Supplemental Information

Phospho-dependent Accumulation of GABA<sub>B</sub>Rs at Presynaptic Terminals after NMDAR Activation

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Phospho-dependent accumulation of GABA$_B$Rs at presynaptic terminals after NMDAR activation

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**Abbreviated title** – Mobility of presynaptic GABA$_B$Rs during excitatory transmission
Supplemental Figures

A.

|                | eGFP | QD655 | Merge | Merge+DIC |
|----------------|------|-------|-------|-----------|
| R1a<sup>BBR2</sup> +BTX-B +QD655 | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| R1b<sup>BBR2</sup> +BTX-B +QD655 | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| R1a<sup>BBR2</sup> +BTX +QD655 | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| R1b<sup>BBR2</sup> +BTX-B | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |
| eGFP +BTX-B +QD655 | ![Image](image17) | ![Image](image18) | ![Image](image19) | ![Image](image20) |

B.

![Bar graph](image21)

**Figure S1**
Figure S1. Specificity of QD labeling (related to Figure 1).

(A) Images of neurons expressing the bungarotoxin binding site (BBS) tag in R1a\textsuperscript{BBS} or R1b\textsuperscript{BBS} with R2 and eGFP showing labeling with quantum dots (QDs) only when incubated in 4 µg/ml bungarotoxin biotin (BgTx-B) for 2 min followed by 10 pM QD 655 coupled to streptavidin (QD) for 1 min at 37°C. Neurons were not labeled with QDs when they were incubated with unlabeled bungarotoxin (lacking the biotin linker) or with just BgTx-B. In addition, neurons expressing just eGFP showed no staining when incubated in BgTx-B and QDs, confirming the high specificity of the labeling reaction. Scale bar = 10 µm. (B) Bar-chart of bound QDs for the conditions in (A) plus untransfected cells.
Figure S2
Figure S2. Expression levels of recombinant GABA\(_B\)Rs and Synaptophysin-eGFP (related to Figures 1 and 2)

(A) Representative 100 µM baclofen (bac)-activated K\(^+\) currents recorded from untransfected (UTF), eGFP transfected (GFP), and eGFP, R1a (R1a) or R1b (R1b) and R2 transfected hippocampal neurons at 9, 12 and 14 days in vitro (DIV). Neurons were transfected at 7 DIV then left for 2, 5 and 7 days before whole-cell recording. (B) 100 µM baclofen (bac)-activated K\(^+\) current densities from untransfected (UTF), eGFP transfected (GFP), and eGFP, R1a (R1a) or R1b (R1b) and R2 transfected hippocampal neurons at 9, 12 and 14 DIV. Note that the current densities evoked by baclofen are not different between untransfected, eGFP only transfected, and eGFP and R1a/R1bR2 neurons at all days tested using one-way ANOVA; however, there is a trend towards increased K\(^+\) current in R1a/R1bR2 cDNA transfected cells at 14 DIV. (C) Images showing expression of GABA\(_B\)Rs in untransfected, neurons transfected with cDNAs for eGFP (without (-) R1a, R2) or eGFP with R1a and R2 (+ R1a, R2) at 14 DIV. Neurons were transfected at 7 DIV and fixed at 14 DIV, permeabilised and labeled using primary antibodies against GABA\(_B\)R2 and secondary antibodies conjugated to Alexa Fluor 594 before imaging. The bar-chart shows the mean fluorescence intensity of intracellular GABA\(_B\)Rs in GFP-positive dendrites. P<0.05, two-tailed unpaired t test, n = 7-8. Scale bar 10 µm (D) Images of hippocampal neurons expressing synaptophysin-GFP colocalized with the inhibitory postsynaptic marker, gephyrin, and the excitatory postsynaptic marker, PSD-95 from Figure 2A. Arrowheads show the close apposition of the synaptophysin-eGFP puncta with endogenous gephyrin and PSD-95. The bottom panel shows high resolution images of the synaptic contacts between synaptophysin-eGFP and postsynaptic markers for the arrowheads depicted above. Scale bars 20 µm and 5 µm.
Figure S3
Figure S3. Deletion of the first Sushi Domain reduces lateral diffusion of axonal-extrasynaptic GABA\textsubscript{5}Rs (related to Figure 3).

