

Legends to Supplementary Figures

Supplementary Figure S1. PRAF2 interacts with GB1 in the ER but does not modulate GB2 cell surface expression.

(a) PRAF2 selectively interacts with GB1 (b isoform, lower arrowhead) compared to GB2 (upper arrowhead). HEK-293 cells were transfected with the indicated plasmids and processed as in (Figure 1a). Lower panel: densitometric analysis of GB1-GFP or GB2-GFP co-immunoprecipitated with PRAF2; Unpaired t- test *** $p < 0.001$ (N=3). (b) Glycosylation profile of GB1 interacting with PRAF2. HEK-293 cells were transfected with the indicated plasmids. Cell lysates (upper panel, 5% of the input) or material immuno-precipitated with anti-V5 antibodies (as in Figure 1a, lower panel), were incubated overnight at 37°C with buffer alone or buffer containing glycosidases: Endo H (50.000 U/ml, final), cleaves glycans added in the ER, which cannot be cleaved anymore after additional glycosylation occurring in the Golgi apparatus; PNGase (50.000 U/ml, final), cleaves all added N-glycans. In the presence of GB2, minimal amounts of GB1 were deglycosylated by Endo H in the cell lysate, whereas after immuno-precipitation of PRAF2, co-IPd GB1 appeared mostly sensitive to the enzyme. (c) Hippocampal neurons infected with the AAV-PRAF2-GFP as in (Figure 2b), or with a control AAV-GFP virus were fixed and stained with the anti-GB2 antibody without permeabilization (see Supplementary Figure S3b for GB2 antibody specificity). Wide-field or confocal IF images (at mid distance between the bottom and the top of the cells for confocal images) were taken. Note that no labeling was observed in these non-permeabilized neurons with an antibody directed against the intracellular C-tail of GB2 (not shown). Lower graph: Quantitation of surface-associated GB2 immunoreactivity in GFP-containing neurons (n=30). AAV-PRAF2-

GFP: 50.0 ± 14.9 ; AAV-GFP: 50.1 ± 15.8 (NS). Bar $10 \mu\text{m}$. (d) Surface GB2 expression analyzed by FACS in non-permeabilized cells expressing stable amounts of GB2 (measured by FACS analysis on permeabilized cells, not shown) and increasing amounts of PRAF2-V5.

Supplementary Figure S2. Electrophysiological study in hippocampal neurons

(a) Hippocampal neurons in culture for 7 days were transfected with control-scrambled or PRAF2-specific siRNAs (Si1 and Si2). After one week, the efficacy of PRAF2 gene silencing was determined by immunoblot experiments on $50 \mu\text{g}$ of total cell lysates using anti-PRAF2 antibodies. Actin immunoreactivity was used as loading control. (b) Sample current clamp recordings of spontaneous action potential discharge in basal conditions, in the presence of $0.2 \mu\text{M}$ baclofen and after washing, from non-transfected neurons (control) or neurons transfected with PRAF2 siRNAs (PRAF2 siRNA). (1-3) Traces are from 3 independent preparations of neurons (control and PRAF2 siRNA comparisons were done on the same preparation).

Supplementary Figure S3. Specificity of antibodies and [3H]-CGP54626 for GABA_B subunits.

Immunofluorescence studies were conducted on HEK-293 cells transfected with the constructs for GB1-GFP or GB2-GFP. (a) The GFP-associated fluorescence was compared to the anti-GB1 antibody-associated fluorescence. Arrowheads indicate a non-transfected cell. Cells expressing GB2-GFP and the non-transfected cell were not labeled by the anti-GB1 antibody. (b) The GFP-associated fluorescence was compared to the anti-GB2 antibody-associated fluorescence. Cells expressing GB1-GFP were not labeled by the anti-GB2 antibody. (c) Competition radioligand binding

assays with [³H]CGP54626 and unlabeled CGP54626 were conducted on membranes (100µg per assay), prepared from GB1-GFP or GB2-GFP transfected HEK-293 cells, expressing equivalent amounts of recombinant receptors. Only background levels of bound [³H]-CGP54626 were measured in membranes from GB2-GFP-expressing cells.

Supplementary Figure S4. Receptor cell-surface export analysis

HEK-293 expressing Myc-GB1-YFP alone or co-expressed with HA-GB2 and/or PRAF2-V5 were processed as described in the “Receptor cell-surface export analysis” section of “Supplementary Experimental Procedures”. The Figure shows a representative example of FACS analysis and calculation of surface GB1 in HEK-293 cells expressing Myc-GB1-YFP in the absence or presence of HA-GB2. CY5 and YFP signals correspond to surface and total Myc-GB1-YFP, respectively. Gated cells were distributed in 4 groups: double negative (non-transfected, group 1), YFP-positive and CY5-negative (group 2), YFP- and CY5-double positive (group 3), YFP-negative and CY5-positive (group 4, this last group being routinely close or equal to zero). The total amount of expressed receptor was used for experiment calibration. This value was equivalent ($\pm 10\%$) for all experiments. It was calculated by adding the products of the % of cells expressing YFP multiplied by the mean YFP-associated fluorescence intensity of groups 2 and 3. The amount of surface GB1 corresponded to the % of the cells in group 3 multiplied by the mean CY5 signal of the group, this product corresponding to the arbitrary units indicated in the figures. Thus, in cells expressing Myc-GB1-YFP alone (left panels), total GB1 expression was $(59,79\% \times 40816,73) + (10,72\% \times 150274,57) = 40482$ whereas GB1 surface expression corresponded to $10,72\% \times 942,27 = 100,8$ A.U. In cells co-expressing Myc-GB1-YFP

and HA-GB2 (right panels), total GB1 expression was $(39,57\% \times 12069,01) + (33,48\% \times 108604,78) = 40979$, whereas GB1 surface expression corresponded to $33,48\% \times 5498,88 = 1841$ A.U.

Supplementary Figure S5. Exogenous PRAF2 and GB2 modulate subcellular distribution of GB1 in HEK-293 cells.

HEK-293 cells were transiently transfected with various combinations of plasmids encoding GB1-GFP, HA-GB2 or PRAF2-V5. Triton-permeabilized cells were labeled with anti-V5 (to detect exogenous PRAF2), anti-HA (to detect exogenous GB2) and primary anti-BIP antibodies, as indicated, and then with the appropriate secondary antibodies before confocal immunofluorescence analysis. Co-localization was determined as in [Figure 1](#). **(a)** In the absence of GB2, $92.9 \pm 1.3\%$ (mean \pm SEM, $n=40$) of GB1-GFP and $98,1 \pm 0.5\%$ ($n=10$) of the PRAF2-V5/GB1-GFP co-localized signal overlapped with the anti-BIP signal. **(b, c)** in the presence of exogenous HA-GB2, $95.4 \pm 0.9\%$ of GB1-GFP was colocalized with HA-GB2, mostly in the peripheral area of the cells ($n=20$). **(d, e)** The redistribution of GB1-GFP observed in the presence of HA-GB2 was counteracted by the simultaneous expression of exogenous PRAF2, the GB1-GB2 colocalization dropping to $53.4 \pm 3.2\%$ ($n=20$). Scale bar: $10\mu\text{M}$. **(f-h)** Quantitative analysis of GB1 subcellular distribution. Confocal images of the cells above (optical magnification: 60x, digital zoom, 1x, resolution: 10 pixels/ μm , laser intensity set at 20% of the maximum) were acquired. Quantitative analysis of GB1-GFP distribution was achieved by measuring the fluorescence intensity along an axis passing across each examined cell from one side of the plasma membrane to the other, outside the nuclear area, in the same focal plane of each analyzed cell (dashed line). Fluorescence values (expressed as the % of the

maximal value in the examined field) were plotted pixel by pixel from left to right. n=20 to 40 per condition.

Supplementary Figure S6. Distribution and quantitative analysis of exogenous PRAF2 in the VTA of AAV-PRAF2+GFP injected mice.

(a) Imaging of the virus diffusion and characterization of the infected neurons of the VTA. Mice that received bilateral stereotactic injection of 500nL of AAV-PRAF2+GFP virus in the VTA were sacrificed. Coronal sections of the brains were analyzed using Tyrosine Hydroxylase (TH, the rate-limiting enzyme in the synthesis of catecholamines) antibodies (red) to identify dopaminergic VTA neurons. GFP fluorescence revealed the distribution of the AAV-PRAF2+GFP virus, relatively to the targeted VTA. Panels “a” and “b” show higher magnification of the corresponding boxed areas. Overlays were used to appreciate colocalization. Arrowheads, indicate virus-infected dopaminergic neurons, whereas asterisks indicate non-infected dopaminergic neurons. In panels “b” the arrows indicate infected non- dopaminergic neurons. VTA: ventral tegmental area; SNR: substantia nigra reticulata; SNC: substantia nigra compacta; cp: cerebral peduncle. Bars: 200 μ m in low-magnification images, 20 μ m, in panels “a” and “b”. (b) Quantitation of AAV-PRAF2+GFP-induced PRAF2 expression in mouse VTA. Locomotor activity of mice that received bilateral injection of either AAV-GFP (# 1,2,3) or AAV-PRAF2+GFP virus (# 4,5,6,7) in the VTA, was recorded during one hour. Mice were then sacrificed and PRAF2 and tubulin expression quantitated by immunoblot analysis of VTA extracts (duplicates). Lower panel: average tubulin-normalized PRAF2 values were plotted against the locomotor activity of each corresponding mouse. r = Pearson correlation coefficient. (c) Changes in PRAF2 expression during hippocampal neuron maturation in culture.

Hippocampal neurons were lysed after the indicated number of days in culture (DIV) and used for immunoblot analysis of PRAF2 and GB1 expression as in [Figure 1](#). After normalization, densitometric analysis (Image J software) showed that PRAF2 concentration increased progressively up to 20 times from DIV5 and DIV8.

Supplementary Figure S7. Quantitative analysis of GB1 and PRAF2 in the VTA of unilaterally injected mice.

