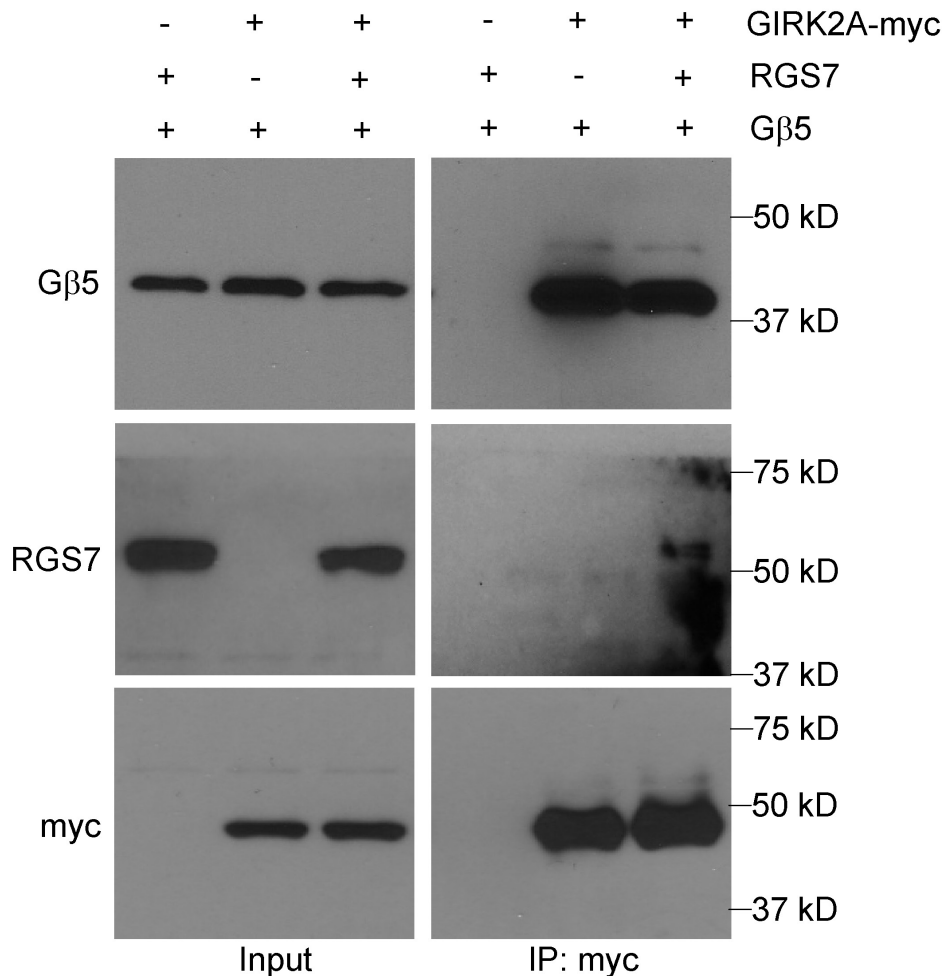


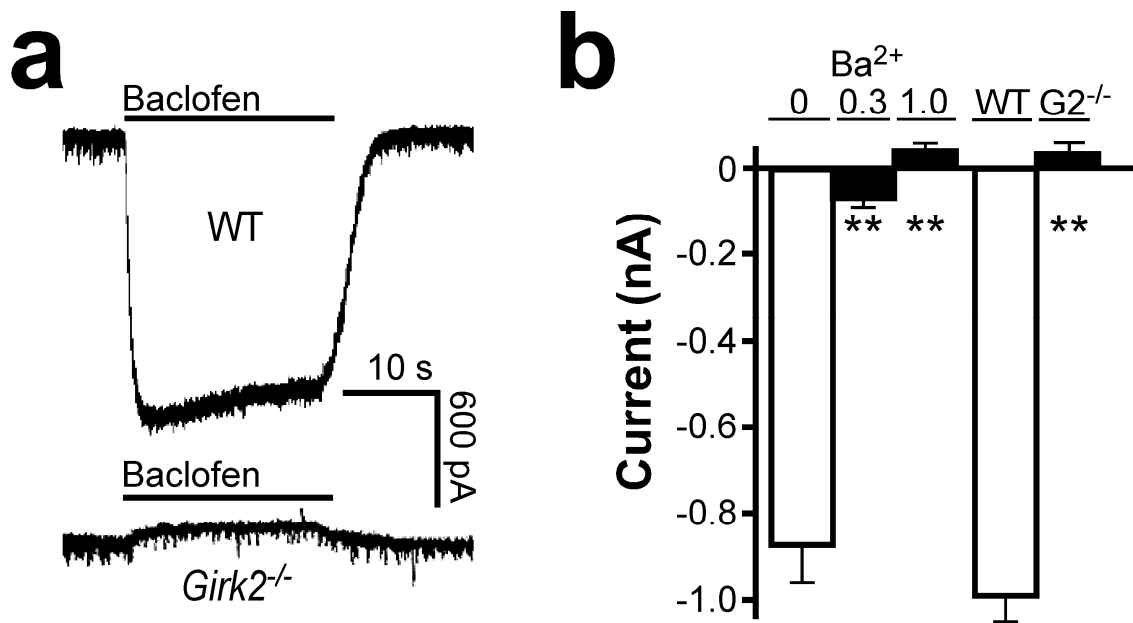
“Gβ5 recruits R7 RGS proteins to GIRK channels to regulate the timing of neuronal inhibitory signaling” Keqiang Xie, Kevin L. Allen, Saïd Kourrich, José Colón-Saez, Mark J. Thomas, Kevin Wickman, Kirill A. Martemyanov

