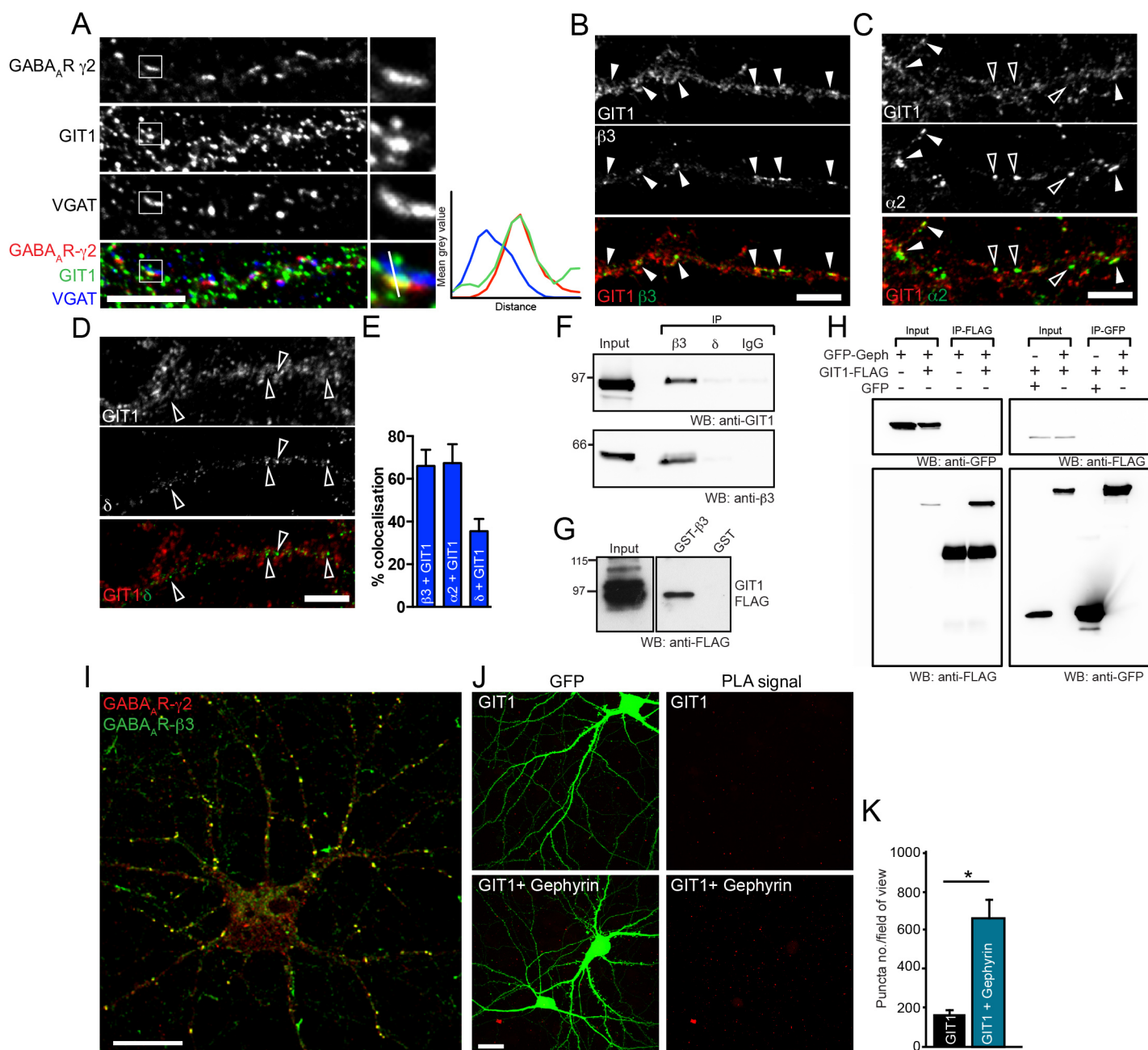


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Supplemental Information

**GIT1 and  $\beta$ PIX Are Essential  
for GABA<sub>A</sub> Receptor Synaptic Stability  
and Inhibitory Neurotransmission**

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### Figure S1, Related to Figure 1; GIT1 localisation in neurons

(A) Deconvolved image of a neuronal dendrite labelled with antibodies to GABA<sub>A</sub>R-γ2 (red), GIT1 (green) and VGAT (blue), scale bar=5μm.