(a) VTA-selective GB1 KO in GB1fl/fl mice. GB1fl/fl mice were unilaterally or bilaterally (not shown) injected with AAV CRE-GFP (coding for CRE recombinase-GFP fusion protein) in the VTA, as shown in the diagram, to locally disrupt GB1 expression. Fluorescence imaging of nuclear CRE-GFP is shown at increasing magnification. The Box corresponds to magnified areas. Right panels: protein extracts from the VTA were analyzed by immunoblot with anti-GB1 antibodies. GB1 expression was reduced in the injected VTA comparatively to the non-injected contralateral region. Densitometric analysis of the specific immunoreactive material was carried on 5 samples. *** $p < 0.001$. **(b, c)** Quantitation of PRAF2 expression in the VTA of mice unilaterally injected with 500nL of AAV-PRAF2+GFP **(b)** Red fluorescence corresponds the anti-PRAF2 antibody labeling; GFP fluorescence shows AAV-PRAF2+GFP distribution in coronal sections. Lower panels correspond to the magnification of the “a” squares; Top and bottom bars: 500 and 30 μ m, respectively. Asterisks show infected neurons (GFP, and PRAF2-positive), arrowheads show uninfected neurons. Upper histograms: PRAF2-associated fluorescence, normalized over background PRAF2 values in contralateral non-infected neurons. (Mean \pm SEM of 75 neurons, Unpaired t-test $p < 0.0001$; Lower histograms: expression of the data above as the fold change in infected compared to non-infected neurons (n=75 in each group). **(c)** VTA extracts were processed for

immunoblot experiments with anti-PRAF2 antibodies. Representative immunoblots from 2 mice injected with AAV-GFP in the right VTA and with AAV-PRAF2+GFP in the left VTA. Right histogram: for quantitation of the immunoblots (4 mice, triplicates), the PRAF2 signal was normalized with that of tubulin. Mean density (\pm SEM) is shown, $p < 0.0001$. Data were analyzed using unpaired t-test. As in mice bilaterally injected in the VTA, a 2-3 fold increase of PRAF2 expression was sufficient to induce the phenotypic effects.

Movies

Mice injected unilaterally with the AAV-PRAF2+GFP virus or the control AAV-GFP virus

(Movie S1a) Video file of mice unilaterally injected in the right VTA with 500 nL of AAVPRAF2+GFP virus. After 4weeks recovery, mice locomotor activity was recorded in their home cage. Note the contralateral (to the injection site) pivoting behavior and the hyperactivity of the mice.

(Movie S1b) For comparison, locomotor activity in control mice injected with the AAV-GFP virus.

Supplementary Experimental Procedures

Animals. Adult male Sprague Dawley rats (250–300 g body weight) were housed in agreement with the institutional guidelines for use of animals and their care, in compliance with national and international laws and policies (Council directives no. 87-848, October 19, 1987, “Ministère de l’Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale”, agreement N° 75-974 to M.D., 75-976 to M.B.E). Wildtype 129S2 mice (8-10 week old) used in these experiments were in a 129S2/SvPas (129S2) background (Charles River, France). Only male were used in all experiments. The GB1 knockout, GB1-floxed mice and their control littermates were from the laboratory of B. Bettler and generated on a BALB/c background ¹. Behavioral tests and animal care were conducted in accordance with the standard ethical guidelines (National Institutes of Health; European Communities Directive 86/609 EEC). All mice were maintained on a 12 light / 12 dark schedule (lights on at 8:00 am), and housed in groups of 3-5 of the same genetic background and sex after weaning. Behavioral studies were carried out between 9:00 am – 3:00 pm). Mice were moved to the testing room in their home cage at least 5 days prior to testing to allow habituation to the environment and stayed there until the end of the experiments. Mice were randomly assigned to different experimental groups. Groups were of 4 to 10 animals and independent experiments were performed 2 to 3 times. Each time, the observer was blind to experimental conditions being measured.

AAV-mediated local VTA PRAF2 or CRE expression. Adeno-associated virus (AAV9)-expressing GFP ($7,5 \times 10^{12}$ virus molecules/ml), PRAF2 (IRES) GFP (8×10^{12} virus molecules/ml) or CRE-GFP ($\times 10^{12}$ virus molecules/ml) under the control of the synapsin promoter (UNC Vector Core, Dr. R. Jude Samulski, Chapel Hill) were stereotaxically injected into VTA. Depending of the experiment, 200 nL or 500 nL of

the viral solution was injected. Mice were anesthetized with ketamine (50 mg/kg) and xylazine (2 mg/kg) and fixed in a stereotaxic apparatus. A burr hole was drilled above the VTA (coordinates: 3.5 mm posterior to bregma, 0.5 lateral to midline). Stereotaxically guided injections were made through the hole in the dorsal surface of the cranium (3.5 mm deep). Glass capillary tubes were pulled (HEKA pipette puller PIP5) and tips broken to 40 μ m diameter. After 4 weeks recovery and viral expression, the VTA AAV-injected mice were used for behavioral testing or biochemical experiments. Proper viral infection was verified by GFP detection on brain fixed sections. For in vitro studies, hippocampal neurons (DIV 2-3 after plating) were infected with 1 μ l/ml AAV-PRAF2+GFP and incubated 15 days before the experiments.

Basal Locomotor activity and psychostimulant challenge recording. Locomotor activity was measured in a circular corridor with four infrared beams placed at every 90° (Imetronic, France). Counts were incremented by consecutive interruption of two adjacent beams (i.e., mice moving through one-quarter of the corridor). Mice were intraperitoneally (i.p.) injected with a saline solution and individually placed in the activity box for 60 min during 3 days consecutively for habituation before all behavioral motor experiments. Amphetamine (3mg/kg) induced-locomotor activity in AAV/injected mice was recorded during two hours. Saline or baclofen (4 mg/kg) injections were made thirty minutes before amphetamine challenge. Based on initial trials showing that baclofen completely (4 and 5 mg/kg; i.p) or partially (2,5 and 3 mg/kg) blocked amphetamine-induced locomotion in mice (Sv129 background) and had no effect on basal locomotor activity, we used the 4 mg/kg dose.

Video tracking set up and data analysis. All experiments were performed using a conditioning chamber (30×30×30cm, L×W×H) made with white plastic. A video camera was set 150 cm above four different chambers for simultaneous video recording (640×480 resolution, 30 frames per second). The video files were then processed using ANY-maze software (Stoelting, Wood Dale, IL, USA). The tracking settings were defined in the protocol pane of ANY-maze. The definitions included the white chamber's floor as background. The animal's center-point detection option was selected to track the position of the mice and the tracking frequency was set to 15 frames per second during ten minutes. The animal's center-point was set to be shown on the computer screen, so that the observer could monitor the tracking quality.

Cell Culture. Neuronal cultures were made as described previously² with some modifications. Hippocampi of rat embryos were dissected at embryonic day 17-18. After trypsinization, tissue dissociation was achieved with a Pasteur pipette. Cells were counted and plated on poly-D-lysine-coated 15-mm diameter coverslips for immunofluorescence and electrophysiological recording, on 4-well dishes for binding experiments, or on 35 mm dishes for western blots experiments at a density of 300 to 500 cells per square millimeter (depending on the experiment), in complete Neurobasal medium supplemented with B27 (Invitrogen), containing 0.5 mM L-glutamine, 10 U/mL penicillin G, and 10 mg/mL streptomycin. The actual number of surviving neurons was routinely half of the neurons plated, after 7 days in culture. Three hours after plating, the medium was replaced by a conditioned medium obtained by incubating glial cultures (70–80% confluency) for 24 hours in the complete medium described above.

Human Embryonic Kidney 293 (HEK-293) cells, obtained from the ATCC[®], were maintained in Dulbecco's modified Eagle's medium, at 37°C in 5% CO₂-enriched humidified atmosphere. Media were supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. Experiments were conducted on unfrozen batches maintained in culture for no more than five passages. Cells were routinely (every 3 months) screened for the absence of mycoplasma infection using the Lonza, MycoAlert™ Mycoplasma Detection Kit.

Plasmids and transfections. Plasmids coding for Myc-GB1-YFP, HA-GB2, Myc-GB1-Luc were kindly provided by Dr. Michel Bouvier (Institute for Research of Immunology and Cancer, IRIC, University of Montreal, Canada). Myc-GB1-ASA, AA, AA/ASA-YFP were prepared by site directed mutagenesis using Myc-GB1-YFP as template and the QuikChange site-directed mutagenesis procedure (Stratagene). The PRAF2-GFP plasmid was prepared from the PRAF2 cDNA, a generous gift of Prof. Karin Moelling (Institute of Medical Virology, University Zurich, Switzerland), which was subcloned in frame in the pEGFP-N1 vector (Clontech), RLuc-PRAF2 was generated by subcloning in frame the cDNA for a humanized form of Renilla Luciferase (Biosignal; PerkinElmer Life Sciences) upstream of PRAF2 cDNA. PRAF3 cDNAs were amplified by reverse PCR and subcloned in frame, either in the phRLuc-C1 vector encoding the humanized Renilla Luciferase or into the pEYFP-N1 vector (Clontech). HEK-293 cells were transfected using GeneJuice Transfection Reagent (Novagen) according to manufacturer instructions. Hippocampal neurons were transfected using Lipofectamine 2000 (Invitrogen) following manufacturer instructions. PRAF2 cDNA was subcloned in frame in AAV synapsin promoter IRES

GFP plasmid, kindly provided by Dr. Luc Maroteaux and Imane Moutkine (Institut du Fer-à-Moulin, INSERM U839, Paris, France).

Inhibition of Protein Expression by RNA Interference. To silence the expression of PRAF2, hippocampal neurons were transfected with 40nM of each siRNA with Lipofectamine 2000 (Invitrogen) 7 days after plating (DIV7). Two small-interfering RNAs ON-Target plus (S1: 5'-GAUCGAGAGCAUCGGUCUA-3') (S2: 5'-CUUCACUGCGCCUGAGAAA-3') targeting Rat PRAF2 from Dharmacon (Thermo Fisher Scientific, Inc.), were used. An off-target siRNA (siCONTROL siRNA from Dharmacon) were used as controls. Assays were performed 7 days after the transfection and efficacy of knockdown was assessed by immunoblotting using whole cell lysates.

