Supporting Information

SI Materials and methods

cDNA and plasmids – myc-tagged R1a, flag-tagged R2, BBS-tagged R1a, and eGFP cDNAs have been previously described (1, 2). Myc-tagged R1b was kindly provided by Andreas Couve. The amino acids encoding for the BBS (WRYYESSLEPYPD (3)) were inserted adjacent to the myc tag, which resides six amino acids from the start of the mature R1b protein, using an inverse PCR strategy and CCCTGGAGCCCTACCCTGACCCGCGGCCTCACCCGCGGGTCCCC (forward) and AGCTCTCGTAGTATCTCCATAGGTCTTCTTCTGATATTAG (reverse) primers. Two arginines (R806, R808) which form the –RSR- endoplasmic reticulum (ER) retention motif in the C-terminus coiled-coil domain of R1b were replaced by alanines using site-directed mutagenesis with:
GCCTCCGCGCGCCACCCACCGACACCCC (forward) and
GAGCTGCTGCCGAGACTGGAG (reverse) primers to create $R1b^{BBS\text{-}ASA}$. A dileucine motif (4) in the R1b C-terminus coiled-coil domain (L773, L774) was replaced by alanines in $R1b^{BBS\text{-}ASA}$ to create $R1b^{BBS\text{-}ASA\text{-}L773A,L774A}$ using an inverse PCR strategy and GCGGCGGAGAAGGAGAACCGTGAACTG (forward) and CCGGGACTTCTCCTCCTCGTTG (reverse) primers. To study the role of the SDs on $GABA_B$ receptor trafficking, each SD was deleted using inverse PCR strategies. Deletion of the first SD ($R1a^{BBS}\Delta SD1$), removing amino acids G28 to C95, required TCCGAATCTGCTCCAAGTCTTA (forward) and CTTCCGAGGTGCTAGCGTCAGG (reverse) primers. Deleting the second SD ($R1a^{BBS}\Delta SD2$) removed R97 to V185 using GAATCGAACGCCACACTCAGAACG (forward) and
ACACAGCGGCTGGGTGTGTCCAT (reverse) primers.

The BBS was also inserted into the human mGluR2 cDNA in the vector pCDNA3.1+ (Missouri S&T cDNA Resource Center (www.cdna.org)) at the N-terminus, 6 amino acids from the start of the mature protein using TTTAGAACCATATCCAGATGTGCTGACCCTGGAGGGAGAC (forward) and CTACTTTCATAATATCTCCACTTCTTGGCTGGGCCCTCAGC (reverse) primers to create mGluR2^{BBS}. The mGluR2^{BBS}-Sushi domain chimera, (mGluR2^{BBS}-SD), was created by introducing a NheI restriction site next to the BBS by inverse PCR. The N-terminus of R1a from amino acids G16 to N159 containing the two Sushi domains was amplified by PCR with primers containing NheI restriction sites at the end. The mGluR2 cDNA was digested with NheI, 5' dephosphorylated with shrimp alkaline phosphatase (Promega) and ligated with DNA ligase (Roche) to the PCR amplified DNA encoding Sushi domains after NheI digestion. The fidelity of all cDNA constructs was checked by DNA sequencing.

Cell Culture and transfection – HEK293 cells stably expressing Kir 3.1 and Kir 3.2 potassium channels (GIRK cells), were grown in DMEM supplemented with 10% v/v fetal calf serum (FCS), penicillin-G/ streptomycin (100 u/100 µg/ml), 2 mM glutamine, and geneticin (0.5 mg/ml) at 37°C and in 95% air/5% CO2. Cells were seeded on 22 mm glass coverslips (VWR), coated with poly-L-lysine (Sigma) at a density of 5 X 10^{-4} cells/ μm^2 for imaging and electrophysiological studies.

Cultured hippocampal neurons were prepared from E18 Sprague Dawley rat embryos as described previously (4). Briefly, hippocampi were dissociated into a single cell suspension and plated onto 18 or 22 mm glass coverslips (Assistence/VWR) coated with poly-D-lysine (Sigma) in a medium containing MEM,

supplemented with 5% v/v heat-inactivated FCS, 5% v/v heat-inactivated horse serum, penicillin-G/ streptomycin (10 u/10 µg/ml), 2 mM glutamine, and 20 mM glucose (Sigma). After 2 hrs, the media was replaced and the cells were maintained until used for experiments in a media containing Neurobasal-A, supplemented with 1% v/v B-27, penicillin-G/ streptomycin (50 u/50 µg/ml), 0.5% v/v Glutamax, and 35 mM glucose.

GIRK cells were transfected 1 hr after plating using a calcium phosphate method (4) with 4 µg total DNA in the following ratios: R1a, R1b, $R1a^{BBS}$ or $R1b^{BBS}$: R2: eGFP, 1:5:1; R1b or $R1b^{BBS}$: R2, 1:5; $mGluR2^{BBS}$ or $mGluR2^{BBS}$-SD : eGFP, 1:1.
For radioligand binding experiments, cells were electroporated (Biorad Gene Pulser II) with 10 µg DNA for $R1b^{BBS}$: R2 in the ratio 1:3, and then plated in 10 cm dishes at 70% confluence. Neurons were transfected at 8-10 DIV using Effectene (Qiagen) or a calcium phosphate method (5). All media components were acquired from GIBCO/ Invitrogen unless otherwise stated.