(B-D) GIT1 localisation with other GABA<sub>A</sub>R subunits. Neurons labelled with antibodies to GIT1 (red) and β3 (B), α2 (C) and δ (D) and imaged by CLSM; solid arrowheads=overlap, open arrowheads=no overlap, scale bar=5μm.

(E) Quantification of colocalisation from images in B-D. Values are mean ± SEM.

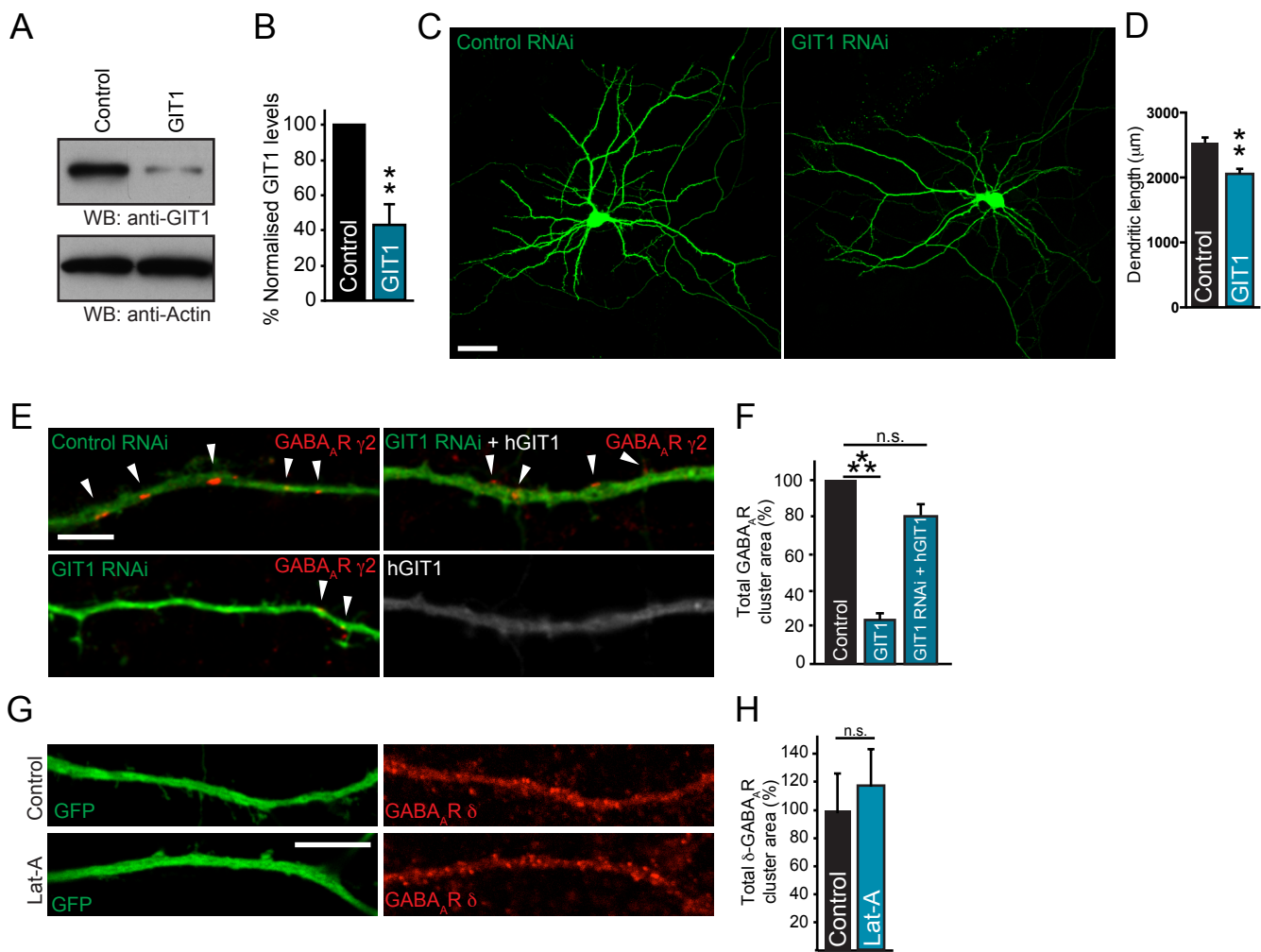
(F) Western blot of coimmunoprecipitation of GIT1 from rat brain lysate by antibodies to the GABA<sub>A</sub>R β3 or δ subunit. The β3 subunit can coimmunoprecipitate GIT1, but the δ subunit cannot.