SUPPLEMENTARY MATERIAL

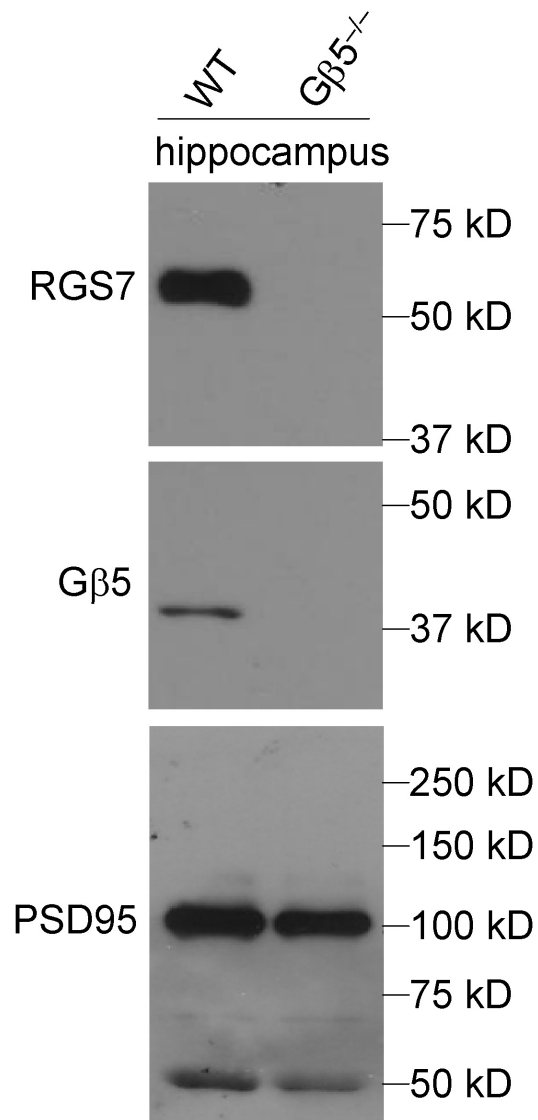
Supplemental Figures



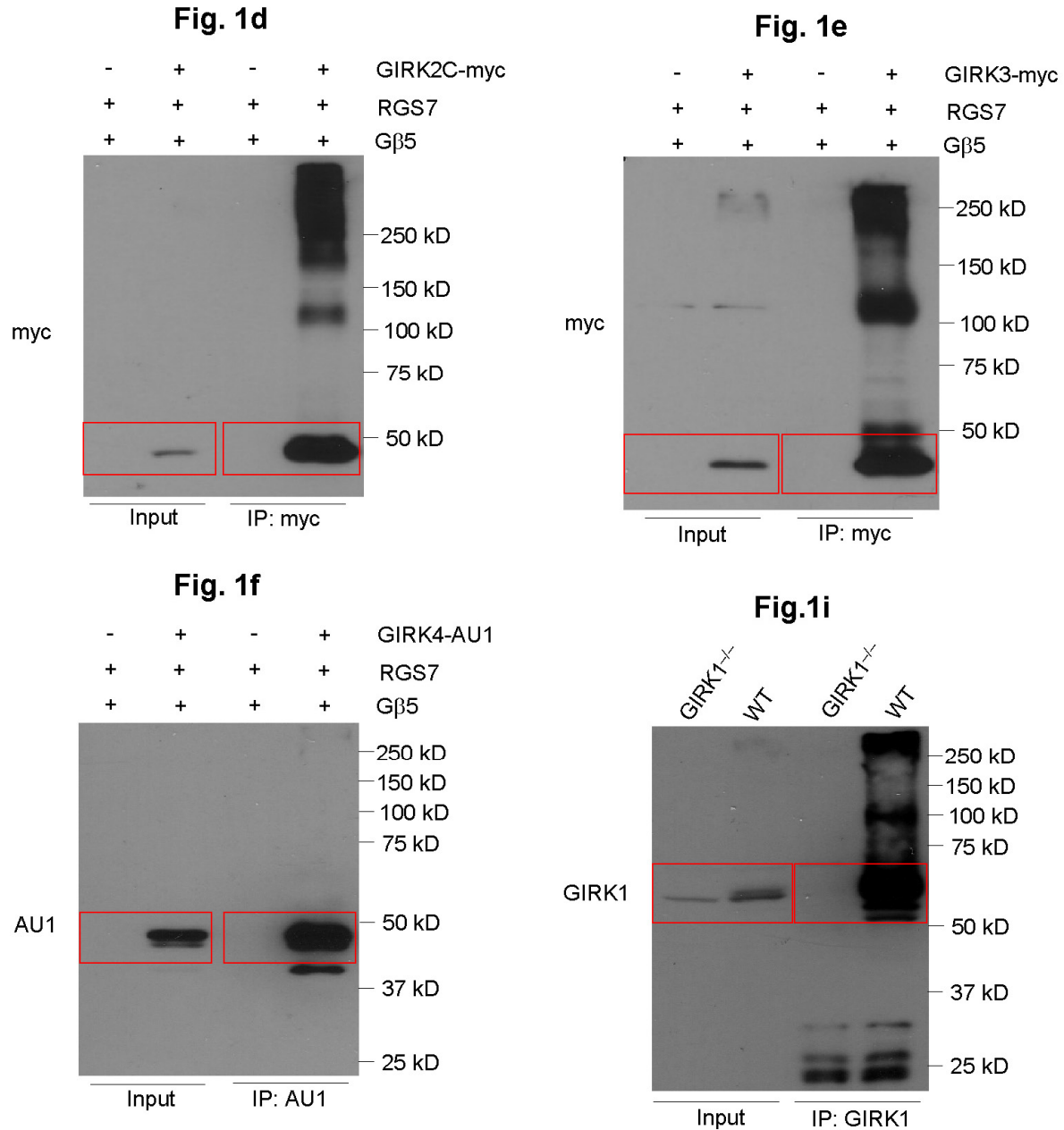
Supplemental Fig. 1. GIRK2 co-immunoprecipitates with Gβ5 in the absence of RGS7. Immunoprecipitations (IP) were conducted with a myc-tag antibody recognizing the c-myc epitope present in GIRK2. Resultant immunocomplexes were probed for the presence of RGS7, Gβ5, and GIRK2 by immunoblotting (IB). Transfected cells without GIRK2-myc construct served as controls for non-specific binding.