α-Bungarotoxin radioligand binding assay - The apparent affinity of BTX for its binding site on the $GABA_B$ $R1b^{BBS}R2$ receptor was determined as described previously using ^{125}I-BTX (2, 4). Briefly, GIRK cells co-transfected with cDNAs encoding for $R1b^{BBS}R2$ were washed in PBS and re-suspended in fresh PBS containing 0.5% w/v bovine serum albumin (BSA; Sigma). Cells were incubated, with gentle agitation, in a total volume of 150 µl containing ^{125}I-BTX (200 Ci/mmol; PerkinElmer) for 60 min at room temperature. Nonspecific binding was determined by the addition of a 1000-fold excess higher concentration of unlabelled BTX (Invitrogen). Radioligand binding was assessed by filtration onto 0.5%

polyethylenimine pre-soaked Whatman GF/A filters, followed by rapid washing with PBS using a Brandel cell harvester. The radiation retained on the filters was assayed in a Wallac 1261 gamma counter. Scatchard analysis and non linear regression was used to obtain B_{max} and K_d values (Origin 6) using:

$y = (B_{max}*X)/ (K_d + X)$, where X is the concentration of ^{125}I-BTX.

For comparison, the same analysis was used for GIRK cells expressing a chimeric α7/5HT3a receptor which is known to retain a high affinity binding site for BTX (2).

Whole-cell patch clamp electrophysiology - Membrane potassium currents activated by GABA were recorded from individual transfected GIRK cells using whole-cell patch clamp recording as indicated previously (6). Patch pipettes (resistances: 3 – 5 MΩ) were filled with the following electrolyte (mM): 120 KCl, 2 $MgCl_2$, 11 EGTA, 30 KOH, 10 HEPES, 1 $CaCl_2$, 1 GTP, 2 ATP, 14 creatine phosphate, pH 7.0. The GIRK cells were constantly superfused with a salt solution containing (mM): 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11 glucose, and 5 HEPES, pH 7.4. To ensure larger $GABA_B$ receptor-activated K^+ currents, the KCl concentration of the external salt solution was increased to 25 mM coupled with a reduction in the NaCl concentration to 120 mM, prior to the application of GABA. This shifted E_K from -90 to -47 mV. The peak amplitude GABA-activated K^+ currents were now inward at a holding potential of -70 mV. Membrane currents were recorded from cells 48–72 hr after transfection and filtered at 5 kHz (-3dB, 6th pole Bessel, 36 dB/octave) before storage onto a Dell Pentium III computer for analysis with Clampex 8. Changes >10% in the membrane input conductance or series resistance resulted in the recording being discarded.

GABA concentration-response curves were compiled by measuring the potassium current (I) for each GABA concentration applied at 3-min intervals. The current amplitudes were normalized to the maximum GABA response (I_{max}) and the concentration response relationship fitted with the Hill equation:

$I/ I_{max} = [(1 / 1 + (EC_{50} / A)^n)]$,

Where A is the concentration of GABA, EC_{50} is the GABA concentration causing 50% of the maximum response and n is the Hill slope.

Fixed cell internalisation and membrane insertion assays - Internalisation and membrane insertion of $R1b^{BBS}R2$ was studied as described previously (2). For internalisation, this involved incubating GIRK cells co-trasnsfected with cDNAs encoding for $R1b^{BBS}$ (or $R1a^{BBS}$), R2 and eGFPs in 3 µg/ml BTX-AF555 for 10 min at room temperature to label the surface receptors. The cells were washed three times in Krebs to remove the BTX-AF555 and incubated in Krebs in a 37ºC incubator for different times at the end of which the cells were washed and fixed in 4% w/v paraformaldehyde (PFA; Sigma) in PBS for 5 min and quenched with 5% w/v NH_4Cl in PBS for 5 min. After washing (3x), the cells were mounted on glass slides with glycerol.

For membrane insertion, GIRK cells expressing $R1b^{BBS}$ (or $R1a^{BBS}$), R2 and eGFP were incubated with 20 µg/ml unlabelled BTX (UL-BTX; Invitrogen) for 10 min at room temperature to saturate the surface receptors. The cells were washed 3x in Krebs to remove the UL-BTX and incubated in 3 µg/ml BTX-AF555 at 37ºC for different times at the end of which the cells were washed, fixed and mounted as described above.

A Zeiss Axioskop LSM510 upright confocal microscope with 3 laser illumination sources (488nm, 543nm, and 643nm) and a Meta head was used with a Plan Neofluor x40 oil DIC objective (NA 1.3) for imaging. A mid optical slice was optimised and acquired as a mean of 4 scans in 8 bits and stored for further analysis.

Live cell imaging - Live transfected GIRK cells and hippocampal neurons (12-14 DIV or 19-21 DIV), were imaged using the Zeiss LSM 510 Meta confocal microscope and an Achroplan x40 water DIC objective (NA 0.8) as described previously (4). Cell surface receptors with the BBS were labelled on GIRK cells by incubation in 3 µg/ml BTX-AF555 at room temperature (RT) for 10 min and transfected hippocampal neurons were similarly incubated in 1mM d-tubocurarine (d-TC; Sigma) for 5 min followed by incubation in 3 µg/ml BTX-AF555 in Krebs for 10 min. Labelled cells were superfused with Krebs in a recording chamber at 30-32°C or RT. At the first time point selected for imaging (t = 0), the top and bottom of the cells were determined using a rapid z-scan and the mid optical slice was optimised and imaged as a mean of 4 scans in 8 bits using the 543 nm Helium-Neon laser with a 560nm long-pass filter for imaging BTX-AF555 and the 488 Argon laser with a 505-530 nm band-pass filter for imaging eGFP. The t = 0 point in live cell internalisation experiments was the first time point at which an image was acquired after labelling BBS containing receptors with BTX-AF555, having washed off the excess BTX-AF555, mounted the cells in the imaging chamber, and optimised the imaging settings. For subsequent time points all settings, including detector gain, amplifier offset, optical slice thickness and laser intensity, were unaltered from those used at t = 0. Any minor changes due to z-drift were corrected by adjusting the position on the z-axis by comparison against the GFP fluorescence at t = 0. When the morphology

of the cells changed significantly, the images were discarded and not used for analysis.