(G) Western blot of pull-down from FLAG-GIT1 transfected COS7 cell lysates with the GST-β3 intracellular domain, or GST only control.

(H) Western blot of coimmunoprecipitation from transfected COS7 cell lysates.

(I) CLSM of neuron colabelled for GABA<sub>A</sub>R with antibodies to β3 (green) and γ2 (red) subunits, scale bar=20μm.

(J,K) Proximity ligation assays with antibodies to GIT1 alone or GIT1 and gephyrin, scale bar=20μm. Values are mean ± SEM.



### Figure S2, Related to Figure 2; Characterisation of GIT1 RNAi

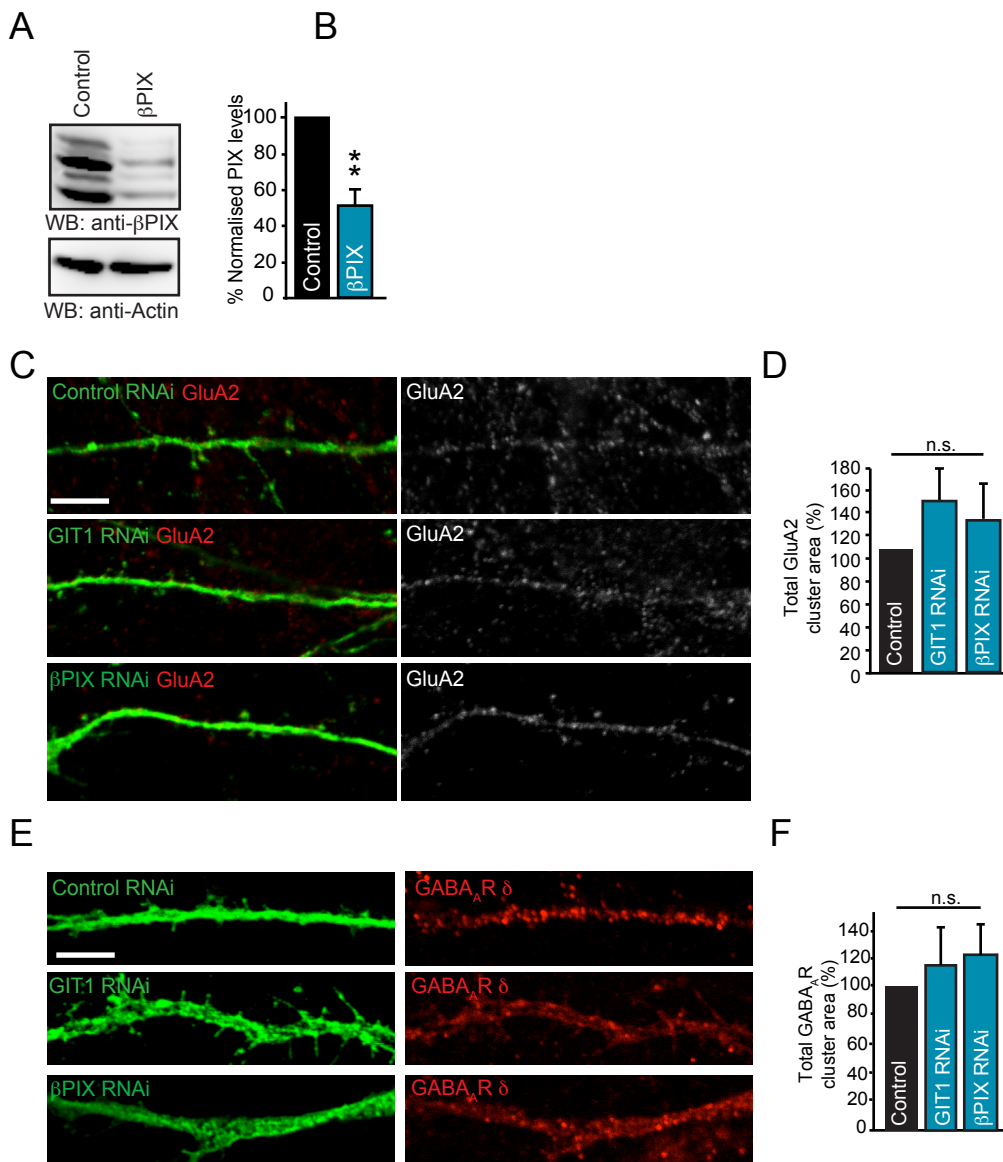
(A) Western blotting of cortical neuron lysates transfected with either GIT1 or control RNAi constructs.