Antibodies, Drugs, and Receptor Ligands. The following monoclonal antibodies (mAb) or polyclonal antibodies were used for immunofluorescence labeling, FACS analysis or immunoblotting: Anti-PRAF2 rabbit polyclonal antibody (Bioworld Technology Inc, JM4/PRAF2, #L166), Anti-GABA B receptor 1 mouse monoclonal antibody (abcam, #ab55051), Anti-GABA B receptor 2 (N-terminal) rabbit polyclonal antibody (sc-28792, Santacruz biotechnology) Anti-GABA B receptor 2 (C-terminal) rabbit polyclonal antibody (ab75838, Abcam), anti-GM130 mouse monoclonal antibody (BD Biosciences), anti-KDEL mouse monoclonal antibody (10C3, Stressgen), anti-calnexin goat polyclonal (C-20, Santa-Cruz Biotechnologies), anti-myc mouse monoclonal antibody (clone 9E10, Roche), anti-HA (hemagglutinin) monoclonal rat antibody (clone 3F10, Boehringer Mannheim), anti-GFP mouse monoclonal antibody (clones 7.1 and 13.1, Roche), anti-V5 antibody (Invitrogen;

R96025). Dr. Patricia Gaspar (Institut du Fer-à-Moulin, INSERM U839, Paris, France) kindly provided the rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody. The following secondary antibodies were used for immunofluorescence labeling, immunoblotting or FACS analysis: anti-mouse IgG (H+L), anti-rabbit IgG (H+L) and anti-rat IgG (H+L) coupled to Horse Radish Peroxidase (Jackson ImmunoResearch Laboratories), and anti-mouse IgG (H+L) and anti-rabbit IgG (H+L) and anti-goat IgG (H+L) coupled to Alexa-488, Alexa-568 or Alexa-647 (Invitrogen) and Cy5 (Rockland). [3H]CGP 54626 was purchased from ARC (American Radiolabeled Chemical Inc.) and CGP 54626 hydrochloride was purchased from Tocris Bioscience. Coelenterazine h, the substrate of Renilla luciferase was from Interchim (France). D-amphetamine (Sigma-Aldrich, Saint-Quentin Fallavier, France) and baclofen (Tocris Bioscience, USA) were slowly dissolved in 0.9% (wt/vol) NaCl solution (saline). All drugs were administered i.p. (0.1ml/10g body weight).

Immunofluorescence experiments (cell culture). Hippocampal neurons coverslips were proceed for immunofluorescence at DIV8. After 2 washes with PBS+ (Containing 0.1 mM CaCl₂ and 0.1 mM MgCl₂) at 37°C, cells were fixed for 20 min at room temperature in PBS containing 4% PFA and 4% Sucrose and permeabilized for 5 min in PBS containing 0.2% Triton X-100. Cells were incubated with primary antibodies at the recommended concentration in PBS containing 3% BSA for 1 hr. After 3 washes in the same buffer, Alexa-conjugated secondary antibodies as indicated in figure legends, were added for 1 hr. HEK-293 cells growing on coverslips were fixed in 4% PFA-PBS and quenched with 50 mM NH₄Cl for 10 min. A 1% BSA-0.2% Triton X-100-PBS solution was used to block and permeabilize cells. Cells were incubated with primary and Alexa-conjugated secondary antibodies. Coverslips were

mounted in DAPI-containing medium (SlowFade Gold antifade reagent). Image acquisition was performed on a laser-scanning confocal spinning-disk microscope (63X oil immersion lens) equipped with a CoolSnap HQ2 CCD or a wide-field microscope Zeiss Axiovert. Images were collected using the Metamorph Application and were processed with the Image J 1.43u software. Co-localization analyses were done with the Image J plugin JACOP using Pearson's coefficient or overlap coefficients K1&K2. For triple co-localization analysis, co-localization clusters from PRAF2-V5 (blue) and GB1-GFP (green) were first generated with a co-localization plugin (Pierre Bourdoncle, Institut Cochin) generating 8bits images only including co-localized points. The « co-localized points » images and the BIP red channel were then processed with the JACOP plugin to quantify the co-localization of PRAF2/GB1 in the RE.

Tissue preparation and immunofluorescence. Mice were anesthetized with pentobarbital (30 mg/kg, i.p.; Sanofi-Aventis) and perfused transcardially with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.5. Brains were postfixed overnight in the same solution and stored at 4°C. Fifty-micrometer-thick sections were cut with a Vibratome (Leica) and stored at -20°C in a solution containing 30% (v/v) ethylene glycol, 30% (v/v) glycerol, and 0.1 M sodium phosphate buffer, until they were processed for immunofluorescence. Brain regions were identified using a mouse brain atlas. Sections were incubated overnight with primary antibody (PRAF2 or TH) in PBS, 0,5% Triton, 3% BSA solution at 4°C. Sections were then washed in PBS solution and incubated two hours with secondary antibody (Alexa 647-conjugated antibodies, 1:1000, invitrogen). Finally, sections were cover-slipped in anti-fading mounting medium (moviol-DABCO 25 mg/ml).

Immunofluorescence analysis. Confocal microscopy and image analysis were performed at the Institut du Fer-à-Moulin Imaging Facility. Labeled images from each region of interest were obtained bilaterally using sequential laser-scanning confocal microscopy (Olympus Fluoview). Optical density from isolated neuron was measured with image-J. The regions of interest were drawn as a circle of 2 μm diameter. Specific background values in the extracellular space were subtracted for each cytoplasmic optical density measure.

BRET assays. BRET-donor saturation assays allow establishing the specificity of the interaction between BRET partners (shape of the saturation curve) and the BRET₅₀ value (see below), which reflects the propensity of their interaction. Maximal BRET values (BRET_{max}), which depend on the distance and on the relative orientation of BRET donor and acceptor, provide little information in the present context where different proteins are compared. The expression level of the BRET-donors, determined in preliminary studies, was the lowest amount of expressed proteins compatible with detectable and robust BRET signals. Based on this, HEK-293 cells were seeded at a density of 500,000 cells/well of a 6-well plate and were transfected with DNA constructs coding for BRET donors and increasing amounts of the BRET acceptor plasmids or BRET competitor plasmids in the case of BRET displacement experiments. The total amount of transfected DNA was maintained constant by adding appropriate amounts of empty vector DNA. Cells were washed (PBS), detached (PBS-EDTA 10mM), centrifuged (1,200 x g for 5min) and resuspended in Hank's-balanced salt solution. After addition of the luciferase substrate, coelenterazine h (5 $\mu\text{M}/10^5$ cells), luminescence and fluorescence were

measured (see below) by using the Mithras fluorescence-luminescence detector (Berthold Bad Wildbad, Germany). BRET ratios were calculated as described previously³⁸. Briefly, the BRET ratio is the fluorescence signal (filter 530±12.5 nm) over the Rluc signal (filter 485±10 nm) measured simultaneously. The readings were repeated three to six times to obtain average values. The specific BRET ratio was calculated by subtracting from the mean BRET ratio value above the background BRET ratio, which corresponds to the signal obtained with cells expressing the BRET-donor alone (not expressing the BRET-acceptor). BRET results were expressed in milli-BRET units (mBRET) by multiplying the values x 1,000 and plotted as a function of YFP/Rluc, in which YFP represents the actual amount of expressed BRET acceptor and Rluc the amount of BRET donor in each sample. To quantify the amount of BRET-acceptor in each well, the fluorescence was measured at 530±12.5 nm after external excitation at 480 nm. To normalize the background fluorescence and luminescence values from experiments conducted in different conditions (different times, different constructs), BRET saturation curves were obtained by plotting BRET values as a function of $[(YFP-YFP0)/YFP0] / [Rluc/Rluc0]$, indicated YFP/Rluc in the figures] where YFP is the specific fluorescence associated with the BRET acceptor in each sample, YFP0 corresponds to background fluorescence measured in cells not expressing the BRET-acceptor, Rluc is the luminescence value associated with the BRET donor in each sample and Rluc0 to the average luminescence value in cells expressing the BRET-donor alone. The amount of BRET-donor in each well was controlled by measuring luminescence values at 485±10 nm after coelenterazine h addition. In case of significant variation (difference of 30% or more from the average value) the corresponding experimental points were excluded from the final plot. Data were fit using a non-linear regression equation assuming a

single binding site (GraphPad Prism) to estimate $BRET_{max}$ and $BRET_{50}$ values (the YFP/Rluc value for half-maximal BRET). In some experiments the BRET saturation experiments were conducted in the presence of variable concentrations of competitors, all the other experimental conditions remaining unchanged.

Receptor cell-surface export analysis. To study the impact of PRAF2 co-expression on GB1 targeting at the cell surface, HEK-293 cells were seeded at a density of 500.000 per well in the appropriated culture medium and were transiently cotransfected with increasing concentrations of HA-GB2 or Luc-PRAF2 and a construct coding for GB1 wild type displaying the myc epitope at the N-terminus and fused C-terminally to the YFP. Empty vector, pCDNA3.1 was used to maintain identical the total amount of transfected DNA. 48 h after transfection, cells were harvested. The level of expression of the constructs was measured by FACS, immunoblotting or luciferase assay. Typically 70-80% of the transfected cells expressed myc-GB1-YFP; in the case of lower % of transfected cells the samples were not analyzed further. To determine cell surface GB1 expression, cell aliquots were stained with a primary antibody directed against the extracellular myc epitope and a CY5-labeled secondary antibody. After washing and fixation in 2% PFA, GB1 cell surface was analyzed by Cytomyics FC500 FACS analyzer (Beckman Coulter). For each point of transfection, 10.000 cells were sorted. **A representative example of analysis and calculation is shown in the Supplementary Figure 4.**

Binding assay. Quantification of GB1 sites at the surface of rat primary hippocampal neurons was performed with radioligand binding assays. Briefly, rat hippocampal neurons were transfected with siRNAs directed against PRAF2 (S1, S2) or scrambled control at DIV 7-8. For AAV-PRAF2+GFP infection, neurons were infected

at DIV 2-3 with 1 μ l/ml of viral solution. One or two weeks after transfection or infection, respectively, neurons were incubated for 20 min at RT in 0.25 mL of binding buffer (120 mM NaCl, 50 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 1mM EDTA) containing 20 nM [³H]CGP54626 (60 Ci/mmol). Non-specific binding was determined in the presence of 10 μ M unlabeled CGP54626 hydrochloride. After 3 ice-cold washes with the binding buffer, the membrane bound [³H]CGP54626 was recovered by incubation for 10 min at 37°C in 0.2 mL of 0.5 M NaCl, 0.2 M acetic acid buffer, then transferred into plastic vials containing 4.5mL of Aquasolv scintillation fluid (New England Nuclear, Boston, MA, USA) for radioactivity counting. Data were normalized to control siRNA-treated cells or AAV-GFP infected cells and analyzed using GraphPad Prism software.