(A) Cumulative probability distributions of diffusion coefficients ($D$) for presynaptic and axonal-extrasynaptic N-terminal Sushi Domain deleted (\textDelta SD1) R1a$^{\textDelta SD1}$R2 receptors. Inset shows median $D$ and IQR. (B) Cumulative probabilities for $D$ of presynaptic \textDelta SD1 receptors compared to R1aR2 (wild-type) controls. Inset shows median $D$ and IQR. (C) Cumulative probabilities for $D$ of axonal-extrasynaptic wild-type or \textDelta SD1. (D) Box-plot showing the IQR and median $D$ for axonal-extrasynaptic wild-type and \textDelta SD1. (E) Cumulative probability distributions of $D$ for presynaptic and axonal-extrasynaptic R1aR2 (wild-type) or C-terminal Sushi Domain deleted (\textDelta SD2) R1a$^{\textDelta SD2}$R2. (F) Box-plot showing the IQR and median $D$ for presynaptic (Syn) and axonal-extrasynaptic (Esy) wild-type (WT) and \textDelta SD2. NS – not significant, **P<0.01, ***P<0.001 KS test.
Figure S4. Baclofen causes a larger reduction in the mobility of axonal-extrasynaptic GABA\(_B\)Rs (related to Figures 2 and 4).

Cumulative probability distributions for presynaptic (A) and axonal-extrasynaptic (C) R1aR2 diffusion coefficients in the presence of 30 µM glutamate (Glu) or 100 µM baclofen (Bac). Box-plot showing the IQR and median \(D\) of presynaptic (B) and axonal-extrasynaptic (D) R1aR2 receptors. ***\(P<0.001\), NS – not significant, KS test.
Figure S5. Effects of CNQX and BAPTA on glutamate-induced reduction in GABAR mobility (related to Figures 4 and 5).

(A) Cumulative probability distributions of presynaptic and axonal-extrasynaptic R1αR2 diffusion coefficients in control and +30 µM glutamate (Glu) and 10 µM CNQX. (B) Box-plot showing the IQR and median $D$ for presynaptic (Syn) and axonal-extrasynaptic (ESy) R1αR2 in control and +glutamate and CNQX. (C) MSDs for synaptic and axonal-extrasynaptic R1αR2 in control and +glutamate and CNQX. (D) Presynaptic dwell times in control and in the presence of glutamate or glutamate + CNQX. (E) Cumulative probability distributions for presynaptic GABA$_B$R diffusion coefficients in control and +30 µM glutamate with (+Glu and DMSO) or without (+Glu) pre-treatment with vehicle or BAPTA-AM (+Glu and BAPTA). Neurons were pre-incubated in either vehicle or BAPTA-AM for 20 min at 37°C and imaged in 30 µM glutamate in the presence of vehicle or BAPTA-AM. (F) Box-plot showing the IQR and median diffusion coefficients for the data in A. *P<0.05, **P<0.01, ***P<0.001, NS – not significant; one-way ANOVA, KS test.
Figure S6
Figure S6. Accumulation of cell surface GABA_{\alpha}Rs at presynaptic terminals: effect of glutamate (related to Figure 6) and phosphorylation of GABA_{\alpha}R2^{S783} (related to Figure 7)

(A) Images showing co-localisation of cell-surface GABA_{\alpha}R1a with synaptophysin-eGFP in hippocampal neurons at 14 DIV. Cells were transected at 7 DIV using cDNAs encoding for R1a^{BBs} with either wild-type (WT) or mutant (S783A) R2, and synaptophysin-eGFP, and at 14 DIV incubated in control Krebs or 30 \mu M glutamate (+Glu) for 5 min at 37°C. Neurons were then fixed and incubated in 4 \mu g/ml bungarotoxin Alexa Fluor 555 for 10 min at room temperature and imaged. Scale bar = 2 \mu m. (B) Cumulative probability distribution for cell surface presynaptic R1a^{BBs} fluorescence normalized to synaptophysin-eGFP for GABA_{\alpha}Rs containing WT R2 in control and +Glu. (C) Cumulative probability distribution for cell surface presynaptic R1a^{BBs} fluorescence normalized to synaptophysin-eGFP for mutant receptors containing R2^{S783A} in control and +Glu. (D) IQR and median of normalized cell surface presynaptic fluorescence for WT (R1aR2) and mutant (R1aR2^{S783A}) receptors in control and +Glu. Normalised F_{median} WT control = 0.38 (n = 1094), F_{median} WT +Glu = 0.42 (n = 2174), F_{median} mutant control = 0.32 (n = 1444), F_{median} mutant +Glu = 0.31 (n = 1901). (E) Co-localization of GABA_{\alpha}R2 p783 and synaptophysin (Syn) in hippocampal neurons in control Krebs or 30 \mu M glutamate (+Glu) for 5 min at 37°C. At 14 DIV, neurons were incubated in glutamate, fixed, permeabilised and labeled using primary antibodies against phospho-783 and synaptophysin. The right hand panel shows the zoomed up co-localization in the arrowheads. (F) Cumulative probabilities for presynaptic p783 GABA_{\alpha}R2 fluorescence normalized to Syn fluorescence in control and +Glu. (G) Median values for normalized p783 GABA_{\alpha}R2 presynaptic fluorescence in control and +Glu. ***P<0.001, KS test. Scale bar = 5 \mu m
Figure S7. Reduced basal expression of GABABR2S783A (related to Figure 7).

