Supporting Information

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SI Materials and Methods

Electrophysiology. Recordings were started 5–10 min after stable whole-cell access was obtained. mIPSCs were recorded in the presence of 200 nM tetrodotoxin (Sigma-Aldrich), 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (Sigma), and 20 μM 2-D-aminophosphonopentanoic acid (Sigma). Synaptic currents were recorded using an Axopatch 200B amplifier (Molecular Devices), filtered at 2 kHz, sampled at 5 kHz, digitized (Digidata 1320A; Molecular Devices), and stored for off-line analysis. Access resistance 10–18 Mohm; (80% compensation) was monitored using a −5 mV voltage, applied every 120 s, and data from cells were discarded when >15% change occurred. Miniature events were analyzed using pClamp 9.2 (Clampfit, Axon Instruments), MiniAnalysis 6.0.3 software (Synaptosoft), and IGOR Pro (Wavemetrics) using pooled population data and expressed as mean ± SEM. The decay phase was fitted with a mono-exponential function (τ decay). Rise time was also analyzed by comparing the mean 10–90% rise time.

Fluorescent Bgt Labeling and $GABA_AR$ Endocytosis Assay. $^{BBS}\beta3$ and $^{BBS}\beta3^{S408/9A}$ constructs that contain an N terminally inserted pHluorin reporter and the minimal α-bungarotoxin (Bgt) binding site peptide (BBS) and are functionally silent were used for these experiments (2, 9). HEK-293 cells were labeled with rhodamine labeled Bgt (Rhoda::Bgt) at a final concentration of 3 μg/mL for 5 min at 37°C, then washed extensively in PBS and placed in fresh equilibrated medium containing 30 μg/mL unlabeled Bgt at 37°C (to label any newly inserted receptors with a non fluorescent Bgt), allowing measurement of Rhoda::Bgt loss due to receptor endocytosis. Hippocampal neurons were pretreated for 10 min with 150 μM tubocurarine (Sigma Aldrich) at 37°C to block endogenous acetylcholine receptors (2, 8) then incubated with Rhoda::Bgt at a final concentration of 3 μg/mL with 150 μM tubocurarine for 5 min at 37°C, then washed extensively and placed in fresh equilibrated medium containing 30 μg/mL unlabeled Bgt and 150 μM tubocurarine at 37°C to label any newly inserted receptors with a non fluorescent Bgt, allowing measurement of Rhoda::Bgt loss due to receptor endocytosis. The cells were then fixed under nonpermeabilizing conditions at varying time points, subjected to immunocytochemistry with anti-GFP antibodies and examined by confocal microscopy. For HEK-293 cell endocytosis, 10-μm sections of plasma membrane were analyzed for average Rhoda::Bgt fluorescence intensity. For neuronal experiments, Rhoda::Bgt average fluorescence intensity on a 20-μm length of 2–3 proximal dendrites/neuron was measured and normalized to pHluorin construct expression levels in the same region. The remaining surface Rhoda::Bgt was calculated according to the following formula: $100 \times [I_{t20}/I_{t0}]$ where I_t represents the average fluorescence intensity at the given time point. Image acquisition and data analysis was performed blinded. All Bgt derivatives were obtained from Invitrogen.

Image Acquisition and Analysis of Fixed HEK-293 Cells and Neurons. Confocal images of immunostained cells and neurons were taken using a 60× oil immersion objective, acquired with Bio-Rad software on a Bio-Rad Radiance 2100MP confocal microscope and analyzed with MetaMorph (Molecular Devices) software. Surface anti-GFP staining of $^{BBS}\beta3$ and $^{BBS}\beta3^{S408/9A}$ and synaptic quantification was done as described previously (9). To quantify fluorescence intensity of surface $^{BBS}\beta3$ and $^{BBS}\beta3^{S408/9A}$ synaptic staining, images of neurons were thresholded to a point at which dendrites were outlined. Synapsin staining was thresholded to a set value and kept constant for control and test neurons. Next a 20-μm section along a given proximal dendrite was selected, and an 8-bit binary image (exclusive) was made of the synapsin staining in the outlined dendrite. We then subtracted away all surface $\beta3$ subunit staining that did not colocalize with the binarized synapsin staining. As a result, only surface $\beta3$ subunit staining that colocalized with synapsin remained, and the average fluorescence intensity, total fluorescence levels, and dendritic area covered by these synaptic receptor populations were determined. For extrasynaptic measurements, the binarized synapsin regions colocalizing with $\beta3$ subunit staining were subtracted from the dendritic region before intensity measurements were made.