Image Analysis – Confocal images were analysed using ImageJ (version 1.40g) as described previously (4). For every soma or dendrite three regions of interest (ROI) were selected to include: the total area of analysis (ROI-1), the intracellular compartment (ROI-2) and the surface membrane (area between ROI-1 and ROI-2; ROI-3; Fig S7). The mean fluorescence was determined for each of the ROIs. Background fluorescence was ascertained by imaging a region of the coverslip that lacked cells. This was subtracted from each ROI to provide a mean corrected fluorescence value. For each live cell, the mean corrected fluorescence per unit area (μm^2) at each time point was then normalised to the mean corrected fluorescence per unit area at t = 0. Spines were analysed by drawing ROIs around individual spine heads that could be clearly distinguished from the eGFP fluorescence at t=0 and following the fluorescence over time. Spine structural plasticity was not observed frequently and in cases where spine morphology changed significantly, the images were discarded from analysis. All values collected at different time points, were fitted with a single exponential function using Origin (ver 6).

Photobleaching Profile – The photobleaching profile of BTX-AF555 bound to $R1b^{BBS}R2$ receptors was measured as described previously (4). To determine the extent of photobleaching in live GIRK cells expressing $R1b^{BBS}$ (or $R1a^{BBS}$), R2, and eGFP, receptors were tagged with BTX-AF555 by incubating with 3 µg/ml BTX-AF555 for 10 min at room temperature followed by washes (3x) in Krebs. The cells were exposed to four scans (pixel time 1.6 µs) performed consecutively every 6 s up

to a total of 100 scans (total laser exposure time per pixel - 64 µs) at 12-14°C. A loss of 15% fluorescence intensity was evident over 100 scans. However, this rate of scanning far exceeds that used in all of the live cell experiments, which required four scans at 5 time points over 1 h, giving a total of just 20 scans. Therefore, photobleaching was negligible (<5 %) and did not affect the surface fluorescence measurements.

List of abbreviations

ANOVA	Analysis of variance
$GABA_B$	γ-aminobutyric acid (type B receptor)
BBS	α-bungarotoxin binding site
BTX	α-bungarotoxin
BTX-AF555	α-bungarotoxin coupled to Alexa Fluor 555
CNS	Central nervous system
d-TC	d-tubocurarine
ER	Endoplasmic reticulum
GIRK	G-protein-coupled inward-rectifying potassium channel
mGluR2	Metabotropic glutamate receptor type 2
NMR	Nuclear magnetic resonance
nAChRs	α7 nicotinic acetylcholine receptors
R1 / R2	$GABA_BR1$ / $GABA_BR2$ subunits
RT	Room temperature
SD	Sushi Domain
UL-BTX	Unlabelled α-bungarotoxin

References

1. Arancibia-Carcamo IL, et al. (2009) Ubiquitin-dependent lysosomal targeting of $GABA_A$ receptors regulates neuronal inhibition. *Proc.Natl.Acad.Sci.U.S.A* 106: 17552-17557.

2. Wilkins ME, Li X, & Smart TG (2008) Tracking cell surface $GABA_B$ receptors using an α-bungarotoxin tag. *J Biol Chem* 283: 34745-34752.

3. Harel M, et al. (2001) The binding site of acetylcholine receptor as visualized in the X-Ray structure of a complex between α-bungarotoxin and a mimotope peptide. *Neuron* 32: 265-275.

4. Hannan S, et al. (2011) γ-Aminobutyric Acid Type B ($GABA_B$) Receptor Internalization Is Regulated by the R2 Subunit. *J.Biol.Chem.* 286: 24324-24335.

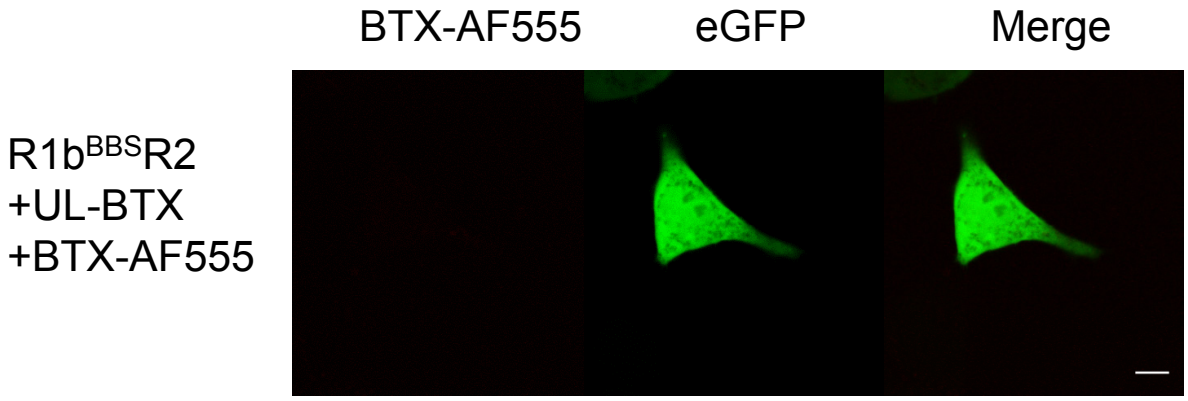
5. Xia Z, Dudek H, Miranti CK, Greenberg ME (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *J.Neurosci.* 16: 5425-5436.

