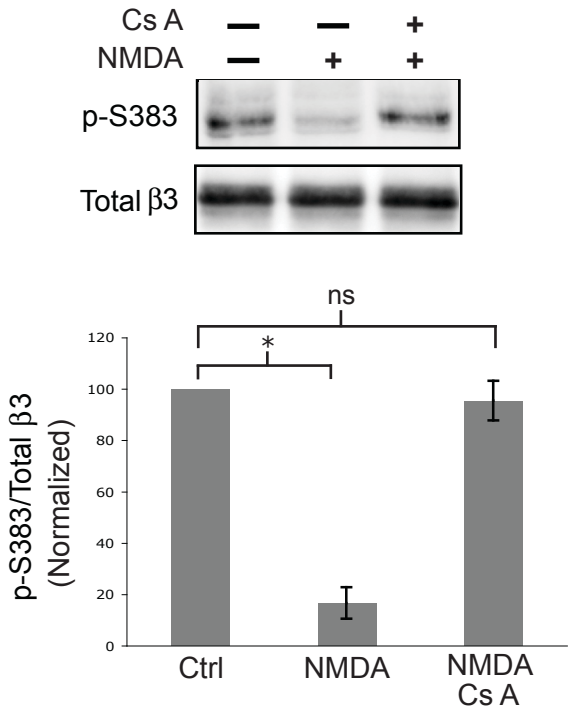

Supplmentary Figure 1.

Application of NMDA induces de-phosphorylation of β3S383 by Calcinuerin.

Hippocampal neurons (16-21 div) were treated with or without NMDA (50 μM) and 1 μM cyclosporin A (Cs A, a calcinuerin inhibitor) for 5 minutes as indicated. Cs A was added 1 minute prior to the application of NMDA. Neuronal lysates (20 μg) were immunoblotted with anti-p-S383 IgGs and anti-β3 IgGs as indicated. Graph represents the ratio of the p-S383 signal to the total β3 signal normalized to control (Ctrl) values (mean ± S.E; *p<0.05; ns: not significantly different; n=3). Compared to control, β3S383 phosphorylation was reduced by 83.5 ± 6% by NMDA application and by 4.67 ± 7% following co-application of NMDA with Cs A.

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Supplementary methods

Constructs: ^{pHBBS}β3S383A and ^{pHBBS}β3S383D were made from ^{pHBBS}β3WT using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) with the following mutagenic primers, ^{pHBBS}βS383A forward primer:
5′-CCAGTATAGGAAACAGgcCATGCCCAAGGAAGGGCATGGGCGG-3′,
reverse primer: 5′-CCGCCCATGCCCTTCCTTGGGCATGgcCTGTTTCCTATACTGG-3′.
^{pHBBS}β3S383D forward primer:
5′-CCAGTATAGGAAACAGgaCATGCCCAAGGAAGGGCATGGGCGG-3′, reverse primer: 5′-CCGCCCATGCCCTTCCTTGGGCATGtcCTGTTTCCTATACTGG-3′. All mutations were verified by DNA sequencing. Rat CaMKIIWT and CaMKIIK42R cloned into the plasmid pcDNA3 were a generous gift from Trevor Smart (UCL).

Neuronal Cell Culture, Nucleofection and Transfection. SH-SY5Y cells (ATTC No. CRL-2266) were cultured in a 1:1 mixture of Eagle's Minimum Essential Medium (Invitrogen) and F12 Medium (Invitrogen) plus 10% fetal bovine serum (Invitrogen). Hippocampal neurons were obtained from embryonic day 18 (E18) rats and cultured in Neurobasal medium (Invitrogen) supplemented with 0.5 mM glutamine and 2% B-27 supplement (Saliba et al., 2009). Dissociated E18 rat hippocampal neurons or SH-SY5Y cells were nucleofected with 3 μg of plasmid DNA per 2 x 10^6 cells using the Amaxa rat neuron Nucleofector™ Kit and the cell line Nucleofector™ kit respectively (Saliba et al., 2009; Saliba et al., 2007). 60 mm poly-l-lysine coated dishes were seeded with 0.4 x 10^6 hippocampal neurons/dish and used in experiments after 16-21 days *in vitro* unless otherwise sated. Hippocampal neurons (15 div) were transfected with cDNAs encoding ^{pHBBS}β3WT, ^{pHBBS}β3S383D, CaMKIIWT and CaMKIIK42R, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Following transfection, neurons were incubated in neurobasal medium for a further 24 hours before fixing and immunofluorescence labeling.