(B) Summary graph of knockdown with GIT1 RNAi with GIT1 expression normalised to actin (GIT1 levels reduced to  $43.0 \pm 11.4$  % of control,  $**p=0.002$ ,  $n=4$  experiments, values are mean  $\pm$  SEM).

(C,D) Analysis of dendrite length in neurons transfected with control or GIT1 RNAi. (C) Confocal images of representative neurons, scale bar= $40\mu\text{m}$ . (D) Bar graph of dendritic length,  $**p=0.006$ ,  $n=15$  cells. Values are mean  $\pm$  SEM.

(E,F) Coexpression of human GIT1 (hGIT1) with GIT1 RNAi rescues the effect of the GIT1 knock-down surface GABA<sub>A</sub>R clusters ( $n=3$  experiments, 12-15 cells,  $***p=3 \times 10^{-5}$ ,  $n.s. p=0.089$ ). Values are mean  $\pm$  SEM.

(G,H) 30 min latrunculin-A treatment of neurons has no effect on extrasynaptic GABA<sub>A</sub>R clusters ( $p=0.68$ ,  $n=15$  cells). Values are mean  $\pm$  SEM.



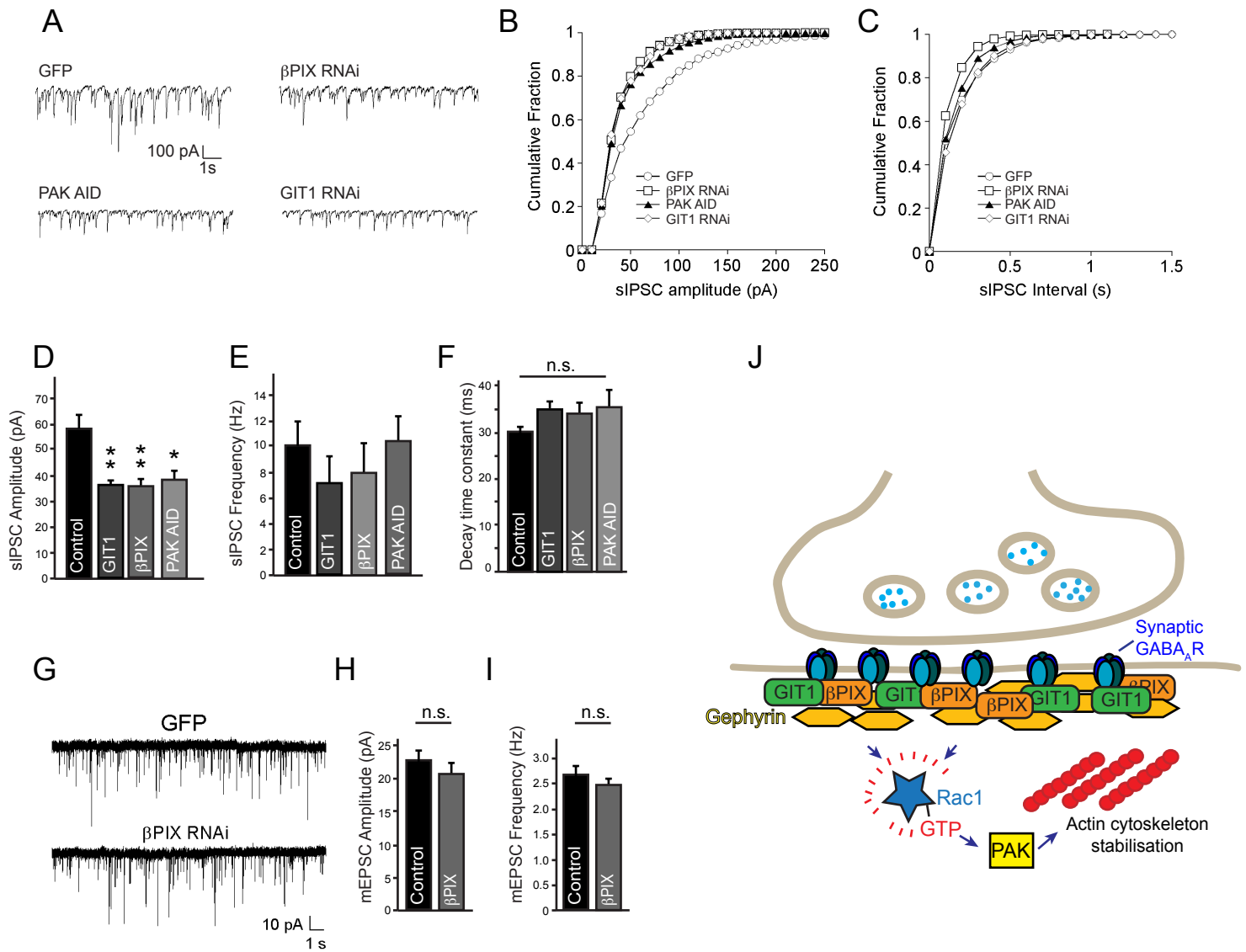
### Figure S3, Related to Figure 3; Characterisation of βPIX RNAi