(A) Images showing co-localisation of WT GABABR2 and R2S783A with synaptophysin in hippocampal neurons at 14 DIV. Cells were transected at 7 DIV using cDNAs encoding for R1a, with either WT or mutant R2, and with synaptophysin-eGFP, fixed at 14 DIV, permeabilised and labeled using primary antibodies against R2 and secondary antibodies conjugated to Alexa Fluor 594 before imaging. (B) Cumulative probability distribution for presynaptic GABABR2 fluorescence normalized to synaptophysin-eGFP for WT GABABR2 or GABABR2S783A. (C) IQR and median of normalized GABABR2 presynaptic fluorescence for WT GABABR2 or GABABR2S783A. F_{median} WT = 0.23 (n = 3127). F_{median} mutant = 0.19 (n = 6089). ***P<0.001 MW test. Scale bar = 5 µm.
Table S1. Median Diffusion Coefficients of GABABRs (related to Figure 1-5, 7)

| Receptor | Drug | Median $D$ ($\mu m^2 s^{-1}$) | Number of Receptors | Number of Neurons |
|----------|------|-------------------------------|---------------------|-------------------|
| Cell surface receptors not localized to synapses |
| R1aR2   | Control | 0.12                         | 1529                | 32                |
| R1aR2   | Baclofen | 0.10                         | 3262                | 48                |
| R1bR2   | Control | 0.07                         | 804                 | 22                |
| R1bR2   | Baclofen | 0.09                         | 3061                | 52                |
| Cell surface receptors localized to presynaptic terminals |
| R1aR2   | Control | 0.078                        | 445                 | 115               |
| R1bR2   | Control | 0.108                        | 276                 | 86                |
| R1aSD1R2 | Control | 0.11                         | 295                 | 27                |
| R1aSD2R2 | Control | 0.081                        | 574                 | 47                |
| R1aR2S783A | Control | 0.076                        | 264                 | 38                |
| R1aR2   | Baclofen | 0.063                        | 515                 | 114               |
| R1bR2   | Baclofen | 0.11                         | 397                 | 59                |
| R1aSD1R2 | Baclofen | 0.08                         | 211                 | 34                |
| R1aSD2R2 | Baclofen | 0.071                        | 322                 | 47                |
| R1aR2   | Glutamate | 0.048                        | 203                 | 69                |
| R1aR2   | Glutamate + TTX | 0.05                         | 324                 | 48                |
| R1aR2   | Glutamate + CNQX + APV | 0.079                        | 197                 | 38                |
| R1aR2   | Glutamate + CNQX | 0.043                        | 205                 | 23                |
| R1aR2   | NMDA Control | 0.045                        | 532                 | 59                |
| R1aR2   | NMDA Glutamate Control | 0.037                        | 198                 | 20                |
| R1aR2   | NMDA | 0.019                        | 234                 | 40                |
| R1aR2   | NMDA Glutamate + APV | 0.047                        | 485                 | 36                |
| R1aR2   | Glutamate + BAPTA Vehicle | 0.05                         | 204                 | 40                |
| R1aR2   | Glutamate + BAPTA | 0.07                         | 347                 | 56                |
| R1aR2S783A | Glutamate | 0.077                        | 261                 | 56                |
| Cell surface receptors localized to axonal extrasynaptic membranes |
| R1aR2   | Control | 0.124                        | 1834                | 115               |
| R1bR2   | Control | 0.129                        | 1203                | 86                |
| R1aSD1R2 | Control | 0.11                         | 532                 | 27                |
| R1aSD2R2 | Control | 0.09                         | 1202                | 47                |
| R1aR2   | Baclofen | 0.075                        | 2740                | 114               |
| R1bR2   | Baclofen | 0.13                         | 1814                | 59                |
| R1aR2   | Glutamate | 0.105                        | 865                 | 69                |
| R1aR2   | Glutamate + TTX | 0.095                        | 2584                | 48                |
| R1aR2   | Glutamate + CNQX + APV | 0.089                        | 2722                | 38                |