6. Kuramoto N, et al. (2007) Phospho-Dependent Functional Modulation of $GABA_B$ Receptors by the Metabolic Sensor AMP-Dependent Protein Kinase. *Neuron* 53: 233-247.

Table S1 – Rates and extents of $GABA_B$ receptor internalisation

Cells	Construct	Temperature	Rate (min) (No. of cells)	Extent (%)
Live GIRK	$R1b^{BBS}R2$	22-24°C	14.6 ± 1.4 (9)	23.9 ± 2.1
	$R1b^{BBS\text{-}ASA}$	22-24°C	8.6 ± 1.1 (8)	25.9 ± 2
	$R1b^{BBS\text{-}ASA\text{-}AA}$	22-24°C	14.1 ± 1.9 (8)	21.8 ± 1.7
Fixed GIRK insertion	$R1a^{BBS}R2$	37°C	26.5 ± 2.4 (9)	-
	$R1b^{BBS}R2$	37°C	12.4 ± 2.9 (9)	-
Fixed GIRK internalization	$R1a^{BBS}R2$	37°C	11.9 ± 2.9 (7-9)	34.6 ± 4.3
	$R1b^{BBS}R2$	37°C	8.7 ± 0.4 (7-11)	21.4 ± 3.2
Neuron	$R1a^{BBS}R2$ Soma	30-32°C	16.3 ± 2.5 (7)	30.7 ± 2.2
	$R1b^{BBS}R2$ Soma	30-32°C	9.4 ±1.5 (12)	18.1 ± 1.2
	$R1a^{BBS}R2$ Dendrite	30-32°C	19.4 ± 3.3 (7)	36.3 ± 2.7
	$R1b^{BBS}R2$ dendrite	30-32°C	9.2 ± 0.9 (10)	22.8 ± 2.5
	$R1a^{BBS}R2$ spines	30-32°C	11.4 ± 3.1 (78)	32.8 ± 1
	$R1b^{BBS}R2$ spines	30-32°C	6.6 ± 1 (75)	30.6 ± 1
	$R1a^{BBS}\Delta SD1R2$	30-32°C	6.5 ± 1.8 (9)	19.2 ± 2
	$R1a^{BBS}\Delta SD2R2$	30-32°C	7.9 ± 1.3 (12)	19.4 ± 2.3
	$mGluR2^{BBS}$	30-32°C	29.8 ± 4.5 (8)	41.2 ± 3
	$mGluR2^{BBS}$-SD	30-32°C	42.3 ± 8 (7)	64.5 ± 2.2

GIRK cell



Hippocampal neuron

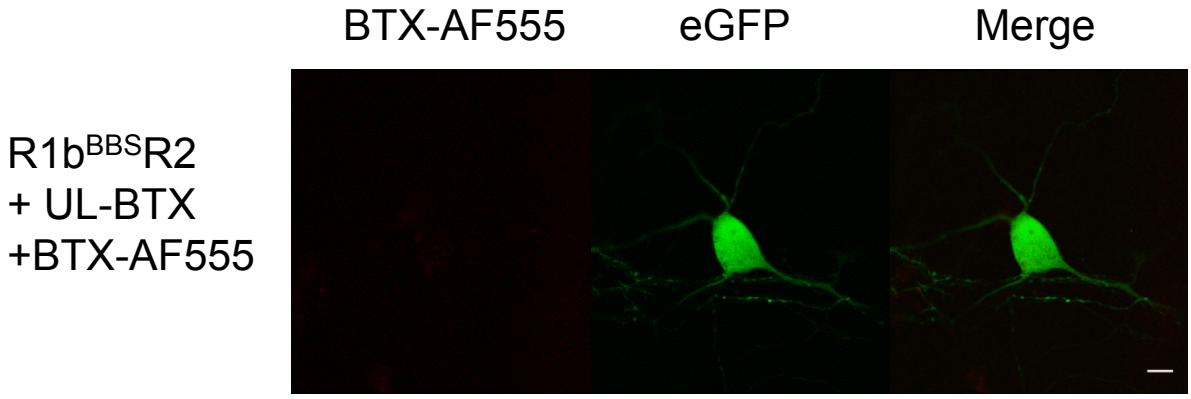


Fig S1. Specific binding of BTX-AF555 to R1b^{BBS}R2 receptors. GIRK cells and hippocampal neurons, pre-incubated in 1mM d-tubocurarine for 5 min at room temperature, expressing R1b^{BBS}, R2 and eGFP, were incubated with 3 µg/ml unlabelled BTX (UL-BTX) followed by 3 µg/ml BTX-AF555. Scale bar = 5 µm.