Binding assays were also performed on membranes, prepared from GB1-GFP or GB2-GFP transfected HEK-293 cells (grown in 10 cm diameter dishes), expressing equivalent amounts of recombinant receptors. After a single wash with PBS, cells were scraped in 10 ml of PBS on ice, centrifuged for 5 min at 1,000 x g. Pellets were dissociated mechanically, lysed in 2 ml of binding buffer (50 mM Tris HCl, 10 mM MgCl₂, 0.1 mM EDTA, pH 7.4) and centrifuged for 30 min at 10,000 x g. Membrane-containing pellets were resuspended in binding buffer. Binding assays were performed in the wells of 96-well plates (250 μ l per well, final volume) containing 100 μ g of membranes, 20 nM (final concentration) of [³H]CGP54626 (60 Ci/mmol) and increasing concentrations of unlabeled CGP54626 hydrochloride. Membranes were harvested by rapid filtration onto Whatman GF/B glass fiber filters (Whatman) pre-soaked with cold saline solution, washed three times with ice-cold buffer, and filters were processed as above. Three independent experiments were performed in duplicate.

Deglycosylation Experiments.

Endoglycosidase H (Endo H) and N-glycosidase F (PNGase F) were purchased from New England Biolabs. All necessary buffers and reagents (NP-40) were supplied. Manufacturer's instructions were followed for the enzymatic digestion. However, due to the aggregation tendency of GB1 receptor above 70°C, denaturation reactions were carried out at 70°C. Briefly, total protein lysates of transfected HEK293 cells were denatured in 0.5% SDS and 40 mM DTT for 10 min at 70 °C and then digested with Endo H overnight at 37 °C, in 50 mM sodium acetate, pH 6. Digestion of denatured proteins with PNGase F was performed in 0.05 M Tris buffer, pH 8.0, supplemented with 1% Nonidet P-40. Digestions were terminated by adding electrophoresis sample buffer. After elution from protein G-Sepharose with 0.5% SDS and 40 mM DTT for 10 min at 70 °C, GB1 receptor co-immunoprecipitated with PRAF2 was treated as the total lysates.

Electrophysiology. *On hippocampal cells in culture.* On the day of recording, the culture medium was replaced with patch-clamp bath solution at least 10 min before recording. All recordings were performed at room temperature on hippocampal neurons at DIV 14. The recorded neurons were continuously perfused at a rate of 2 mL/min throughout the experiment with gassed (95% O₂, 5% CO₂) bath solution. The patch pipettes were made of thin-walled borosilicate glass capillaries with a BB-CH horizontal pipette puller (Mecanex, Geneva, Swiss). The initial input resistance of the recording pipettes was 3-6 MΩ. To record action potentials, pipettes were filled

with 140 mM K-gluconate, 3 mM EGTA, 1 mM MgCl₂, 10 mM HEPES, 2 mM ATP-Mg, pH 7.3 (adjusted with 1M KOH), 290-300 mOsm/L. The bath solution consisted of 124mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM Glucose, pH 7.3, 300-310 mOsm/L. Coverslips containing the neurons were fixed on a chamber mounted on the stage of an upright microscope (Leica DM LFSA, Germany). After obtaining a high-resistance gigaohm seal and achievement whole-cell access, action potentials were recorded at room temperature using an Axopatch 1D amplifier (Axon Instruments, Union City, CA, USA), digitized using a Digidata 1200 interface (Axon Instruments) and stored on a computer. The pClamp6 software from Axon Instruments (Fetchex 6.0.4 for action potential recording) was used to drive storage of current data into data files. Whole cell capacitance and series resistance (using only cells with <30 MΩ) were compensated (>80%) before experimentation. The change in series resistance over the course of each recording was monitored, and recordings with a greater than 20% change in series resistance were excluded from the final data analysis. Off-line analysis was performed using Mini Analysis Program version 6.0.7 (Synaptosoft) and Microsoft Excel software.

In VTA slices. C57Bl6 mice of 3 to 4 weeks old were stereotactically injected unilaterally in the VTA with AAV-GFP control or AAV-Prpf2-GFP virus. After three weeks, mice were tested for their locomotor activity and the same mice were anesthetized (Ketamine/Xylazine) and decapitated for slice physiology. Subsequently the brain was isolated and horizontal slices of 250 μm thickness containing the VTA were prepared in ice-cold carbogenated (95% O₂ / 5% CO₂) slicing medium of the following composition (in mM): cholineCl (110); glucose (25); NaHCO₃ (25); MgCl₂ (7); ascorbic acid (11.6); Na⁺-pyruvate (3.1); KCl (2.5); NaH₂PO₄ (1.25); CaCl₂ (0.5).

Afterwards slices were allowed to recover in the same medium at 30 °C for 10 minutes before being stored at room temperature in 95% O₂/5% CO₂-equilibrated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124; NaHCO₃ 26.2; glucose 11; KCl 2.5; CaCl₂ 2.5; MgCl₂ 1.3; NaH₂PO₄ 1. Slices were kept at 30–32 °C in a recording chamber superfused with 2.5 ml/min ACSF. GFP positive neurons in the VTA were identified using an upright microscope connected to an LED source at 490 nm (Olympus France; CoolLed, UK). Only putative dopamine neurons were recorded from, as only large (>20 pF) *h*-current positive neurons were selected for experiments. Recordings of the holding current were made in ACSF, using an internal solution containing (in mM): 140 K-gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl₂, 4 Na₂ATP, 0.3 Na₃GTP and 10 sodium creatine-phosphate. GABA_BR currents were evoked by application of 15 μM baclofen (in 1eq of NaOH) and were blocked by 5 μM CGP54626 hydrochloride (dissolved in water) (Tocris Cookson, UK). Data analysis was performed in Igor Pro-6 (Wavemetrics, USA). Differences in baclofen-induced GABA_BR currents between the two groups were statistically assessed using a Students T test on the amplitude of both the maximally evoked current, and on the stable steady-state current. Compiled data are expressed as mean ± s.e.m.

Immunoprecipitation of membrane proteins from rat brains. Whole brains of adult male Sprague Dawley rats were homogenized in a Dounce homogenizer in sucrose buffer (320 mM sucrose, 1 mM NaHCO₃, 1 mM MgCl₂, 50 μM CaCl₂, Complete protease inhibitors from Roche). Nuclei were first removed by centrifugation at 1,400 × g for 10 min. The membranes were pelleted by centrifugation at 20,000 × g for 20 min, then resuspended during one night under rotation in solubilization buffer (400 mM NaCl, 10mM Tris, pH 8.0, 1% Triton X-100,

protease inhibitors). The insoluble material was removed by ultracentrifugation (100,000×g for 60min). The resulting supernatant represents the solubilized membrane protein fraction. Twelve mg of solubilized membrane proteins were incubated with anti-PRAF2 antibody or control IgG under rotation overnight. The immune complexes were incubated for 4hrs with 40µl of protein-A sepharose (GE Healthcare). After centrifugation, (13000xg, for 5min) the immunoprecipitated material was washed 3 times with 0.5mL of solubilization buffer, then eluted in Laemmli buffer and separated on 10% SDS-PAGE gels. After transfer on nitrocellulose (PORTRAN, Whatman) blots were probed with the appropriate antibodies. Immunoblots were revealed by luminescence (ECL-plus; GE Healthcare Life Sciences).

Immunoblot analysis from mice brain and cells in culture. Mice were decapitated and brain regions, including the VTA and the hippocampus were dissected on ice, weighed and homogenized by sonication for 15 s in CHAPS buffer (Tris-HCl 75 mM; EDTA 2mM; MgCl₂ 12 mM ; CHAPS 10 mM). The extraction volumes were 2 ml for all the structures. Samples were then centrifuged at 12,000 x g for 40 min at 4°C and the supernatants were stored at -80°C before used. The antibody dilutions were 1/1000 and 1/5000 for antibodies against PRAF2 and Tubulin, respectively. Primary antibodies were revealed by Fluoprobes 682 goat anti-rabbit or mouse IgG (Interchim, Montluçon, France) at a 1:5000 dilution. Fluorescent immunocomplexes were detected with Odyssey (LI-COR Biosciences, Lincoln, Nebraska). Quantitation was carried out by measuring the average intensity in regions of interest, using the Odyssey software, and data were analyzed with the Prism 3.02 software (GraphPad Software, San Diego, CA). For the experiments aimed at determining the efficacy of

siRNA-dependent knockdown, whole cell lysates were washed with ice-cold PBS and lysed with in 1mL of cold lysis buffer (50 mM Hepes, pH 7.4, 250 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10% glycerol supplemented with protease inhibitors from Roche) and clarified by centrifugation at 13,000 rpm for 20 min at 4°C. Whole-cell lysates were then boiled and analyzed by SDS-PAGE.

Coimmunoprecipitation experiments from transfected cells. V5-tagged wild-type PRAF2 and YFP-tagged wild-type or mutants (ASA, AA, AA/ASA) GB1 constructs were transfected in HEK cells. After twenty four hours, cells were washed in cold PBS, sonicated and solubilized in lysis buffer [75 mM Tris, 2 mM EDTA, 12 mM MgCl₂, 10 mM CHAPS, protease inhibitor cocktail EDTA free, pH 7.4] during 5 hours at 4°C. Lysates were centrifuged at 12,000 g during 30 min at 4°C. Immunoprecipitations were performed using anti-V5 antibody (invitrogen, ref: R96025; 1µg per condition; overnight 4°C incubation) and A/G plus agarose beads (santacruz; sc-2003). Immunoprecipitated proteins and 50-100 µg of total proteins were combined with Laemmli buffer, heated at 70°C for 10 min and run on 12% Bis-Tris gel. Immunoblots were probed with anti-gfp (Roche) and anti-V5 antibodies diluted 1/2000 and immunoreactivity was revealed using secondary antibody coupled to 680nm and/or 800 fluorophores using the Odyssey LI-COR infrared fluorescent scanner. All the Co-IP experiments were conducted on identical amount of proteins (starting material); data were normalized over the quantity of immunoprecipitated PRAF2-V5 and quantity of GB1-GFP or GB2-GFP in the input IB.