Table shows Diffusion coefficients determined from the MSD plots (see Supplemental Experimental Procedures) for wild-type and mutant, BBS-tagged, GABA$_B$R subunits taken from n receptors and n cells.
**Supplementary Movie titles**

Supplemental Movie 1 – Real-time lateral diffusion of Quantum Dot tagged GABA$\text{\textsubscript{A}}$ receptors on neuronal membranes (related to Figure 1)

Supplemental Movie 2 - Real-time lateral diffusion of Quantum Dot tagged GABA$\text{\textsubscript{A}}$ receptors on axonal membranes labeled with synaptophysin-eGFP (related to Figure 2)

Supplemental Movie 3 – Real-time lateral diffusion of Quantum Dot tagged GABA$\text{\textsubscript{A}}$ receptors on presynaptic terminals labeled with synaptophysin-eGFP, along with axonal extrasynaptic and exchanging receptors (related to Figure 2)
Supplemental Experimental Procedures

**Molecular Biology, Antibodies and Drugs** – The GABA\(_B\)R1 isoforms (R1\(_{a\, BBS}\), R1\(_{b\, BBS}\)) containing a bungarotoxin binding site, a flag-tagged GABA\(_B\)R2 (R2), R1\(_{a\, BBS}\) containing a deletion of the N-terminal (R1\(_{a\, \text{SD}1}\)) or C-terminal (R1\(_{a\, \text{SD}2}\)) Sushi Domains, an R2 subunit with a single amino acid substitution from serine 783 to alanine (S783A), and pEGFP-C1, have all been described previously (Hannan et al., 2012; Hannan et al., 2011; Kuramoto et al., 2007). Synaptophysin-eGFP and Synaptophysin-GcAMP6Fast were generous gifts from Yukiko Goda (Riken Brain Science Institute) and Leon Lagnado (University Sussex), respectively.

Antigens were labeled in fixed neurons using mouse primary antibodies against synaptophysin (Abcam ab8049), PSD95 (Neuromab K28/43), gephyrin (Synaptic systems 147 111), a guinea pig antibody against GABA\(_B\)R2 (Chemicon ab5394), and a rabbit antibody against phospho783 (Santa Cruz sc-135695) followed by an goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 555 (Molecular Probes) or goat anti-guinea pig Alexa-Fluor 594 (Molecular Probes) or goat anti-rabbit Alexa-Fluor 555 (Molecular Probes) antibody. All drugs and chemicals were obtained from Sigma unless specified otherwise.

**Cell Culture and transfection** – Dissociated hippocampal cultures were prepared from E18 Sprague-Dawley rat embryos as described previously (Hannan et al., 2011). Briefly this involved dissociation of dissected hippocampi into single cells followed by plating onto 18 or 22 mm glass coverslips (Assistance/ VWR) pre-coated with poly-D-lysine and immersed in a medium containing: minimum essential media (MEM; Life Technologies), supplemented with 5% v/v heat-inactivated fetal calf serum, 5% v/v heat-inactivated horse serum (Life Technologies), penicillin-G/ streptomycin (100 U/100 μg/ml), 2 mM glutamine, and 20 mM glucose. After 2 hrs, the media was replaced and the cells were maintained until used for experiments in a media containing Neurobasal-A (Life Technologies), supplemented with 1% v/v B-27, penicillin-G/ streptomycin (100 U/100 μg/ml), 0.5% v/v Glutamax (Life Technologies), and 35 mM glucose. Neurons were transfected at 7-8 days in vitro (DIV) using a calcium phosphate based method (Hannan et al., 2011).