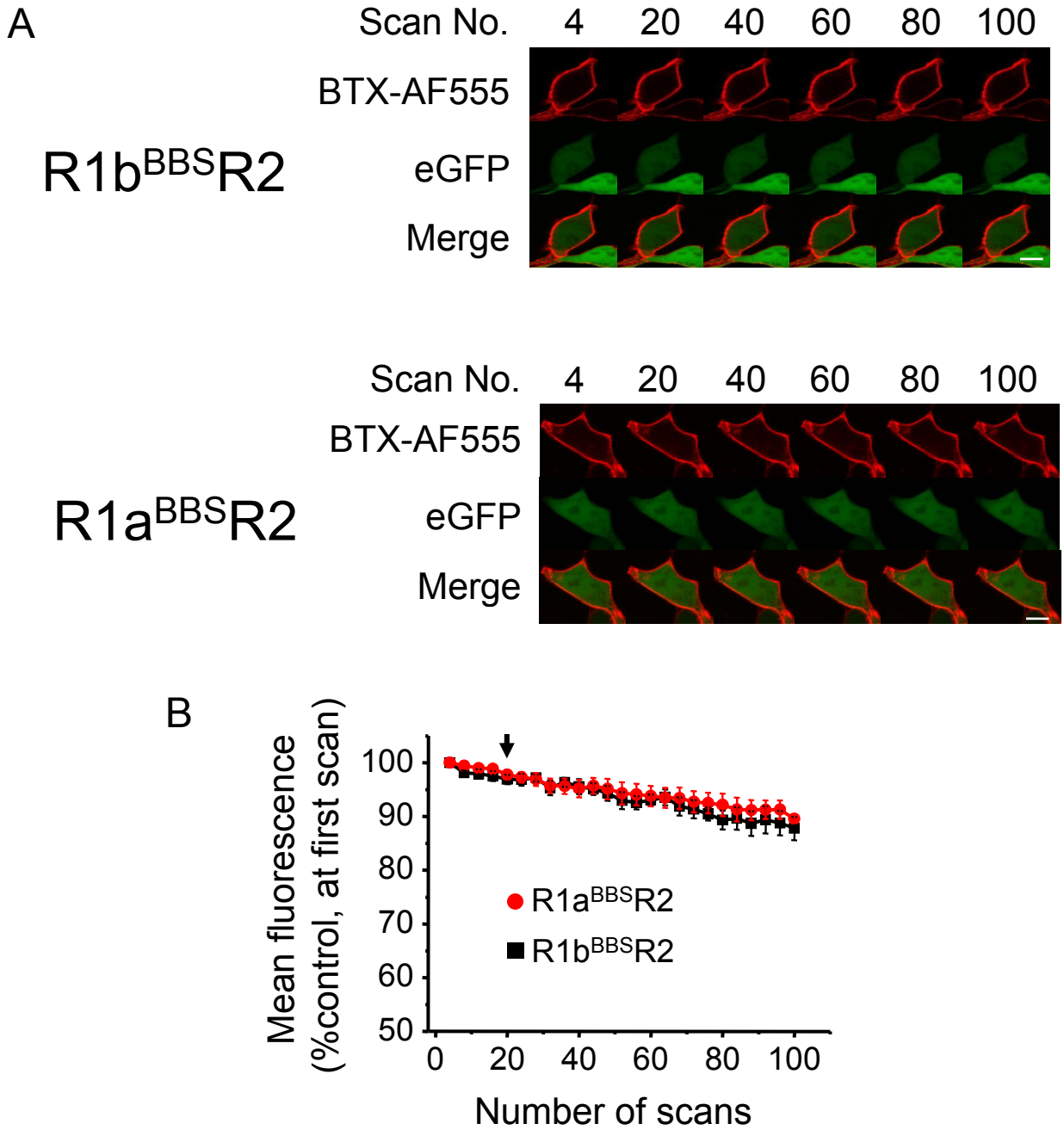


Fig S2. Photobleaching profile of BTX-AF555.
Images (A) and graphs (B) of surface membrane fluorescence decay of GIRK cells expressing R1b^{BBS}R2 (■) or R1a^{BBS}R2 (●) and eGFP, tagged with BTX-AF555 and superfused in Krebs. An average of 4 scans were performed consecutively every 6 seconds to give a total of 100 scans in order to construct the bleaching profile of BTX-AF555 bound to R1b^{BBS}R2 and R1a^{BBS}R2 at 12-14°C. Arrow shows the position of scan 20, the maximum that was used for live cell imaging. Scale bar 5 μm.