Supplemental Fig. 2. GABA_B receptor stimulation evokes a GIRK-dependent response in cultured mouse hippocampal neurons. **a**) Representative currents in hippocampal neurons from wild-type (WT, upper) and *Girk2*^{-/-} (lower) mice evoked by baclofen (100 μM). The bar denotes the duration of baclofen application. Scale bars: 400 pA/10 s. **b**) Baclofen-induced current measured in wild-type neurons in the absence and presence of barium (Ba²⁺, 0-1.0 mM) and in cultures derived from wild-type (WT) and *Girk2*^{-/-} siblings (*n* = 5-13 per group). Note the small outward current evoked by baclofen in wild-type neurons treated with 1 mM Ba²⁺ and neurons from *Girk2*^{-/-} mice, reflecting the modulation of an additional channel type. Symbols: **** *P* < 0.05, 0.01, and 0.001 respectively, vs. control. Error bars are s.e.m.



Supplemental Fig. 3. Absence of RGS7 protein in the hippocampus from $G\beta 5^{-/-}$ mice. Total protein lysates obtained from hippocampal regions of adult wild-type (WT) and $G\beta 5^{-/-}$ mice were probed for the presence of RGS7 and $G\beta 5$ by immunoblotting. The major postsynaptic protein PSD95 served as a loading control.



Supplemental Fig. 4. Un-cropped full-length immunoblotting data for panels presented in Fig. 1. Most blots presented in Fig.1 contained only single specific band. They were cropped to eliminate blank areas that did not contain any other information except as presented. Full-length versions of these blots are available from authors upon request. All blots that contained additional immunoreactivity outside of the cropped area are presented in this figure. Red boxes outline areas of the blots presented in Fig.1 of the main article.

Materials and Methods

Animals. All studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and were granted formal approval by the Institutional Animal Care and Use Committee of the University of Minnesota. All efforts were made to minimize the use of animals in this study, as well as their suffering. All animals used in this study were bred on-site. The generation of *Girk2*^{-/-} mice was described previously¹. *Gβ5*^{-/-} mice were provided by Dr. Jason Chen (Virginia Commonwealth University) and were out-bred onto the C57BL/6 background for 5 generations². All animals used for experiments were littermates derived from crosses between animals heterozygous for the null allele. Mouse genotypes were determined using established PCR-based assays and genomic DNA isolated from small (<0.5 cm) tail biopsies.

DNA Constructs. Cloning of full-length *Gβ5* and *RGS7* was described previously^{3,4}. C-terminal epitope-tagged rat *GIRK1* (*GIRK1*-AU5), mouse *GIRK2* (*GIRK2*-myc), and rat *GIRK4* (*GIRK4*-AU1) expression constructs were generated as described and kindly provided by Dr. David Clapham^{5,6}. The C-terminal myc-tagged *GIRK3* expression construct was generated by PCR using mouse *GIRK3* cDNA as the template⁷; the *GIRK3*-myc cassette was cloned into pCDNA3.1-based plasmid using *NheI* (5') and *HindIII* (3') restriction enzyme sites. The C-terminal coding region of rat *GIRK1* (amino acids 180 – 501), mouse *GIRK2* (*GIRK2C* isoform, amino acids 191 - 425), mouse *GIRK3* (amino acids 157 - 393), and rat *GIRK4* (amino acids 186 - 419) was subcloned into the 3' end of the GST open reading frame of vector pGEX-2T (GE Healthcare) to create GST-*GIRKCT* fusion proteins using PCR primers tagged with *BamHI* linker at the 5' end and an *MfeI* linker at the 3' end.