Biotinylation. Cultured hippocampal neurons were treated with 4-AP, Bay K 8644 or TTX at 37°C then chilled on ice for 5 minutes and washed twice in PBS + 1mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-CM) at 4 °C. Cells were incubated for 12 minutes at 4°C in 1 mg/ml NHS-SS-Biotin (Pierce) dissolved in PBS-CM. To quench un-reacted biotin, neurons were washed two times (5 minutes each wash, at 4°C) in PBS-CM + 25 mM Glycine, then washed twice in PBS and lysed in 100 μl of 1% SDS + 25 mM Tris-HCl pH 7.5, 1 mM EGTA and then diluted with 400 μl of RIPA buffer without SDS (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 2 mM EDTA, plus mammalian protease inhibitor

cocktail - Sigma). Protein concentrations were determined using the micro BCA protein assay kit (Pierce) and equal amounts of solubilized protein were added to immobilized avidin (Neutravidin, Pierce) for 2 hours at 4°C. Avidin beads were washed twice, for 15 minutes at 4°C, in high salt (500 mM NaCl) RIPA buffer, followed by a 15 minute wash at 4°C, in (150 mM NaCl) RIPA buffer. Precipitated biotinylated proteins and total proteins were resolved by SDS-PAGE and β3 was detected using immunoblotting with rabbit anti-β3 IgGs, followed by peroxidase conjugated anti-rabbit IgGs and detection with ECL(Saliba et al., 2009; Saliba et al., 2007). Blots were imaged using the FujiFilm LAS-3000 imaging system and bands quantified with Fujifilm Multi Gauge software.

Immunocytochemistry: Neurons expressing ^{pHBBS}β3WT, ^{pHBBS}β3S383A and ^{pHBBS}β3S383D with or without drug treatments (at 37°C) and with CaMKIIWT or CaMKIIK42R were fixed in 4% paraformaldehyde (22°C) and non-permeabilized neurons were immunolabeled with rabbit anti-GFP IgGs, followed by Rhodamine Red-X conjugated anti-rabbit IgGs. Images were acquired (blind to experimental condition) using a Nikon Eclipse Ti series confocal microscope with a 60× oil immersion objective and NIS-Elements imaging software. The same image acquisition settings were used for ^{BBS}β3WT or ^{pHBBS}β3S383A with or without drug treatments. These images were analyzed using MetaMorph imaging software (Universal Imaging Corporation, Downingtown, USA). A single Z section was taken and the average fluorescence intensity of pHluorin and Rhodamine Red-X staining was measured along 30 μm of 2 or 3 proximal dendrites per neuron, after subtraction of background fluorescence. Data were analyzed from 8-10 neurons for each condition. Analyses were all performed blind to experimental condition.

$GABA_A$ receptor ^{pHBBS}β3WT Insertion Assay: Hippocampal neurons (15 div) expressing ^{pHBBS}β3WT were first labeled with 10 μg/ml unlabeled α-bungarotoxin for 15 minutes at 15 ºC, to block existing cell surface ^{pHBBS}β3WT subunits (Saliba et al., 2009). Neurons were then washed 3 times in PBS at 37 ºC followed by incubation at 37ºC with 1 μg/ml Alexa594-conjugated α-bungarotoxin for various time points (Saliba et al., 2009). All incubations were performed in the presence of 200 μM Tubocurarine to block bungarotoxin binding to endogenous Acetylcholine receptors (Bogdanov et al., 2006). Cells were fixed in 4% paraformaldehyde and nucleofected neurons were identified by pHluorin expression. Confocal images were acquired and analyzed as described above.

Hippocampal slice preparation: All procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Tufts University and Institutional Animal Care and Use Committee. C57Bl6 male mice (8-12 weeks old) were deeply

anesthetized with isoflurane and decapitated at the second cervical vertebrae. The brain was then rapidly removed and rinsed with ice-cold artificial CSF (ACSF) containing 124 mm NaCl, 3 mm KCl, 2 mm CaCl2, 25 mm NaHCO3, 1.1 mm NaH2PO4, 2 mm MgSO4, and 10 mm d-glucose, equilibrated with 95% O2 and 5% CO2 to yield a pH of 7.4. The hippocampus was cut into 350 μm slices using a vibratome (VT1000S; Leica, Deerfield, IL). Hippocampal slices were incubated in ASCF at 32°C for 1 hour to allow recovery before experimentation. Following 4-AP application at 32°C, slices were lysed in 50 μl of 1% SDS, sonicated and diluted with 200 μl of RIPA buffer as described above for cultured neurons.