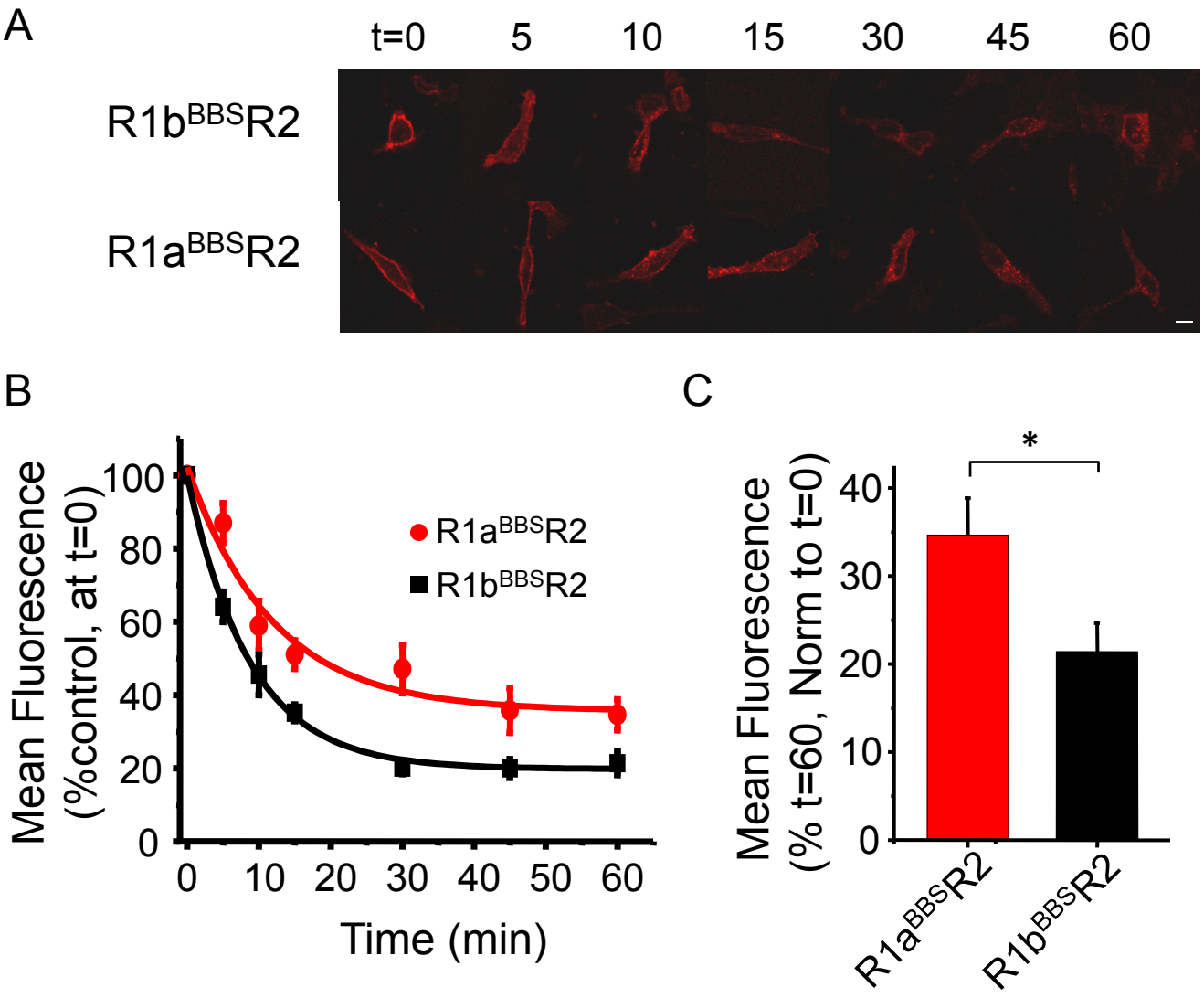


Fig S3. Internalisation of $GABA_B$ receptors.
A, Images of GIRK cells transfected with cDNAs encoding for $R1a^{BBS}R2$ or $R1b^{BBS}R2$ with eGFP incubated in 3 µg/ml BTX-AF555 for 10 min at RT washed and then incubated at 37°C for up to 60 min in Krebs before being fixed. B, Rates of constitutive insertion of $R1a^{BBS}R2$ (●) and $R1b^{BBS}R2$ (■) receptors (n = 7-11). C, Extent of constitutive internalisation for $R1a^{BBS}R2$ and $R1b^{BBS}R2$ receptors. * $P<0.05$. Scale bar = 5 µm.

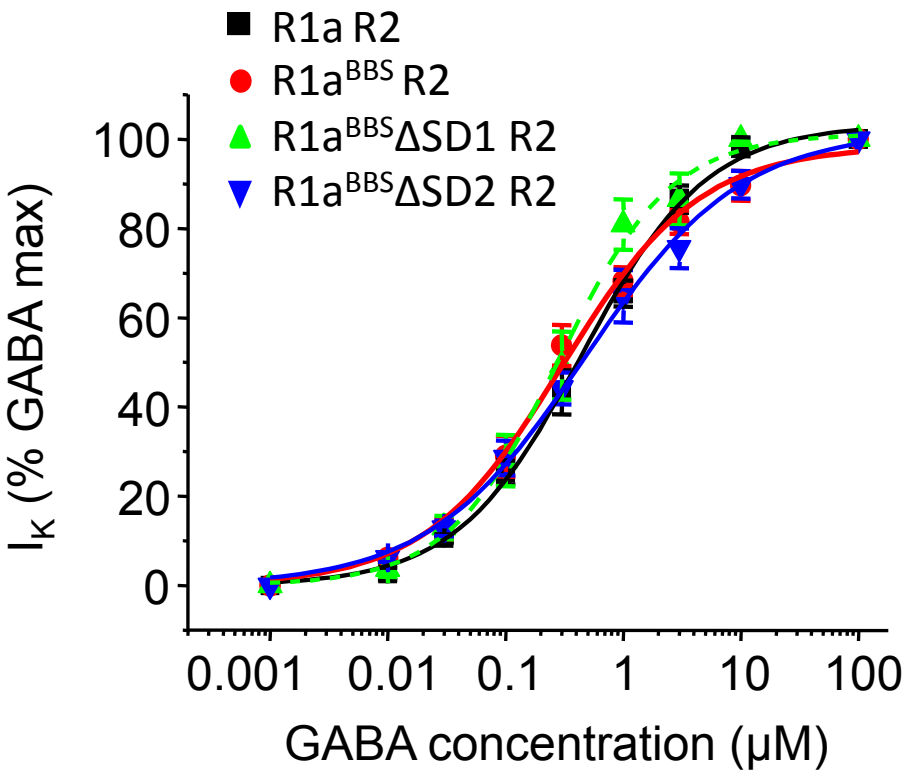


Fig S4. GABA concentration response curves for R1aR2, R1a^{BBS}R2, R1a^{BBS}ΔSD1R2, and R1a^{BBS}ΔSD2R2 expressed in GIRK cells (n = 4 - 11)