**Labeling BBS-containing GABA\(_B\)Rs with QDs** - Hippocampal neurons expressing BBS-containing GABA\(_B\)Rs were incubated in 4 μg/ml biotinylated BgTx (BgTx-B; Life Technologies) for 2 min at 37°C. The cells were washed with Krebs (3x) and incubated in 10 pM Quantum Dot 655 conjugated to streptavidin (QD; Life Technologies) for 1 min at 37°C in QD binding buffer, as described previously (Levi et al., 2011). The buffer contained BSA (2% w/v), sodium azide (1 mM), sucrose (215 mM), and sodium borate (2.5 mM). The cells were washed thoroughly in Krebs and mounted in a recording chamber at 37°C (Solent Scientific) for live cell imaging in Krebs or in Krebs containing vehicle or drugs. All real-time imaging was carried within five min of drug application.

**Real-time imaging of single GABA\(_B\)Rs** - A wide-field imaging setup was used to locate QD labeling in neurons and to image GABA\(_B\)R-QD complexes for single particle tracking. The imaging hardware comprised an Olympus IX71 inverted microscope, with a 60X objective (NA – 1.35; Olympus) and halogen lamp illumination (PhotoFluor-II Metal Halide illumination system). Images were acquired using a back-illuminated cooled electron-multiplying charge coupled device (EMCCD) camera (iXon3 885; Andor Technology). eGFP was imaged with a 457 - 487 nm (Semrock) band-pass excitation filter, a 496 nm long-pass emission filter, and 495 nm
dichroic mirror. QD655 was imaged with a 415 - 455 nm band-pass excitation filter, a 647.5 - 662.5 nm band-pass emission filter, and a 510 nm dichroic mirror. Single images of QD specificity were acquired with optimal exposure (typically 30 - 150 ms) in 16 bits using Cairn-Metamorph Meta Imaging software (Molecular Devices; version 7.7.10) and stored for analysis.

For imaging the real-time movement of single GABA BRs, a suitable region-of-interest (ROI) was selected and an image taken of the eGFP expressing area (typical exposure time ~30 - 150 ms based on levels of eGFP expression). Next, the filters were changed to image QDs without changing the field of view and an image sequence of 300 frames captured at 33 Hz would be taken and stored for later tracking analysis.

Single particle tracking - For GABA BRs, this was undertaken using previously described methods (Bannai et al., 2009; Dahan et al., 2003; Levi et al., 2011; Renner et al., 2009). Single GABA BRs coupled to QD655 via the BBS were identified by their characteristic 'blinking' (Dahan et al., 2003). Matlab (MathWorks) based customised software, SPTrack (Ver5), was used to track the QDs. For every image, in sequence, the centre of the QD spot fluorescence was determined by a 2-D Gaussian fit with a spatial resolution of ~10 – 20 nm. This was undertaken for all QDs. The Gaussian peaks in a given frame were next associated with the Gaussian peaks from the previous frame based on estimated diffusion coefficients and the likelihood of the two Gaussian peaks in consecutive frames belonging to the same QD track. QDs which appear in at least 15 consecutive frames were used for tracking analysis and the shorter ones discarded. The mean square displacement (MSD) of each QD was calculated using the following equation:

$$\text{MSD} (ndt) = (N - n)^{-1} \cdot \sum_{i=1}^{N-n} ((x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2)$$

where $x_i$ and $y_i$ are the spatial co-ordinates of a QD on any image frame $i$, $N$ is the total number of points in the trajectory, $dt$ is the time interval between two successive frames (33 ms), and $ndt$ is the time interval over which the displacement is averaged. From the MSD plot, the diffusion coefficient, $D$, for a QD was calculated by fitting the first two to five points of the MSD plot against time with the following equation:

$$\text{MSD}(t) = 4D_{2-5} t + 4\sigma_x^2$$

where $\sigma_x$ is the QD localization accuracy in one direction. $D$ was determined from the slope of the relationship. Given the inherent noise in CCD imaging systems and the errors in precise pointing accuracy that results, trajectories with $D < 10^{-4}$ $\mu$m$^2$/s were considered immobile.

Synaptic terminals were identified and thresholds set using a multidimensional image analysis plug-in based on MetaMorph (Molecular Devices) software (Racine et al., 2007) or using ImageJ. QD trajectories that co-localised with synaptic markers were defined as synaptic. QD data were subsequently analysed using Origin (Ver 6), Matlab, and SPSS (IBM).