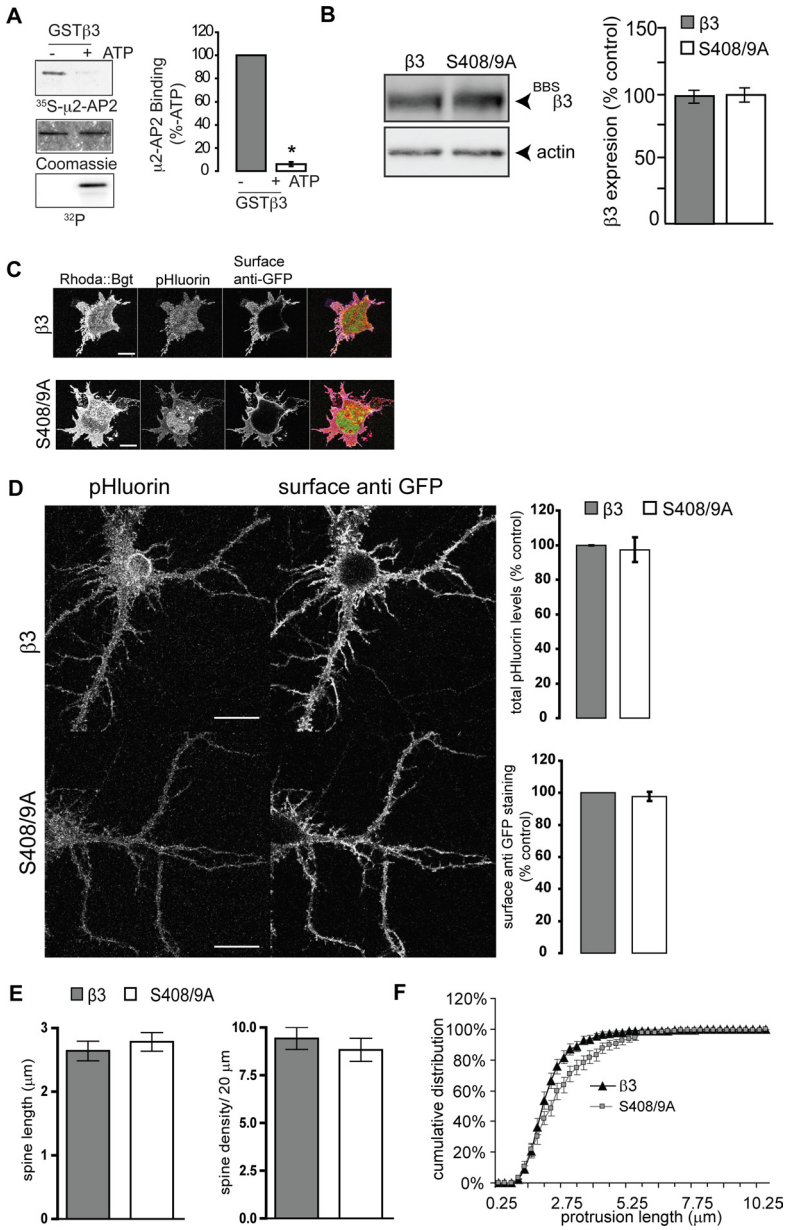

Fig. S1. Characterization of $^{BBS}\beta3$ or $^{BBS}\beta3^{S408/9A}$ expression in HEK-293 cells and neurons. (*A*) GST$\beta3$ fusion protein was subjected to an in vitro kinase assay either in the absence(−) or presence(+) of purified PKA for 30 min. Mock phosphorylated and phosphorylated proteins were separated by SDS/PAGE then transferred to nitrocellulose membrane and overlayed with ^{35}S-AP2-$\mu2$ adaptin. Bound material was visualized by autoradiography (upper panel) and normalized to coomassie stain (middle panel). Data are expressed as a percentage of mock phosphorylated control. To measure the stochiometry of phosphorylation fusion proteins were exposed to PKA in the presence of [^{32}]ATP, followed by SDS/PAGE and exposure to a phospho-imager (lower panel). Under the conditions used phosphorylation of GST$\beta3$ was approximately 0.7 mol/mol. After correction for protein content the level of $\mu2$-AP2 binding was calculated and normalized to that evident in the absence of ATP (100%) ($P < 0.001$; students *t* test, $n = 4$). *, significantly different to control. (*B*) HEK-293 cells transiently expressing $^{BBS}\beta3$ or $^{BBS}\beta3^{S408/9A}$ subunits were lysed in 2% SDS and after correction for protein content extracts were immunoblotted with anti-$\beta3$ antibodies. Expression levels were then normalized to that evident to $^{BBS}\beta3$. (*C*) Transfected HEK-293 cells were labeled with 3 μg/mL Rhoda::Bgt for 5 min, washed, then fixed under nonpermeabilizing conditions and stained for surface $^{BBS}\beta3$ or $^{BBS}\beta3^{S408/9A}$ subunit expression with anti-GFP antibody. Representative confocal images of transfected HEK-293 cells: red, Rhoda::Bgt; green, pHluorin; blue, surface anti-GFP staining. (Scale bars, 10 μm.) (*D* and *E*) Hippocampal neurons were transiently transfected with $^{BBS}\beta3$ or $^{BBS}\beta3^{S408/9A}$ subunits and immunostained with anti-GFP antibodies under nonpermeabilizing conditions. Fluorescence intensity quantification was used to assess wild type $\beta3$ and S408/9A mutant subunit distributions: total construct expression levels were determined from the endogenous pHluorin signal, and anti-GFP antibody staining was analyzed for surface expression levels. (Scale bars, 20 μm.) (*E*) Spine length and density were quantified in hippocampal neurons transiently transfected with $^{BBS}\beta3$ or $^{BBS}\beta3^{S408/9A}$ subunits. Spine length for $^{BBS}\beta3 = 2.78 \pm 0.15$, $^{BBS}\beta3^{S408/9A} = 2.64 \pm 0.15$. The # spines per 20 μm for $^{BBS}\beta3 = 9.42 \pm 0.57$, $^{BBS}\beta3^{S408/9A} = 8.83 \pm 0.60$. (*F*) Cumulative histogram of spine lengths in neurons nucleofected with $^{BBS}\beta3$ or $^{BBS}\beta3^{S408/9A}$ subunits. Histogram shows the distribution frequency ± SEM.