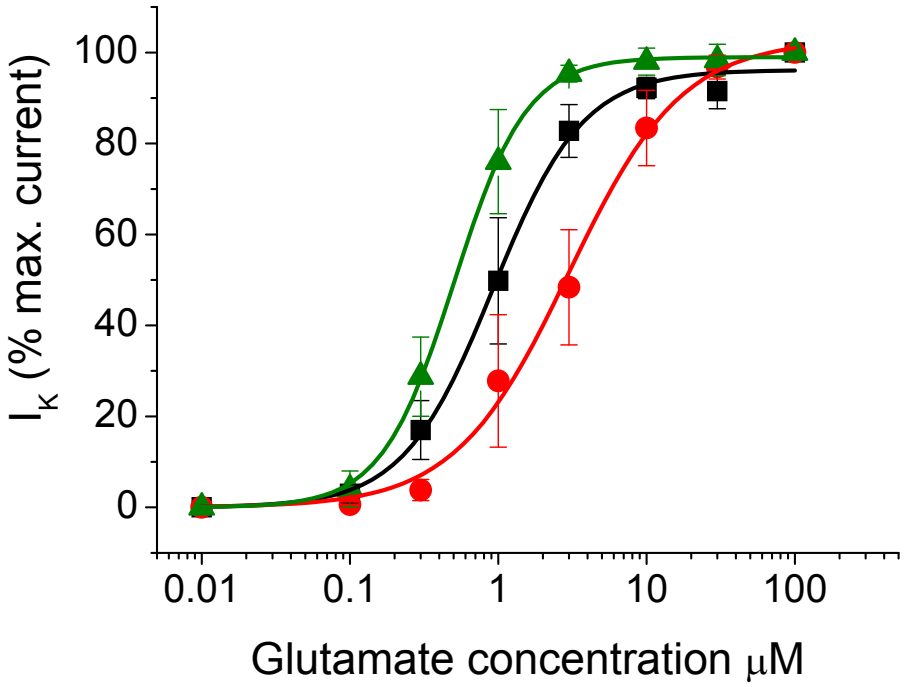


Fig S5. Glutamate concentration response curves for mGluR2 (■), mGluR2^{BBS} (●) and mGluR2^{BBS}+SD (▲) expressed in GIRK cells with EC_{50} values of 0.92 ± 0.07μM, 2.97 ± 0.33μM and 0.50 ± 0.01μM respectively (n = 4-6).