(A) Western blotting of cortical neuron lysates transfected with either βPIX or control RNAi constructs.

(B) Summary graph of knockdown with βPIX RNAi with βPIX expression normalised to actin (βPIX levels reduced to  $51.3 \pm 11.4$  % of control,  $**p=0.002$ ,  $n=5$ ). Values are mean  $\pm$  SEM.

(C,D) Neurons expressing control, GIT1 or βPIX RNAi and labelled with antibodies to GluA2-AMPA receptor subunit showed no change in AMPA receptor cluster area (no significant difference,  $n=3$ , 15 cells). Values are mean  $\pm$  SEM.

(E,F) Neurons expressing control, GIT1 or βPIX RNAi and labelled with antibodies to GABA<sub>A</sub>R-δ subunit showed no change in surface δ-subunit containing receptors (no significant difference,  $n=3$ , 9-13 cells). Values are mean  $\pm$  SEM.



### Figure S4, Related to Figure 6; Effects of disrupting the GIT1/ $\beta$ PIX signalling pathway on inhibitory neurotransmission

(A-E) Whole-cell recordings of sIPSCs from neurons transfected with GIT1 RNAi,  $\beta$ PIX RNAi, GFP-PAK-AID or GFP control.

(A) Representative traces showing a reduction in sIPSC amplitude in neurons expressing GIT1 RNAi,  $\beta$ PIX RNAi or GFP-PAK-AID compared with control neurons.

(B,C) Cumulative distribution plots showing the sIPSC amplitude shifts to smaller sizes in neurons expressing GIT1 RNAi,  $\beta$ PIX RNAi or GFP-PAK-AID, whereas there is no change in sIPSC inter-event interval (C).

(D,E) Summary bar graphs showing average sIPSC amplitude and interval of transfected neurons control neurons:  $58.2 \pm 5.4$  pA, n=11, GIT1 RNAi neurons:  $36.2 \pm 1.8$  pA, n=10, p=0.003,  $\beta$ PIX RNAi neurons:  $36.0 \pm 3.0$  pA, n=11, p=0.004, PAK-AID neurons:  $38.9 \pm 3.1$  pA, n=11, p=0.009. Values are mean  $\pm$  SEM.

(F) Summary bar graph showing average decay time constants for transfected neurons (GFP control, n=27; GIT1 RNAi, n=12, p=0.003;  $\beta$ PIX RNAi, n=17, p=0.004; GFP-PAK-AID, n=19, p=0.12, non-significant). Values are mean  $\pm$  SEM.

(G) Representative mEPSC traces from neurons transfected with GFP and  $\beta$ PIX RNAi. (H,I) Summary bar graphs of average amplitude and frequency of transfected neurons, GFP control, n=8;  $\beta$ PIX RNAi, n=7, p>0.05. Values are mean  $\pm$  SEM.