Statistics. Data were analyzed by two-way ANOVA repeated measures with virus or drug treatment and time as factors. Behavioral and biochemical assays were

analyzed by two or one-way analysis of variance (ANOVA). Bonferroni or t-test were used for post hoc comparisons depending on the experiment. $P < 0.05$ was predetermined as the threshold for statistical significance.

Supplemental References

1. Schuler V, Luscher C, Blanchet C, Klix N, Sansig G, Klebs K *et al.* Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B(1)). *Neuron* 2001; 31: 47-58.
2. Carrel D, Masson J, Al Awabdh S, Capra CB, Lenkei Z, Hamon M *et al.* Targeting of the 5-HT1A serotonin receptor to neuronal dendrites is mediated by Yif1B. . *J Neurosci* 2008; 28: 8063-8073.

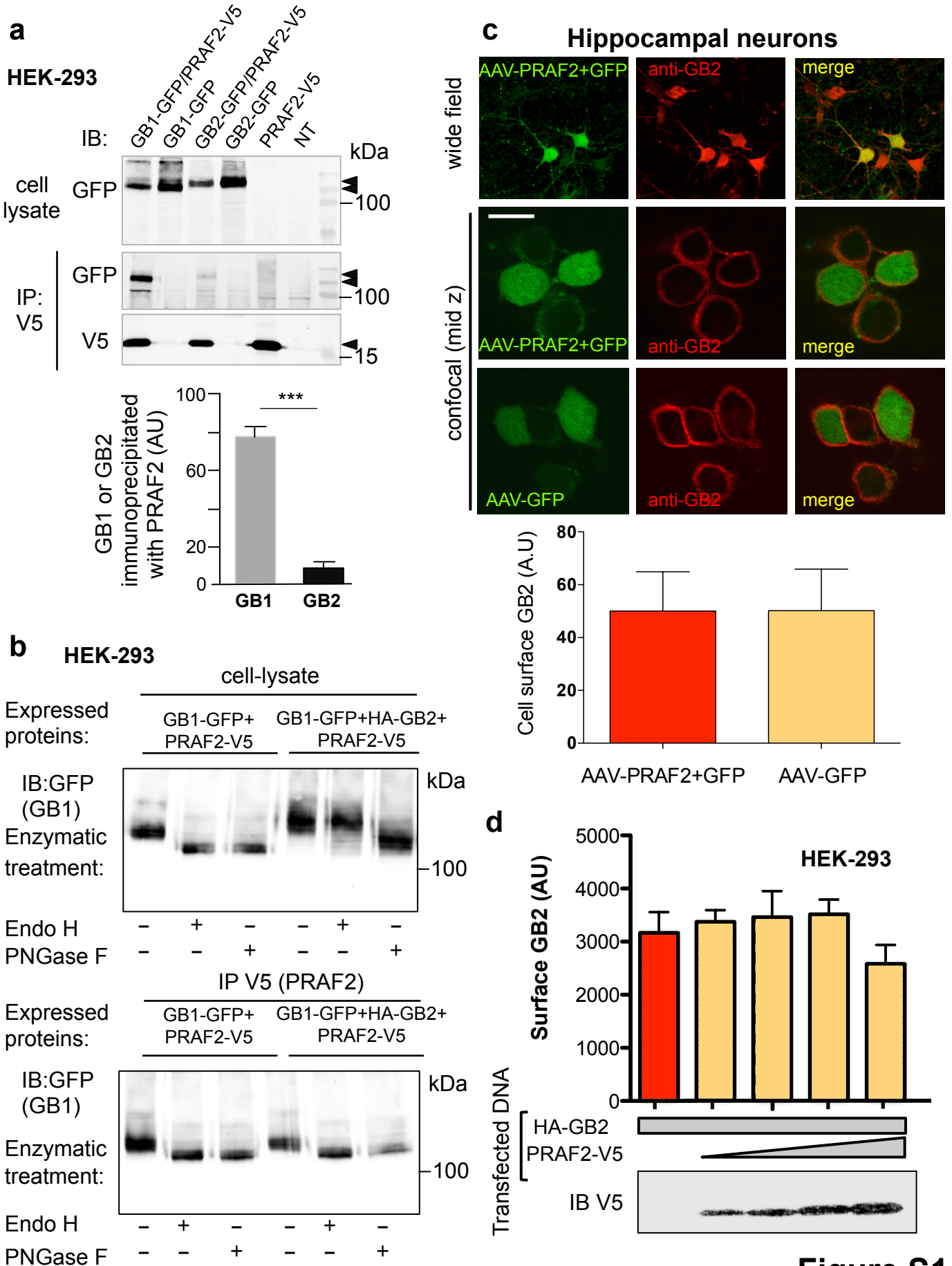


Figure S1

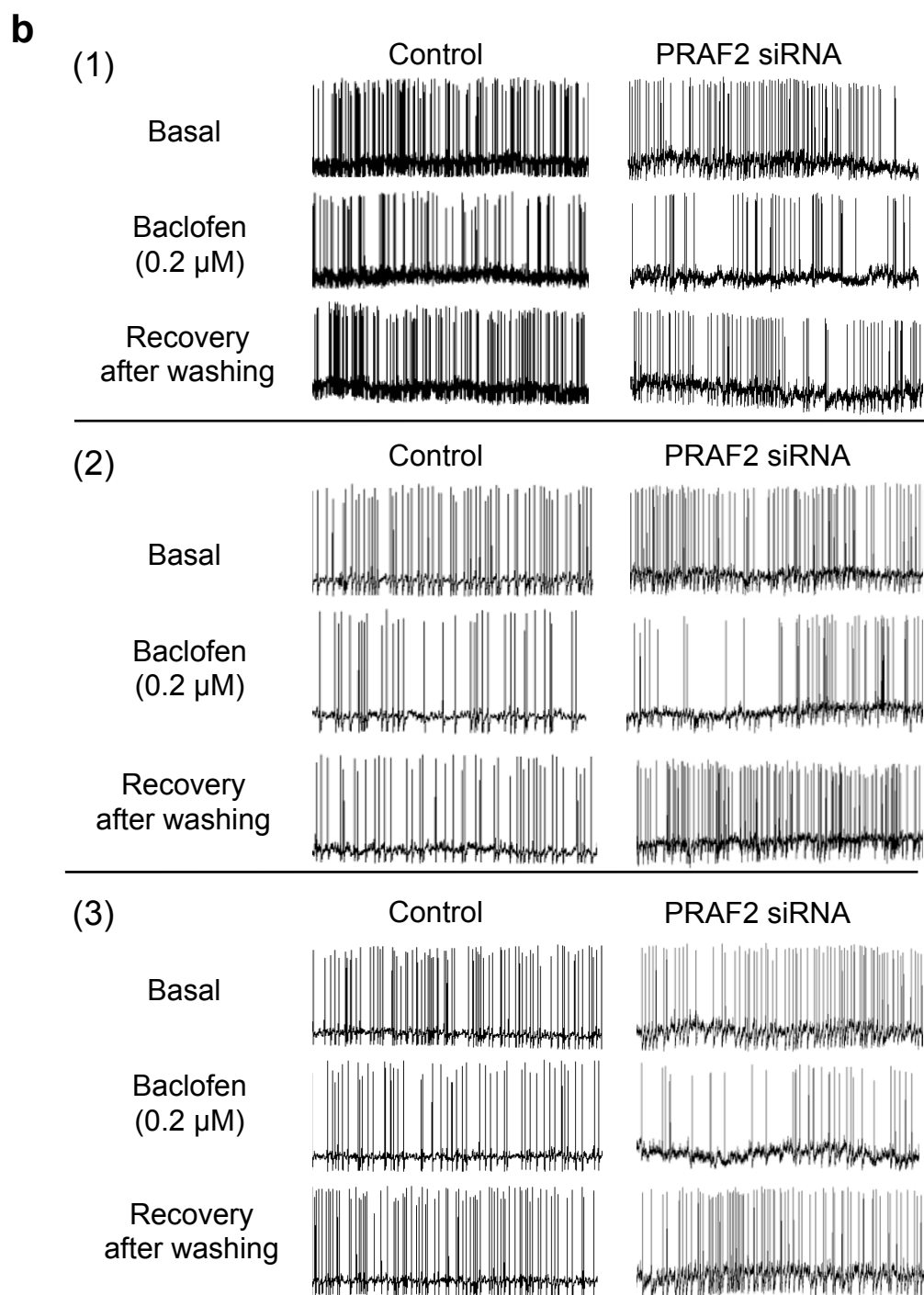
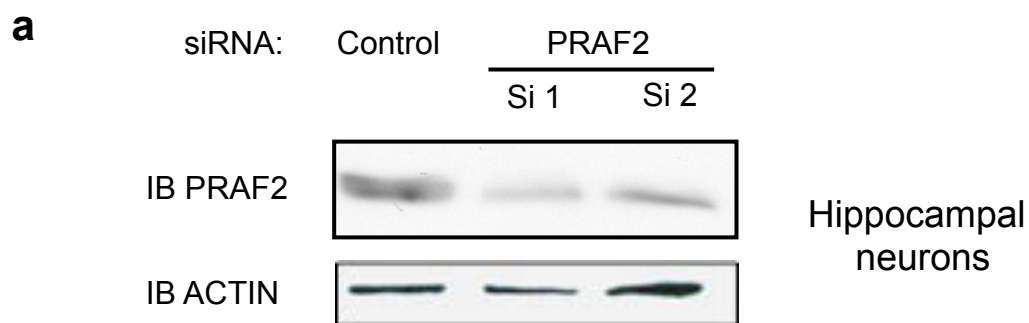