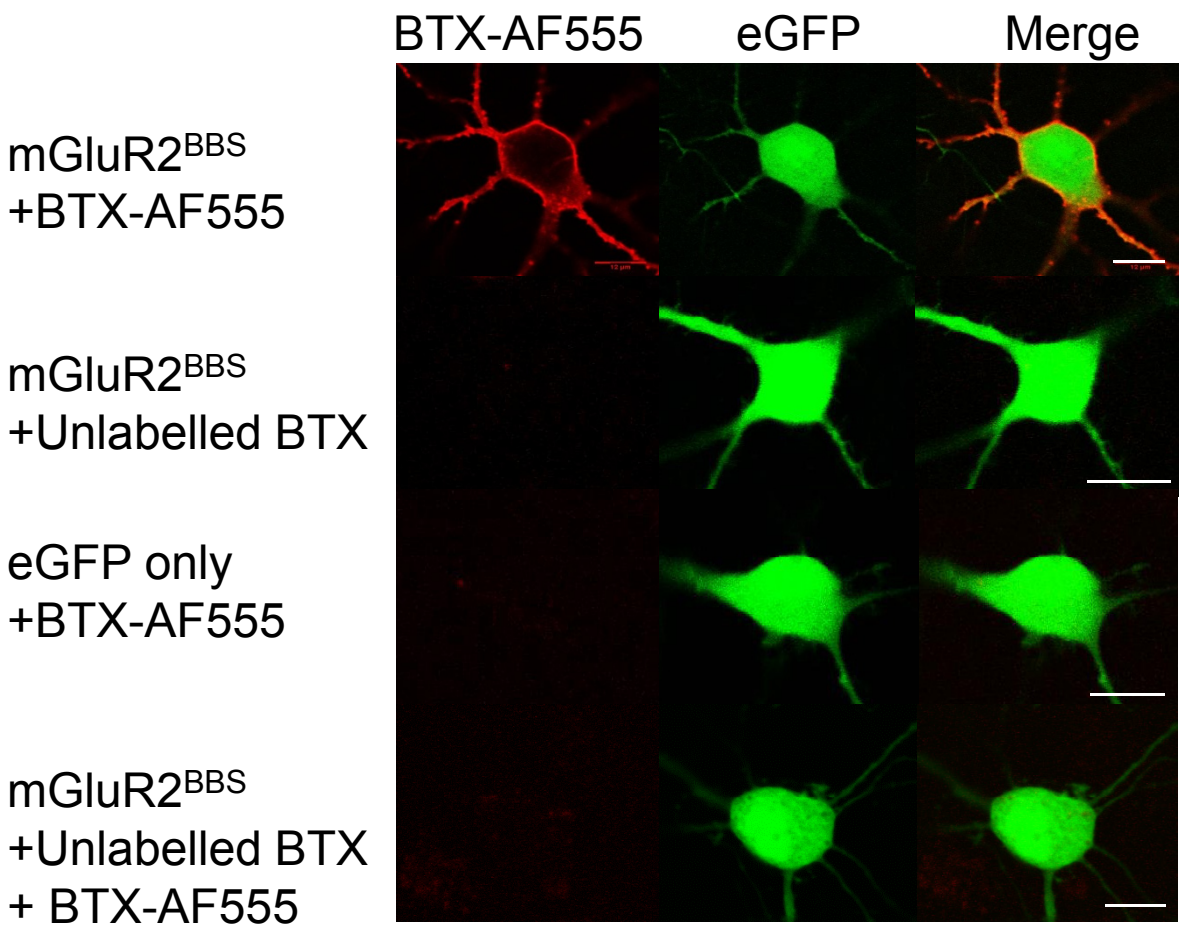


Fig S6. BBS on mGluR2 binds BTX. Cultured hippocampal neurons (14-21 DIV) expressing eGFP with or without mGluR2^{BBS} were incubated in 1 mM d-tubocurarine for 5 min at RT and then with 3 µg/ml BTX-AF555 or 3 µg/ml unlabelled BTX (UL-BTX) or 3 µg/ml UL-BTX followed by 3 µg/ml BTX-AF555. Scale bars = 12 µm.

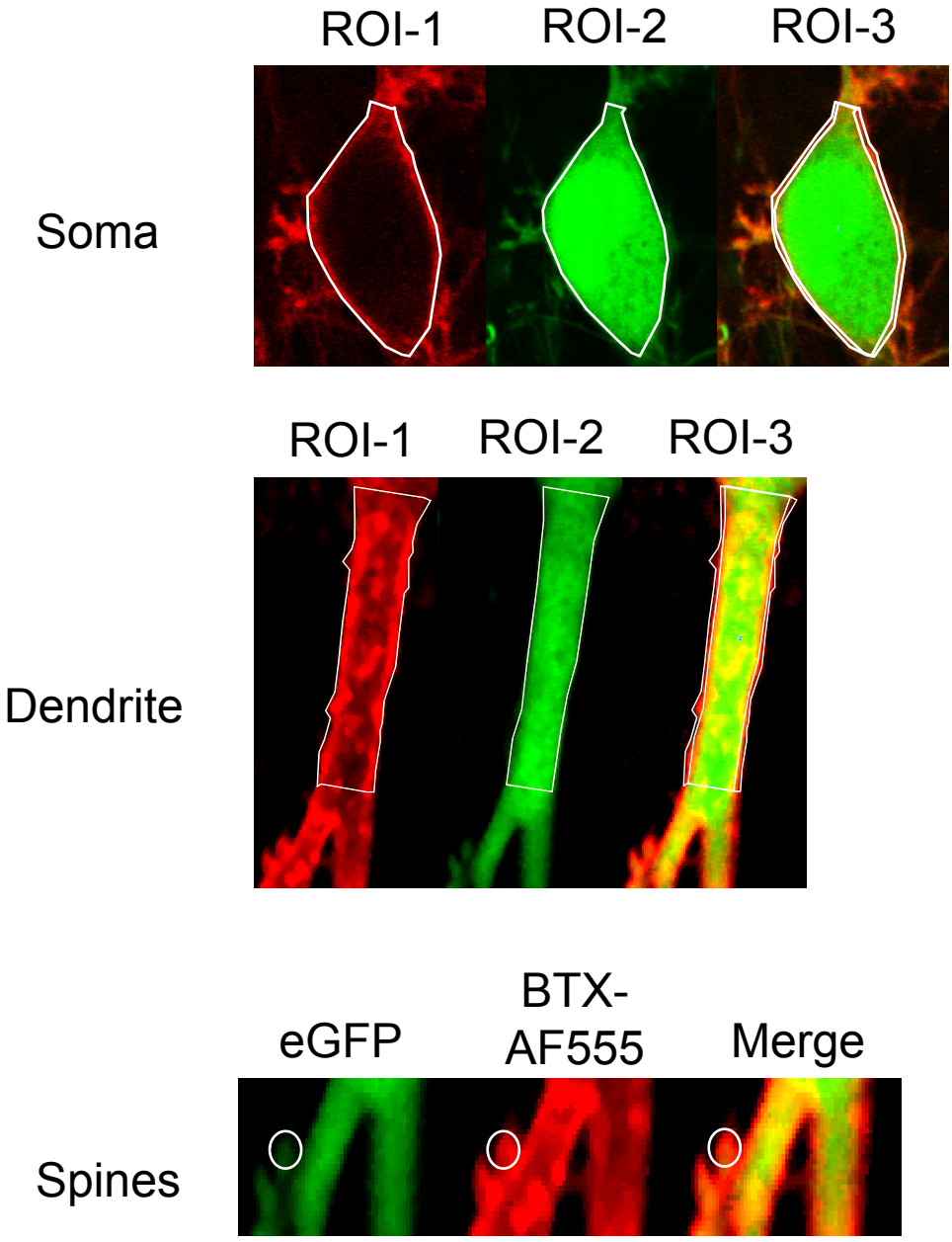


Fig S7. Creating regions of interest.
For each cell soma and dendrite three regions of interest (ROI) were selected: the total area of analysis (ROI-1), the intracellular compartment (ROI-2) and the surface membrane (ROI-3), given by the area between ROIs 1 and 2.
Spines were selected by drawing an ROI around the eGFP image and then transferring the ROI to the BTX-AF555 image