in structures that are not labeled by Rab11-eGFP (arrowheads). Single optical planes of representative neurons visualized with confocal microscopy are shown. (Scale bar, 15 μm.)

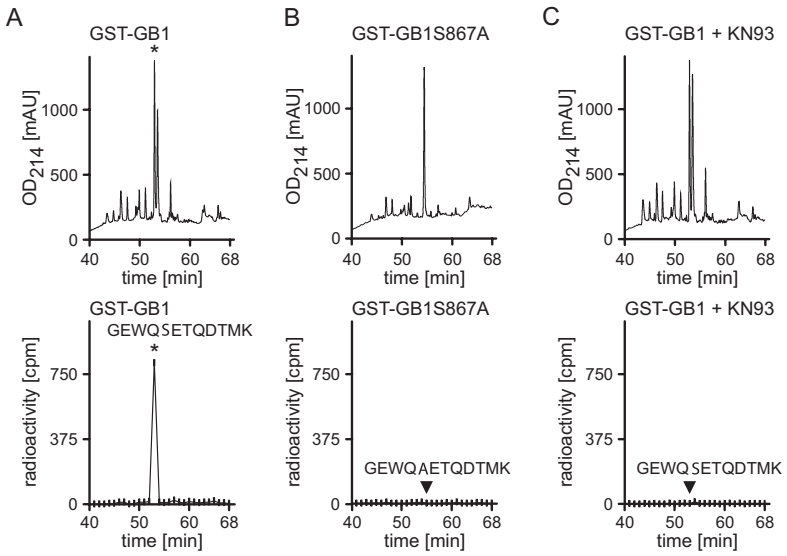

Fig. S3. CaMKII in brain extracts phosphorylates S867 in $GABA_{B1}$. (*A–C*) GST-fusion proteins were incubated with the cytosolic fraction of whole mouse brain extracts in the presence of [γ-^{32}P]-ATP. Proteolytic peptides were separated by RP-HPLC (*Upper*) and the radioactivity in the eluted fractions determined (*Lower*). (*A*) $GABA_{B1}$ peptide GEWQS^{867}ETQDTMK was identified by ESI-MS/MS as a major constituent of the highly radiolabeled fraction (asterisk) of the GST-GB1 effluent. Because the phosphorylation stoichiometry with brain extracts was low, a direct demonstration of phosphorylation at S867 was impossible. (*B*) Following phosphorylation of GST-GB1S867A with brain extracts, no radiolabel was detected in the RP-HPLC fraction containing the peptide GEWQA^{867}ETQDTMK (arrowhead), supporting that brain extracts normally phosphorylate S867. (*C*) No radiolabel was detected in the fraction containing GEWQS^{867}ETQDTMK (arrowhead) after phosphorylation of GST-GB1 in the presence of KN-93, implicating native CaMKII in phosphorylation of S867.

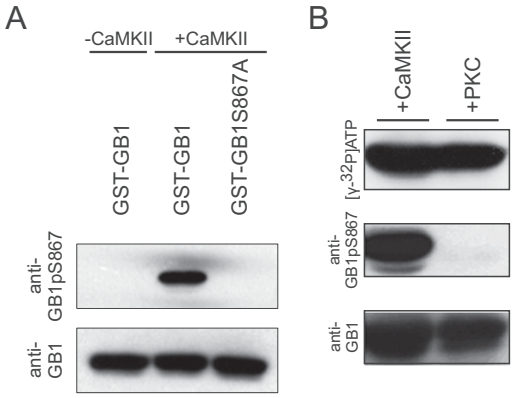

Fig. S4. Validation of the phosphorylation-state specific anti-GB1pS867 antibody. (*A*) Anti-GB1pS867 antibody was generated against the phosphorylated peptide ITRGEWQpS^{867}EAQDT and recognized GST-GB1 but not GST-GB1S867A after phosphorylation with recombinant CaMKII. Unphosphorylated GST-GB1 was not recognized by the anti-GB1p867 antibody. The same blot was probed with an anti-$GABA_{B1}$ antibody (anti-GB1) to demonstrate expression of the GST-fusion proteins. (*B*) In vitro phosphorylation of GST-GB1 with recombinant CaMKII and PKC in the presence of [γ-^{32}P]-ATP. Phosphorylated proteins were separated by SDS/PAGE and exposed to autoradiography or probed with anti-GB1pS867 or anti-GB1 antibodies. Anti-GB1pS867 recognized GST-GB1 after phosphorylation with CaMKII but not after phosphorylation with PKC.