Protein expression and purification. Recombinant complexes of *Gβ5* with His-tagged full-length *RGS7* (mouse) or GGL fragment of *RGS9* (amino acids 193-284) were expressed in Sf9/baculovirus system and purified by Ni-NTA chromatography as described previously^{8,9}. GST-tagged C-terminal domains of *GIRK* subunits were expressed in BL21(DE3) *E.coli* strain. Cells were induced by 1 mM Isopropyl β-D-1-thiogalactopyranoside at density of OD₆₀₀ = 0.6-1.0 and allowed to grow for three hours. Cells were lysed by sonication in PBS-Lysis Buffer (140 mM NaCl, 5 mM KCl, 10 mM dithiothreitol, 20 mM Na₂HPO₄/NaH₂PO₄, pH=7.3) supplemented with Complete (Roche) protease inhibitors. Proteins were solubilized from the particulate fraction by 6 M urea in PBS-Lysis Buffer, dialyzed into the urea-free PBS-Lysis Buffer and purified by affinity chromatography on glutathione-Sepharose 4B beads (GE healthcare) following the manufacturer's recommendations.

Immunoprecipitation and immunoblotting. HEK293T cells were transfected with 2.5 μg of cDNA as indicated per well in 6-well plate using Lipofectamine LTX reagent (Invitrogen) and incubated at 37 °C, 5 % CO₂ for 48 hours. Cells were lysed using 500 μl of detergent buffer: 1xPBS buffer + 150 mM NaCl + 0.5 % *n*-Dodecanoylsucrose containing complete protease inhibitor cocktail (Roche). The homogenate was centrifuged at 15,000 g, 15 min at 4 °C. Supernatant was incubated with specific antibody as indicated and 20 μl 50 % protein G slurry (GE healthcare) on rocker at 4 °C for 1 hr. After 3 washes with detergent buffer, the proteins were eluted from beads with 50 μl 2xSDS sample buffer by boiling for 5 min. Proteins retained by the beads were analyzed with SDS-PAGE and immunoblot by using specific antibodies.

Rabbit anti-RGS7 (7RC1), anti-Gβ5 (ATDG) antibodies were generously provided by Dr. William Simonds (NIDDK/NIH). Anti-AU1 (GeneScript), anti-AU5 (MMS-135R, Covance), anti-c-myc (GeneScript), anti-PSD95 (Chemicon), anti-GIRK1 (Alomone labs) and anti-PLCβ2 (Santa Cruz) antibodies were purchased.

Immunoprecipitation from native hippocampal tissue was performed as follows: Hippocampi from adult mice (~15 mg wet tissue) were homogenized in 10 volumes (w/v) of membrane preparation buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 50 mM EDTA and 1 mM PMSF) by passing through a series of needles with decreasing gauges. The homogenized lysates were centrifuged at 39,000xg for 20 min at 4°C. Pellets were washed twice with 10 volumes of membrane preparation buffer. After final centrifugation, the membranes were dissolved in 0.5 ml of detergent buffer: 1xPBS buffer + 150 mM NaCl + 0.5 % *n*-Dodecanoylsucrose containing complete protease inhibitor cocktail (Roche) for 30 minutes followed by centrifugation at 14,000xg for 15 minutes at 4°C. Resulting supernatants were incubated with 10 μl protein G beads coupled to 5 μg of GIRK1 antibody (Alomone labs). Proteins retained by the protein G beads were eluted with 50 μl of SDS sample buffer after washing the beads three times with the detergent buffer and separated by SDS-PAGE followed by Western blotting detection.