Figure S2

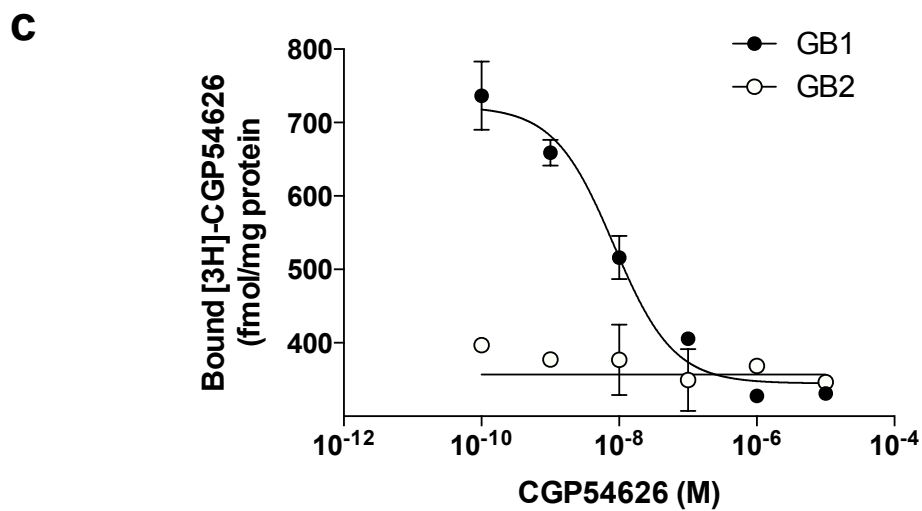
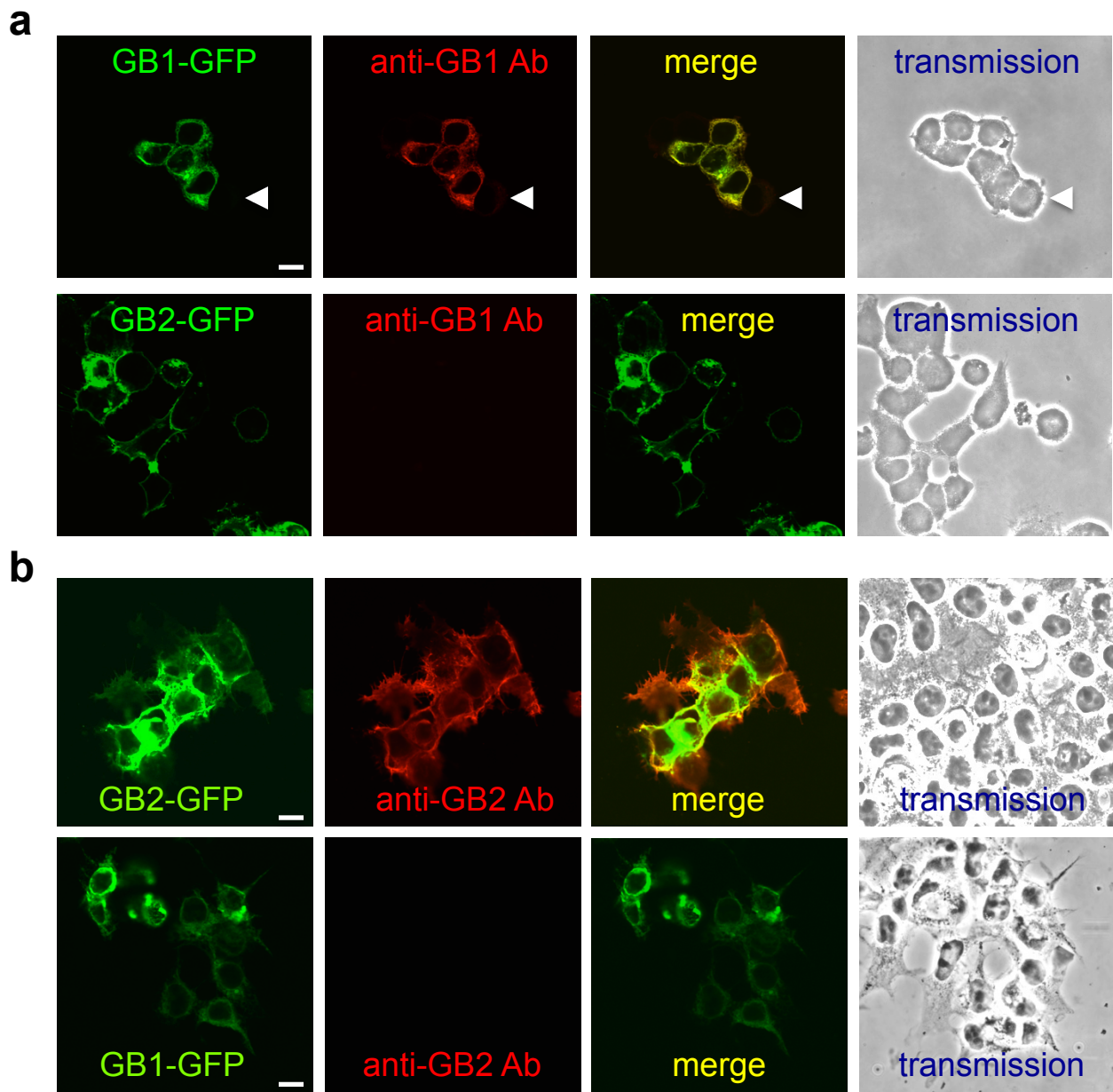
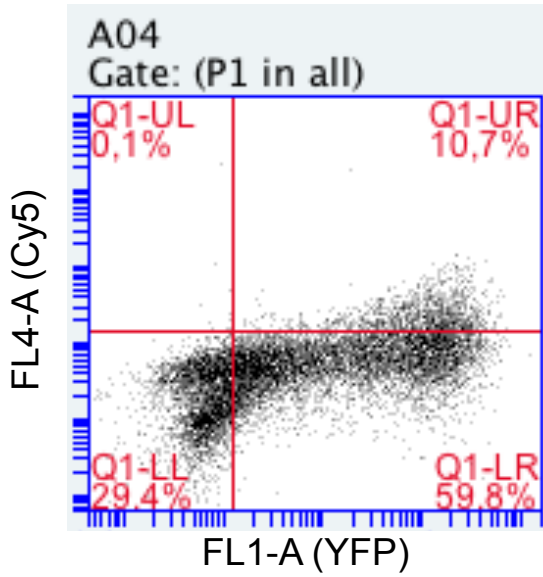
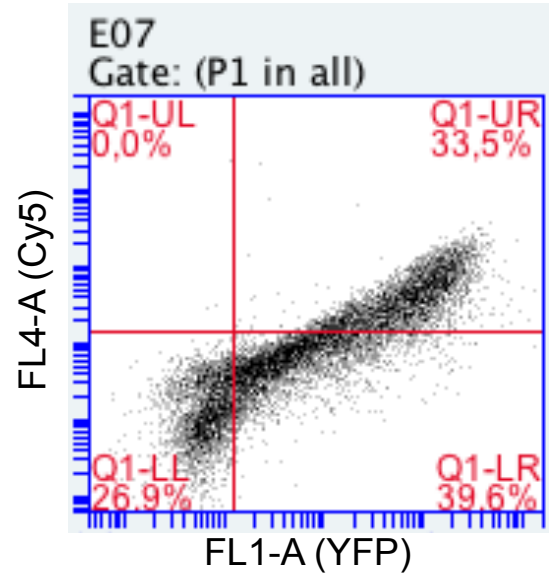


Figure S3

Myc-GB1-YFP



Myc-GB1-YFP+ HA-GB2



Plot 3: A04: Gated on (P1 in all)	Count	% of This Plot	Mean FL1-A	Mean FL4-A
This Plot	10 000	100,00%	52664,41	779,72
Q1-UL	13	0,13%	974,77	20122,92
Q1-UR	1 072	10,72%	150274,57	942,27
Q1-LL	2 936	29,36%	652,23	262,21
Q1-LR	5 979	59,79%	40816,73	603,42

Plot 3: E07: Gated on (P1 in all)	Count	% of This Plot	Mean FL1-A	Mean FL4-A
This Plot	10 000	100,00%	41316,78	2175,69
Q1-UL	4	0,04%	924,75	61497,00
Q1-UR	3 348	33,48%	108604,78	5498,88
Q1-LL	2 691	26,91%	668,46	179,93
Q1-LR	3 957	39,57%	12069,01	661,97

- Q1-LL:** nontransfected cells (group 1)
- Q1-LR:** YFP positives cells (group 2)
- Q1-UL:** Cy5 positives cells (group 4)
- Q1-UR:** YFP and Cy5 positives cells (group 3)