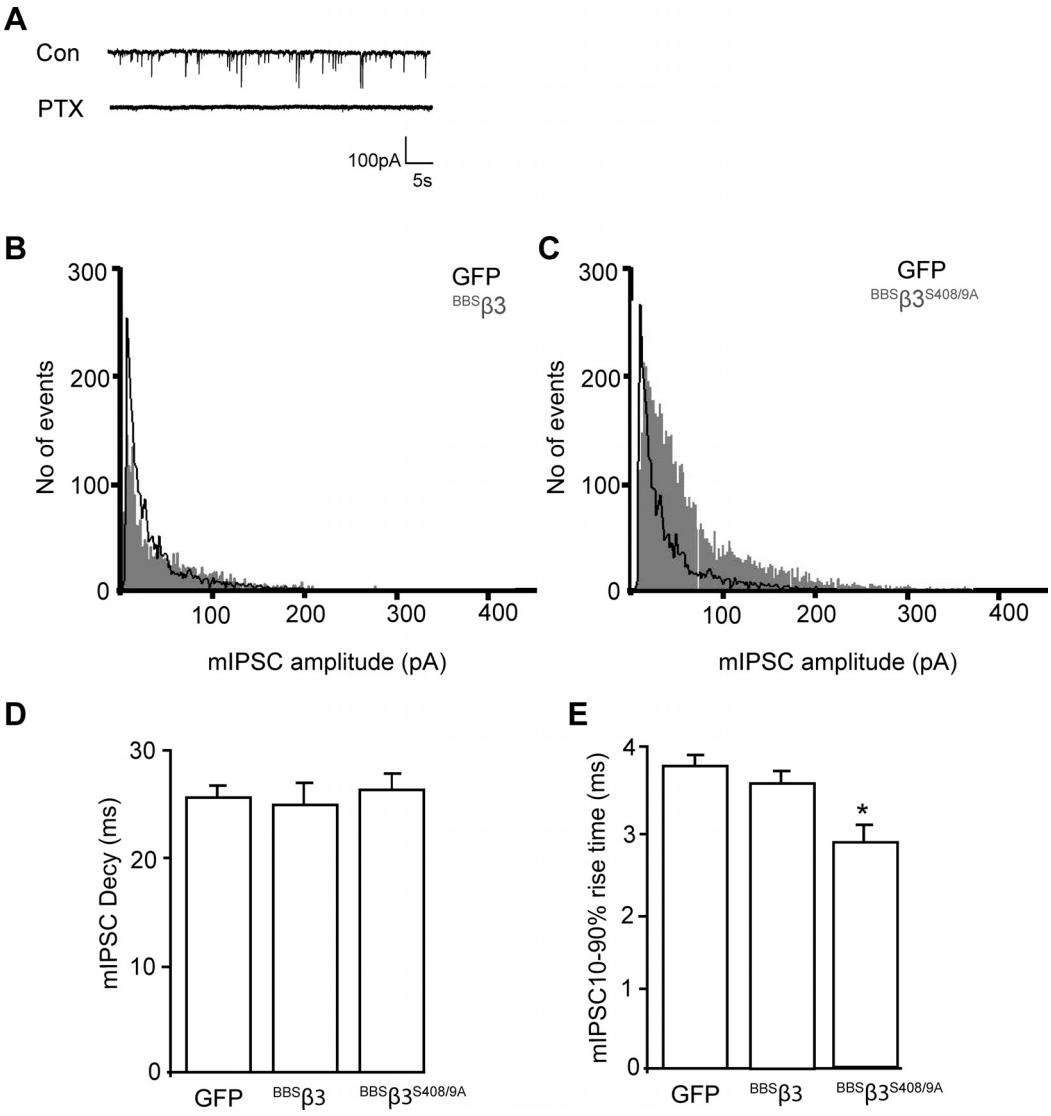

Fig. S2. Comparison of the properties of mIPSCs in hippocampal neurons expressing pHluorin tagged $GABA_AR$ $\beta 3$ subunits. (*A–E*) The properties of mIPSCs were compared in control (GFP) neurons and neurons expressing $^{BBS}\beta 3$ or $^{BBS}\beta 3^{S408/9A}$ subunits. (*A*) representative typical mIPSC events for GFP expressing neurons with control (Con) and PTX-treatments are illustrated. Peak amplitude binned data plots for mIPSCs recorded from neurons expressing GFP (black line), $^{BBS}\beta 3$ (gray), and $^{BBS}\beta 3^{S408/9A}$ subunits (gray) are shown in (*B*) and (*C*), respectively. Note that the distribution for the mIPSCs recorded from $^{BBS}\beta 3^{S408/9A}$ are shifted to higher amplitudes relative to neurons expressing GFP alone. (*D*) Ten percent to 90% decay times were fitted by a single exponential to calculate τ_{decay} from neurons expressing GFP, $^{BBS}\beta 3$, and $^{BBS}\beta 3^{S408/9A}$ respectively ($n = 10$). (*E*) mIPSCs rise time (10–90%) was measured from neurons transfected with GFP, $^{BBS}\beta 3$, and $^{BBS}\beta 3^{S408/9A}$ respectively ($n = 10$). ∗, $P < 0.05$, significantly different from control (Kolmogorov–Smirnov test; $n = 8–10$).