Hippocampal cultures. Primary cultures of hippocampal neurons were prepared using a modified version of a published protocol¹⁰. Briefly, hippocampi were extracted from neonatal (P1-4) pups and placed into an ice-cold HBSS/FBS solution: Hank's Balanced Salt Solution (Sigma; St. Louis, MO), 4.2 mM NaHCO₃, 1 mM HEPES, and 20% FBS. The tissue was washed twice with HBSS/FBS, and then 3 times with HBSS alone. Hippocampi were digested for 5 min with 10 mg/mL Trypsin Type XI (Sigma; St. Louis, MO) in a solution that contained (in mM): 137 NaCl, 5 KCl, 7 Na₂HPO₄, and 25 HEPES, pH 7.2. The tissue was washed twice with HBSS/FBS, 3 times with HBSS alone, and then hippocampi were mechanically-dissociated in HBSS (supplemented with 12 mM MgSO₄) using Pasteur pipettes of decreasing diameter. The neurons were pelleted by centrifugation (600 x g for 10 min at 4°C) and plated onto 8 mm glass coverslips pre-treated with Matrigel (BD Biosciences; San Jose, CA) in 48-well plate. Neurons were allowed to adhere for 30 min prior to adding 0.3 mL of pre-warmed culture medium consisting of Neurobasal (Invitrogen; Carlsbad, CA), 2 mM glutamine and 2% B-27 supplement. Neurons were incubated at 37°C/5% CO₂, and half of the medium was replaced with fresh medium on each of the first 3 days of culture. Neurons were kept in culture for 10-12 days prior to experimental manipulation.

GST pull down assay. The assays were performed as previously described⁴. Briefly, purified recombinant GST fusion proteins or GST control protein (0.25 μM), purified GGL/Gβ5 or RGS7/Gβ5 proteins (0.25 μM) were co-incubated with 20 μl of 50% glutathione agarose beads slurry (GE Healthcare) in binding buffer (20 mM Tris, pH 7.2, 300 mM NaCl, 0.25% *n*-dodecanoylsucrose, 50 μg/ml bovine serum albumin) for 1 hour at 4 °C. The beads were washed with binding buffer 3 times. The proteins were eluted in SDS sample buffer, and proteins retained by the beads were detected by anti-Gβ5 (ATDG) and anti-GST (Z-5, Santa Cruz) antibodies by immunoblotting.

Whole-cell voltage-clamp experiments. Coverslips containing neurons were transferred to a chamber containing a low-K⁺ bath solution (in mM): 145 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5.5 D-glucose, 5 HEPES/NaOH (pH 7.4). Bath solution was perfused continuously through the

chamber (0.5 mL volume) at 1 mL/min throughout the experiments. Neurons were visualized using an Olympus IX-70 microscope. Membrane potentials and whole-cell currents were measured in large neurons (>75 pF) exhibiting pyramidal morphology with hardware (Axopatch-200B amplifier, Digidata 1320) and software (pCLAMP v. 9.0) from Axon Instruments, Inc. (Sunnyvale, CA). Borosilicate patch pipettes (3-5 M Ω) were filled with (in mM): 130 KCl, 10 NaCl, 1 EGTA/KOH (pH 7.2), 0.5 MgCl₂, 10 HEPES/KOH (pH 7.2), 2 Na₂ATP, 5 phosphocreatine, 0.3 GTP. Baclofen (*R*-(+)- β -(aminomethyl)-4-chlorobenzenepropanoic acid hydrochloride) was purchased from Sigma (St. Louis, MO). Baclofen-induced currents were measured at room temperature using a high-K⁺ bath solution (in mM): 120 NaCl, 25 KCl, 1.8 CaCl₂, 1 MgCl₂, 5.5 D-glucose, 5 HEPES/NaOH (pH 7.4). The high-K⁺ bath solution (+/- baclofen) was applied directly to the soma and proximal dendrites with an SF-77B rapid perfusion system (Warner Instruments, Inc.; Hamden, CT). Activation and deactivation kinetics of the holding current response triggered by switching from low-K⁺ to high-K⁺ bath solutions and back again were 198 \pm 13 and 249 \pm 16 ms, respectively (n=9), consistent with values from published studies with analogous designs ^{11,12}.