Figure S4

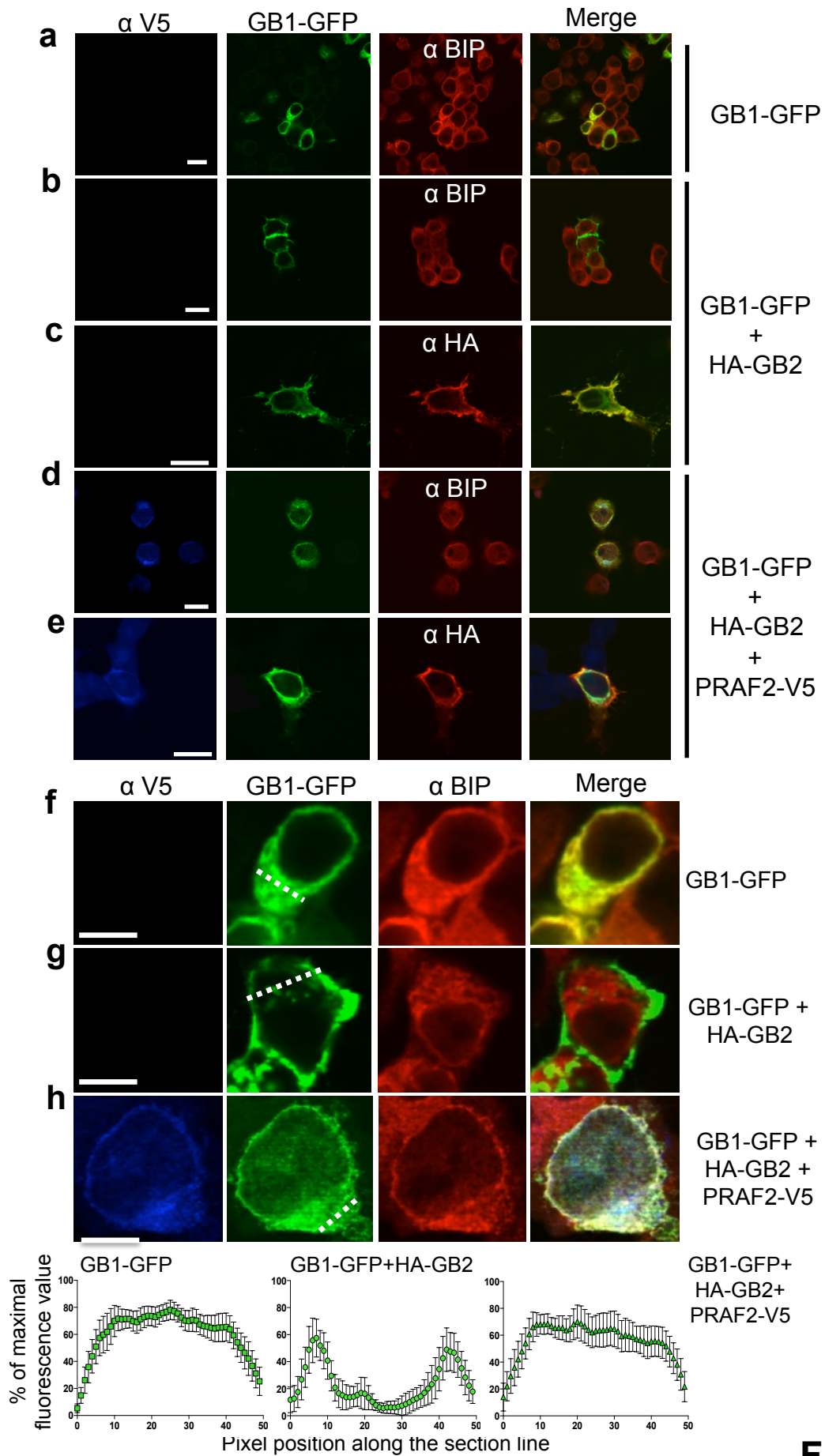


Figure S5

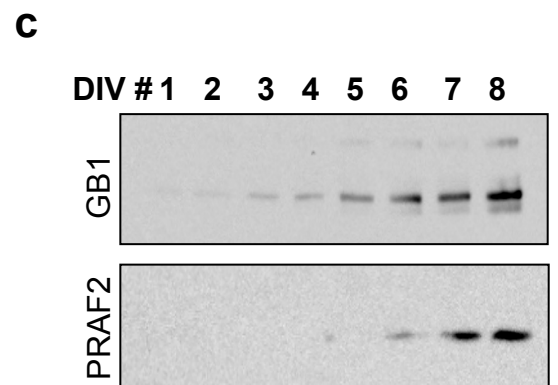
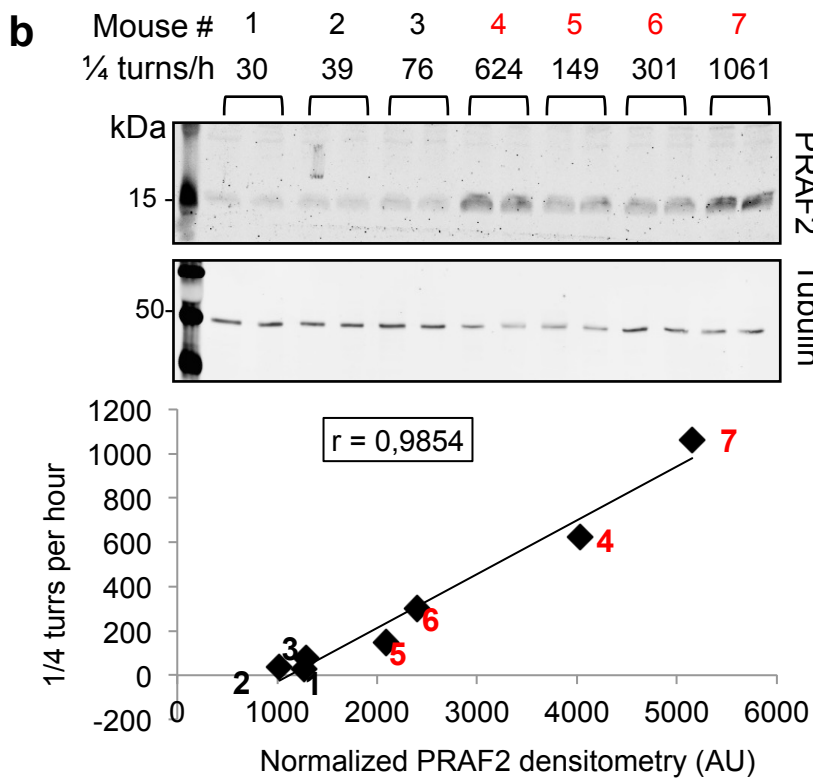
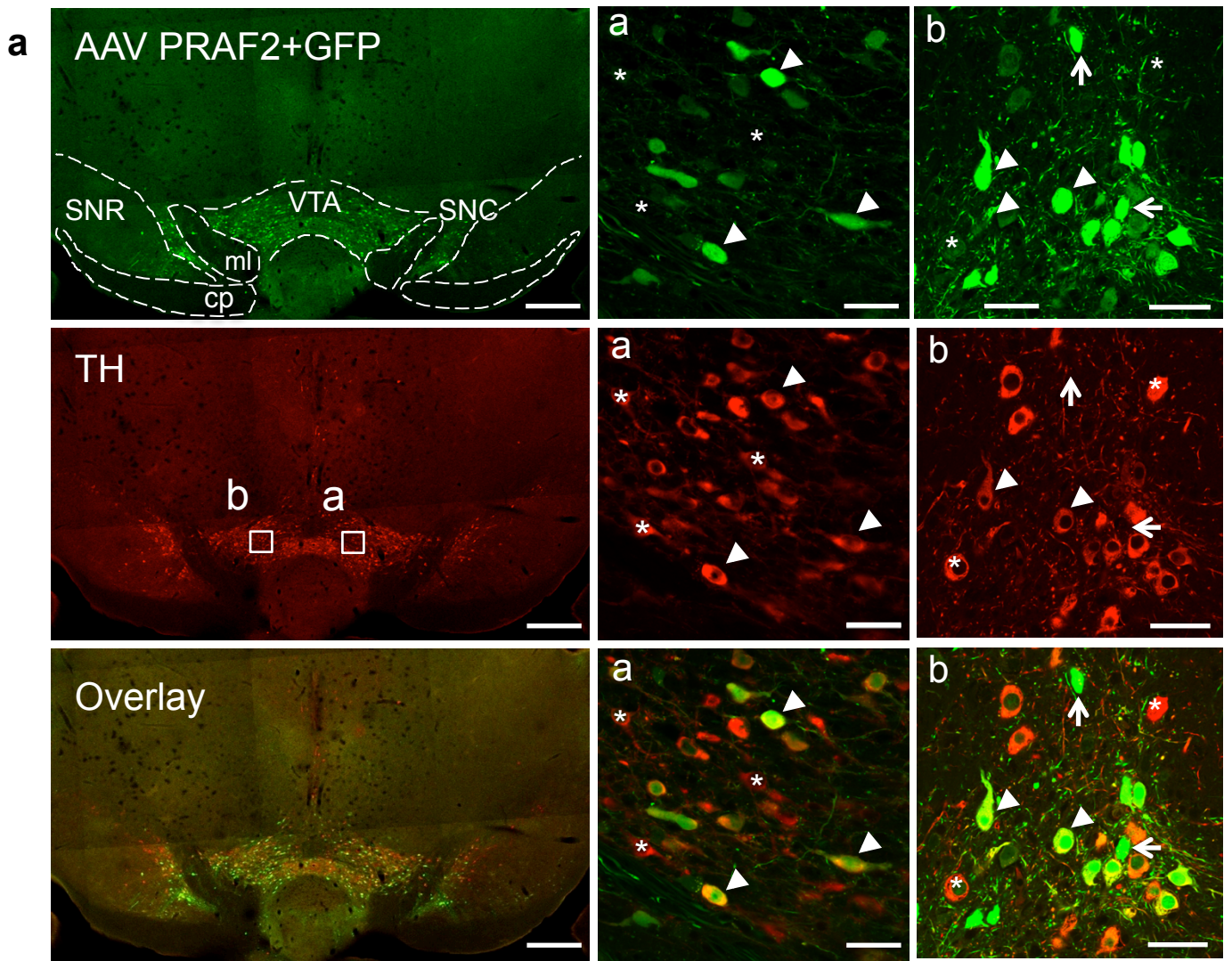