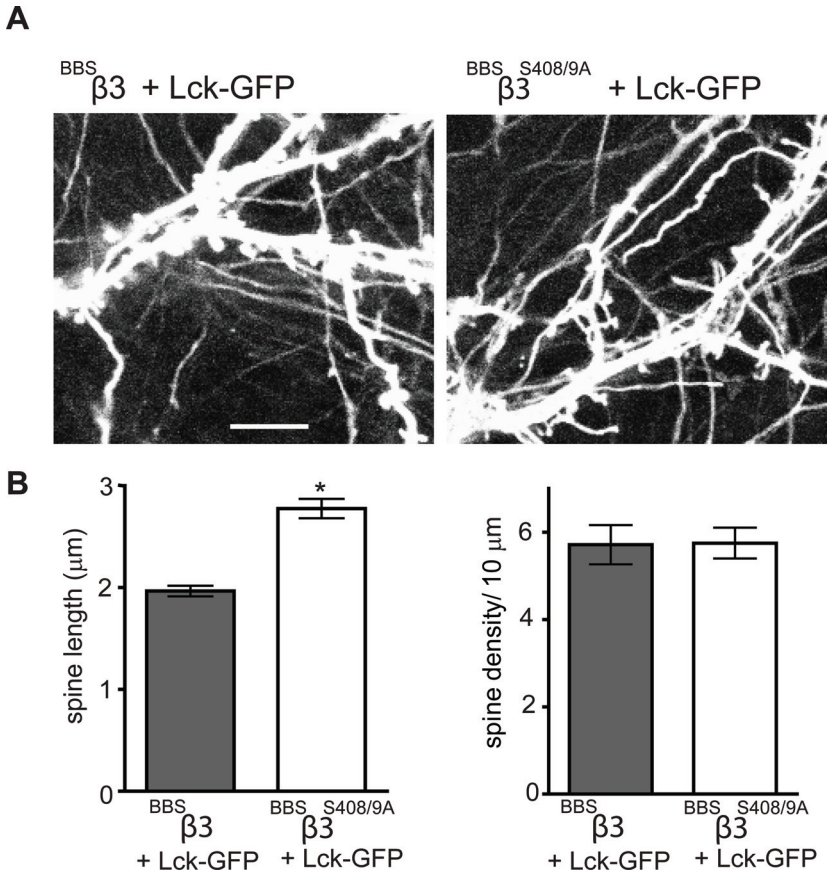

Fig. S3. Spine characterization in $^{BBS}\beta3$ or $^{BBS}\beta3^{S408/9A}$ expressing neurons co-nucleofected with membrane-targeted eGFP (Lck-GFP). (*A*) Live confocal images of $^{BBS}\beta3$ or $^{BBS}\beta3^{S408/9A}$ neurons co-nucleofected with membrane-targeted eGFP (Lck-GFP). Neurons were maintained at 37°C in extracellular Hepes based buffer. (Scale bar = 10 μm.) (*B*) Lck-GFP was used as an independent membrane marker to quantify spine length and density in $^{BBS}\beta3$ or $^{BBS}\beta3^{S408/9A}$ expressing neurons. Spine length for $^{BBS}\beta3$ +Lck-GFP = 1.96 ± 0.05, $^{BBS}\beta3^{S408/9A}$ +Lck-GFP = 2.78 ± 0.10. Spine density per 10 μm for $^{BBS}\beta3$ +Lck-GFP = 5.71 ± 0.45, $^{BBS}\beta3^{S408/9A}$ +Lck-GFP = 5.75 ± 0.35. (spine length, *, $P < 0.05$, significantly different to control, *t* test, 1–2 processes per neuron, 10 neurons per construct, $n = 2$ cultures).