Upon achieving whole-cell access, resting membrane potentials were recorded in current-clamp (I=0) mode and corrected for a 5 mV junction potential. Current responses to the application of the high-K⁺ solution (+/- baclofen) were then measured at a holding potential of -70 mV. All currents were low-pass filtered at 1 kHz, sampled at 2 kHz, and stored on computer hard disk for subsequent analysis. Steady-state current amplitudes were measured for each experiment. Current activation rates were extracted from an exponential fit of the current trace corresponding to the onset of drug effect and the peak evoked current, while deactivation rates were extracted from an exponential fit of the trace corresponding to the return of current to baseline following removal of drug (pCLAMP v. 9.0 software). The Ba²⁺ sensitivity of the evoked current was tested by measuring baclofen-induced current in the high-K⁺ solution, followed 60 s later by a measurement of the baclofen-induced current in a high-K⁺ solution containing either 0.3 or 1 mM BaCl₂. Only those experiments in which the access resistances were stable and low (<15 M Ω) were included in the analysis.

IPSC experiments. Transverse hippocampal slices (300 μ m) from wild-type and G β 5^{-/-} mice (4-5 months old) were obtained by cutting tangentially to the longitudinal axis of the hippocampus. Slices recovered in a holding chamber for at least 1 h before use. During recordings, slices were superfused with ACSF (22–23°C) saturated with 95% O₂/5% CO₂ and containing (in mM): 119 NaCl, 2.5 KCl, 1.0 NaH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂, 26.2 NaHCO₃, and 11 glucose. Kynurenic acid (2 mM) was used to block glutamatergic transmission during recordings. Pyramidal cells in the CA1 field were visualized using infrared-differential interference contrast optics. Synaptically-evoked IPSCs were measured with experimenter blind to genotype at a holding potential of -65 mV using a Multiclamp 700A amplifier (Molecular Devices, Foster City, CA). Recording electrodes (3–5 M Ω) contained (in mM): 120 K-gluconate, 20 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 Na₂ATP, and 0.3 Tris-GTP. Afferents were stimulated at 0.05 Hz by a glass monopolar microelectrode filled with ACSF that was always positioned in the top part of the *lacunosum moleculare* layer exactly below the recording electrode. A range of stimulation intensity (50-300 μ A) was used to identify the maximal IPSC amplitude. Data were filtered at 2 kHz, digitized at 10 kHz, and collected and analyzed using Igor Pro software (Wavemetrics; Lake Oswego, OR). Membrane potentials of CA1 neurons ranged between -70 and -55 mV. Series resistances ranged from 10 to 20 M Ω and input resistances (R_M) were monitored on-line

with a 40 pA/150 ms current injection given before every stimulus. Only cells with a stable R_A ($\Delta < 10\%$) for the duration of the recording were kept for analysis.

Locomotor activity. Locomotion was evaluated in automated Plexiglas open-field environments (Med Associates, Inc., St. Albans, VT) under illuminated conditions, as described¹³. On test day, mice were habituated to testing room environment for 1 hr. Mice were injected with baclofen (5 or 10 mg/kg, *s.c.*) or saline (10 ml/kg, *s.c.*) and then immediately placed in the open-field chambers; activity was then monitored for 3 (5 mg/kg baclofen) or 6 h (10 mg/kg baclofen). Horizontal activity was measured in terms of the total distance traveled in the monitoring period or distance traveled in 5-min intervals.

Statistical Analysis. Data are presented throughout as the mean \pm SEM. Statistical analyses were performed using Prism (GraphPad Software, Inc.; La Jolla, CA) or Minianalysis software (Synaptosoft; Decatur, GA). The impact of genotype (wild-type vs. $G\beta 5^{-/-}$) on baclofen-induced current responses (steady-state current amplitude and density and kinetics) was evaluated using Student's *t*-test or two-way ANOVA, followed by Bonferroni's post hoc test. The impact of external barium on baclofen-induced currents in wild-type neurons was assessed with one-way ANOVA, followed by Tukey's post hoc test. GABA_B-mediated IPSC amplitude, kinetics, and area-under-the-curve were analyzed using two-tailed Student's *t* tests or two-way ANOVA. Motor activity data were analyzed by Bonferroni *t*-test for each dose of baclofen administered. For all analyses, the level of significance was set at $P < 0.05$.

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