Figure S6

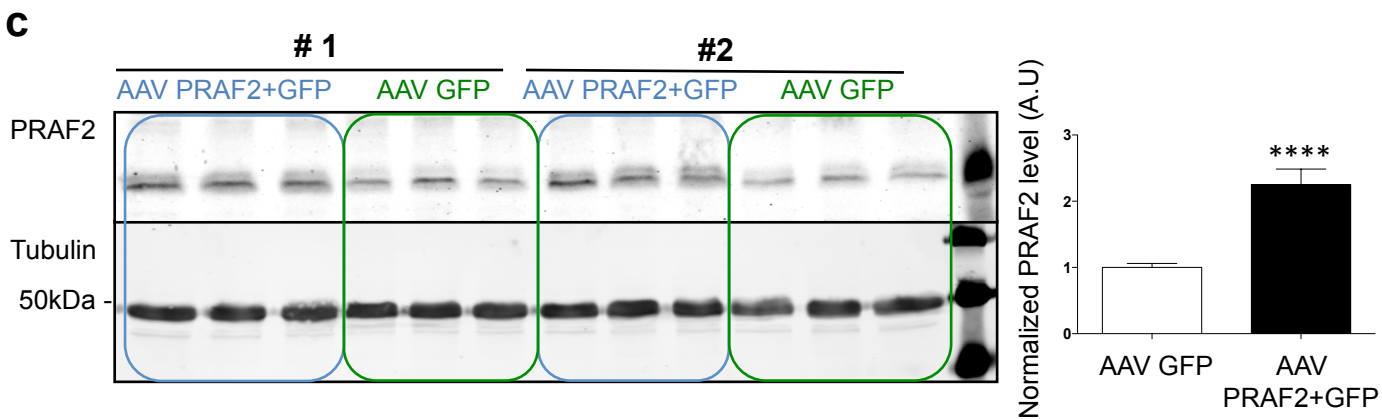
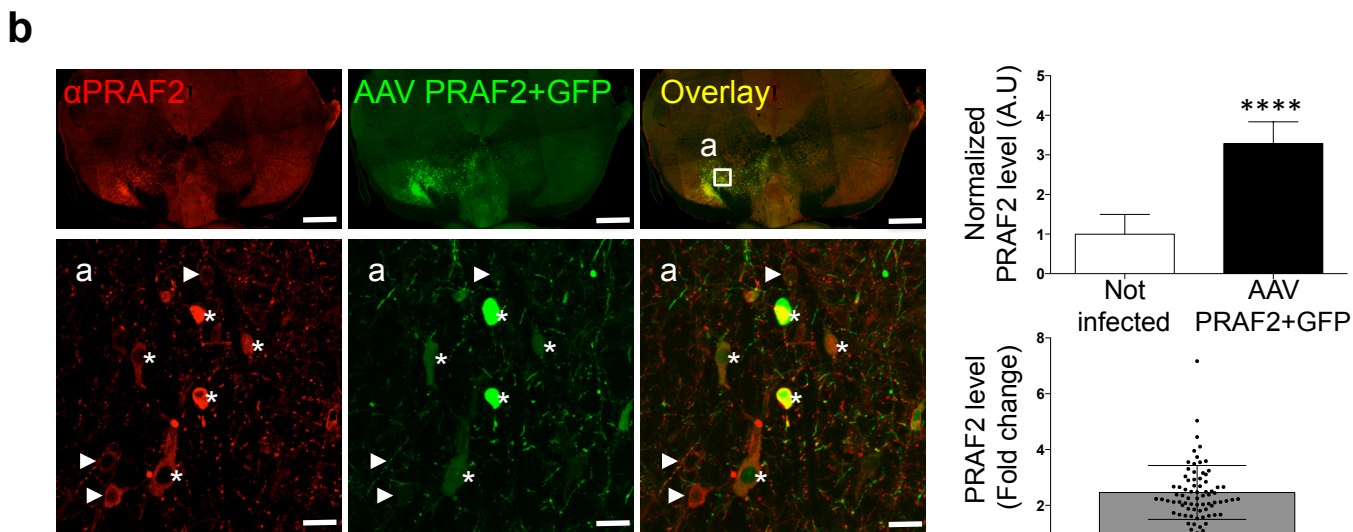
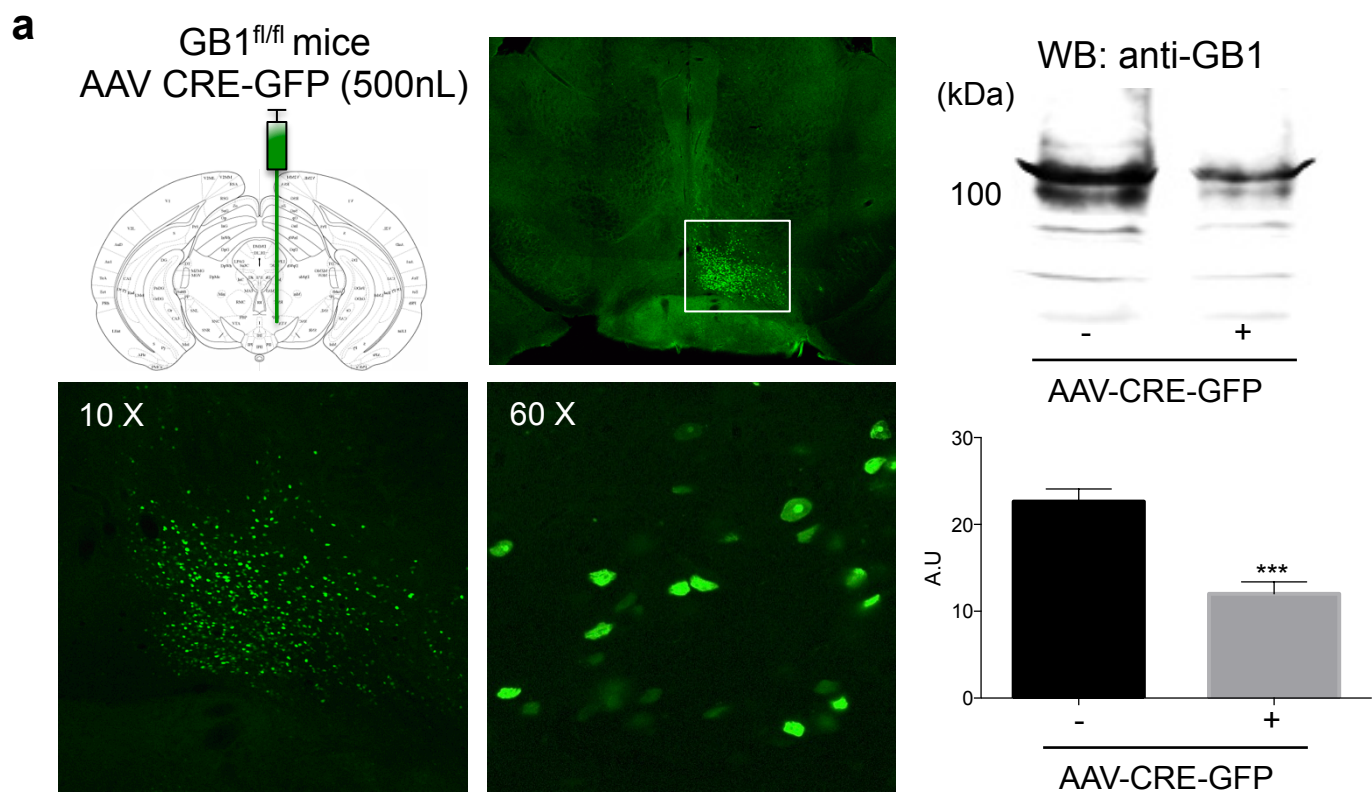


Figure S7

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Summary	N1	N2	t	DF
Baclofen 0µM							
untreated vs. Si-control	0	-19,56 to 19,56	ns	12	9	0	137
untreated vs. Si1-PRAF2	0	-18,11 to 18,11	ns	12	12	0	137
untreated vs. Si2-cPRAF2	0	-22,18 to 22,18	ns	12	6	0	137
Si-control vs. Si1-PRAF2	0	-19,56 to 19,56	ns	9	12	0	137
Si-control vs. Si2-cPRAF2	0	-23,38 to 23,38	ns	9	6	0	137
Si1-PRAF2 vs. Si2-cPRAF2	0	-22,18 to 22,18	ns	12	6	0	137
Baclofen 0.04µM							
untreated vs. Si-control	0,3929	-20,70 to 21,49	ns	12	7	0,04986	137
untreated vs. Si1-PRAF2	24,63	4,381 to 44,87	**	12	8	3,256	137
untreated vs. Si2-cPRAF2	9,75	-15,86 to 35,36	ns	12	4	1,019	137
Si-control vs. Si1-PRAF2	24,23	1,277 to 47,19	*	7	8	2,826	137
Si-control vs. Si2-cPRAF2	9,357	-18,44 to 37,16	ns	7	4	0,9011	137
Si1-PRAF2 vs. Si2-cPRAF2	-14,88	-42,04 to 12,29	ns	8	4	1,466	137
Baclofen 0.2µM							
untreated vs. Si-control	0,873	-21,48 to 23,22	ns	7	9	0,1046	137
untreated vs. Si1-PRAF2	40,03	18,17 to 61,89	****	7	10	4,903	137
untreated vs. Si2-cPRAF2	35,26	10,59 to 59,94	**	7	6	3,826	137
Si-control vs. Si1-PRAF2	39,16	18,78 to 59,53	****	9	10	5,144	137
Si-control vs. Si2-cPRAF2	34,39	11,01 to 57,76	***	9	6	3,938	137
Si1-PRAF2 vs. Si2-cPRAF2	-4,767	-27,67 to 18,14	ns	10	6	0,5572	137
Baclofen 1µM							
untreated vs. Si-control	11,86	-11,10 to 34,81	ns	8	7	1,383	137
untreated vs. Si1-PRAF2	33,67	13,42 to 53,91	***	8	12	4,452	137
untreated vs. Si2-cPRAF2	34	8,715 to 59,29	**	8	5	3,6	137
Si-control vs. Si1-PRAF2	21,81	0,7155 to 42,90	*	7	12	2,768	137
Si-control vs. Si2-cPRAF2	22,14	-3,828 to 48,11	ns	7	5	2,283	137
Si1-PRAF2 vs. Si2-cPRAF2	0,3333	-23,28 to 23,94	ns	12	5	0,0378	137
Baclofen 5µM							
untreated vs. Si-control	-4,222	-27,60 to 19,15	ns	9	6	0,4835	137
untreated vs. Si1-PRAF2	11,78	-12,96 to 36,52	ns	9	5	1,275	137
untreated vs. Si2-cPRAF2	9,778	-19,79 to 39,35	ns	9	3	0,8853	137
Si-control vs. Si1-PRAF2	16	-10,86 to 42,86	ns	6	5	1,595	137
Si-control vs. Si2-cPRAF2	14	-17,36 to 45,36	ns	6	3	1,195	137
Si1-PRAF2 vs. Si2-cPRAF2	-2	-34,39 to 30,39	ns	5	3	0,1653	137

Table 1. Statistical analysis of values in Figure 2e