Cell surface labeling - Hippocampal neurons expressing BBS-containing GABA BRs were fixed in 4% PFA for 10 min and after washing with Krebs (x 3) incubated in 4 µg/ml BgTx Alexa fluor 555 (Life Technologies) for 10 min at room temperature. The cells were washed with Krebs (3x) and mounted for imaging using confocal microscopy.
Confocal Imaging - Confocal images of synaptic protein expression and GABA_9R presynaptic localisation were imaged using an upright Zeiss LSM 510 or Leica SP8 vis confocal microscopes. Images were acquired with optimum gain and offset in 8-bit using a 488 nm laser and 505 - 530 nm band-pass filter for eGFP/Alexa Fluor 488 and a 543 nm laser and 560 nm long-pass filter for Alexa Fluor 555. Confocal images were analysed in ImageJ. To quantify presynaptic GABA_9R and phospho783 labelling levels, images from the synaptophysin channel were thresholded and the built-in ‘Analyze Particles’ function was used to detect and add ROIs to the ROI manager. Next, the ROIs were transferred to the GABA_9R channel and the fluorescence intensity of GABA_9R staining within the synaptophysin ROIs were measured and normalized to the fluorescence intensity from the raw synaptophysin channel. The cumulative probability of the distribution for normalized GABA_9R intensity was used for analysis. For analyzing intracellular fluorescence, longitudinal ROIs were drawn on dendrites identified using eGFP expression and transferred to images on the corresponding GABA_9R channels to measure the GABA_9R fluorescence.

Calcium imaging and analysis – Synaptophysin-GCaMP6 Ca^{2+} transients were image captured in the presence of a brief (<3 min) application of 30 μM NMDA and 10 μM D-serine using the wide-field setup described above. Images of presynaptic terminals were acquired for 15 s at 20 Hz. ROIs were drawn around presynaptic terminals and the fluorescence intensity within individual puncta were measured during the 15 s image sequence using ImageJ. The fluorescence signal (F) was normalised to baseline fluorescence (F_0) to obtain values of ΔF/ΔF_0. ΔF/ΔF_0 peaks were detected and their amplitudes measured in Matlab using the plug-in Peakfinder (Nathanael Yoder, Mathworks). The maximum normalised peak from each presynaptic terminal during the 15 s imaging period and the average normalised Ca^{2+} peak of each terminal have been used for analysis. Ca^{2+} transients less than 3x the signal-to-noise ratio were excluded from the analysis.

Whole-cell patch-clamp recording – G-protein-coupled inwardly-rectifying K^+ (Kir) channel currents elicited in response to GABA_9R activation by 100 μM baclofen, were recorded from hippocampal neurons in culture at 9, 12 or 14 DIV, using patch clamp electrophysiology. Recordings were performed on: untransfected neurons or those transfected at 7 DIV with eGFP only or with eGFP, R1a^{BBS} or R1b^{BBS} and R2. Transfected cells were identified by eGFP expression. Patch pipettes (4-5 MΩ) fabricated from thin-walled borosilicate glass (GC-150TF-10; Harvard Apparatus, Kent, UK), were filled with internal solution (mM: 120 KCl, 2 MgCl_2, 11 EGTA, 30 KOH, 1 CaCl_2, 1 GTP, 2 ATP, pH 7.4). Neurons were continuously perfused with a Krebs solution containing (mM): 140 NaCl, 2.5 CaCl_2, 4.7 KCl, 1.2 MgCl_2, 11 glucose, and 5 HEPES, pH 7.4 and voltage-clamped at -70 mV in the whole-cell configuration, with series resistance compensation (~40%) applied. Membrane currents consecutively generated by 20 sweeps of -10 mV hyperpolarising steps were recorded with an Axopatch 200B amplifier (Molecular Devices, California, USA) at 10 kHz filtering. Currents were recorded using repeat trains of sweeps. Data were discarded if series resistance changed by > 10%. To increase the size of GIRK currents and to convert them to inward currents whilst holding at -70 mV, prior to baclofen application, the KCl concentration was increased to 25 mM and the NaCl concentration reduced to 120 mM in the Krebs solution. This changed E_K from approximately -92 mV to -47 mV. In addition, 2 mM kynurenic acid and 100 μM picrotoxin were added to the Krebs to block ionotopic glutamate and GABA_A receptors. Peak K^+ current amplitudes were filtered at 5 kHz before storage for analysis with Clampex 10 software. Whole-cell capacitance
was calculated using WinWCP V4.7 software (J Dempster, Strathclyde University) from the area under the membrane current discharge curve elicited by a series of -10 mV hyperpolarizing pulses before capacity compensation was applied. Current density was calculated by dividing the peak amplitude by the whole-cell capacitance.
Supplemental references

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