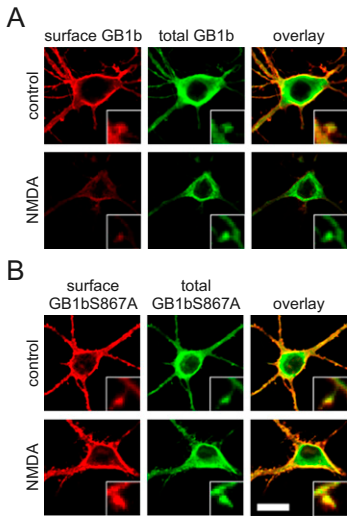

Fig. S5. Surface localization of transfected $GABA_{B1b}$ protein. Cultured hippocampal neurons expressing (*A*) HA-GB1b-eGFP (GB1b) or (*B*) HA-GB1bS867A-eGFP (GB1bS867A) together with transfected $GABA_{B2}$ were treated at DIV14 as indicated. Surface GB1b or GB1bS867A protein was fluorescence-labeled with anti-HA antibodies before permeabilization. Total GB1b or GB1bS867A protein was fluorescence-labeled with anti-eGFP antibodies after permeabilization. Alanine mutation of S867 prevented the NMDA-induced reduction of surface GB1b protein (quantification of data presented in Fig. 4*D*). Single optical planes captured with a confocal microscope are shown. (Scale bar,15 μm.) (*Insets*) Representative spines at higher magnification.

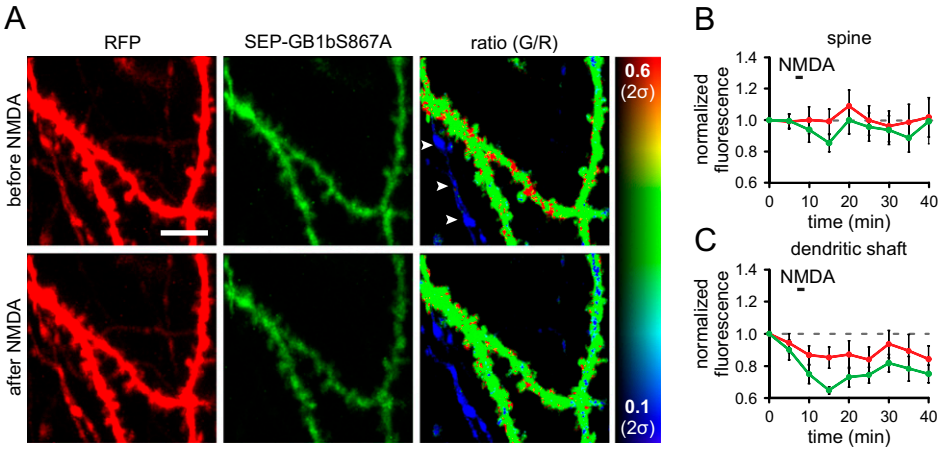

Fig. S6. SEP-GB1bS867A protein in dendritic spines and shafts is refractory to NMDA-induced internalization. (*A*) Red fluorescence (R), green fluorescence (G), and G/R ratio images of dendrites expressing freely diffusible RFP and SEP-GB1bS867A before and after NMDA application. NMDA application does not lead to decrease in green fluorescence in spines and evokes only minor internalization in shafts. G/R ratio is coded in rainbow colors and is scaled to encompass 2 SDs (2σ) of the average dendritic ratio before NMDA application to allow direct comparison with Fig. 6*A*. Similar to GB1b (7), GB1bS867A is effectively excluded from axons (arrowheads). (Scale bar, 5 μm.) (*B* and *C*) Time course of red and green fluorescence in dendritic spines (*B*) and shafts (*C*) before and after NMDA application. Seven cells were analyzed, Data are mean ± SEM; spines: $n = 34$; dendrites: $n = 7$.

Table S1. Putative phosphorylation sites in the C-terminal domain of $GABA_{B1}$

Residue	Position	Consensus sequence	Experimental evidence	Kinase
Serine	867		This paper	CaMKII
	877			
	878	CK1		
	887			
	909			
	917		(1)	AMPK
	923	PKC		
	934			
	942	CaMKII		
	949			
	953			
Threonine	861	CaMKII/CK2		
	869	GRK		
	872	CK1/GRK/PKC		
	875	CK1		
	879	CK1		
	929	CK2/MAPK	(2–5)	Not specified
Tyrosine	959			

Prediction of phosphorylation sites in the C-terminal domain of $GABA_{B1}$ with PredPhospho (available at http://www.nih.go.kr/phosphovariant/html/predphospho.htm). Kinase(s) for each predicted phosphorylation site were allocated by consensus sequence. Where available, experimental evidence is indicated and the kinase specified.

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