(J) Schematic showing the GIT1/ $\beta$ PIX/Rac1/PAK signalling pathway at inhibitory synapses.

## **Supplemental Experimental Procedures**

### **Antibodies**

The following primary antibodies were used: rabbit anti-VGAT (Synaptic Systems (IF 1:1000)), mouse anti-GAD6 was obtained from GAD6 hybridoma cells (IF, 1:100), mouse monoclonal to GFP (Neuromab)(WB supernatant 1:10, Affinity purified 1:100), guinea pig anti- $\gamma$ 2 (serum,(Kittler et al., 2001)(IF 1:100)), rabbit anti-Myc (SantaCruz) (IP 2 1:200), mouse monoclonal to  $\beta$ 3 (supernatant (WB 1:10) and affinity purified (IF 1:100), Neuromab), mouse monoclonal anti-gephyrin mAb 7a (Connex GmbH) (IF 1:400), rabbit anti-gephyrin (Santa-Cruz) (IP 2ug), rabbit anti-gephyrin (Synaptic Systems, IF 1:500), rabbit anti-Homer (Synaptic Systems, IF 1:500), mouse anti-GIT1 (Neuromab) (WB, supernatant 1:10, IF, affinity purified 1:200), mouse anti-Rac1 (Millipore)(WB, 1:500, IF, 1:200), rabbit anti- $\beta$ PIX (Millipore)(WB and IF, 1:500), Phospho-PAK (T423E)(Cell signalling, IF, 1:500), Alexa633-labelled phalloidin (Molecular probes, IF, 1:500), mouse anti-GluA2 (Millipore, IF, 1:500),  $\delta$ -subunit antibody was a gift from T. Smart and described previously (Jones et al., 1997).

### **cDNA cloning**

MycRac1 N17 (dominant negative construct) was a gift from Aron Jaffe. The GIT1,  $\beta$ PIX and scrambled control RNAi were inserted into the pSUPER vector using previously described sequences (Osmani et al., 2006; Twelvetrees et al., 2010; Zhang et al., 2005). GFP-PAK auto-inhibitory domain (PAK-AID) was made by inserting residues 70-150 of PAK into pEGFP (Clontech). Human CFP-GIT1, PAK-CA (T423E) and GFP- $\beta$ PIX were from Addgene (Addgene plasmids 15223, 12208 and 15234). Human  $\beta$ PIX was generated by cloning the coding sequence into pDest-mCherry-N1 (Addgene plasmid

31907) using the Gateway Cloning System (Invitrogen). Mutations for the SH3 domain and dominant-negative  $\beta$ PIX mutants were introduced by performing site-directed mutagenesis as previously described (Smith et al., 2012).

### **Pharmacological treatments**

Neurons were incubated with the following compounds prior to biotinylation or immunofluorescence assays: EHT (Tocris, 100  $\mu$ M, 1 hr), Jasplakinolide (Millipore, 2 $\mu$ M, 2 hours), IPA-3 (30  $\mu$ M, 1 hour), Latrunculin-A (Tocris, 3  $\mu$ M, 30 min).

### **Neuronal transfections**

For biochemistry, cortical neurons were transfected by nucleofection (AMAXA) before plating (DIV 0) as previously described (Smith et al., 2010). For whole-cell recordings, cortical neurons were transfected by Lipofectamine 2000 and recorded 2–3 days after transfection (Yuen et al., 2011). For confocal imaging hippocampal neurons were transfected by either calcium phosphate or Lipofectamine 2000 transfection at DIV 10-11 and expressed for 2-3 days (Twelvetrees et al., 2010).

### **Slice electrophysiology**

Slices were placed in a perfusion chamber attached to the fixed stage of an upright microscope (Olympus) and submerged in continuously flowing oxygenated ACSF containing CNQX (25 $\mu$ M) and D-APV (25 $\mu$ M). Patch electrodes were filled with the following internal solution (in mM): 100 CsCl, 30 N-methyl-D-glucamine, 4 NaCl, 10 HEPES, 1 MgCl<sub>2</sub>, 5 EGTA, 2 QX314, 12 phosphocreatine, 5 MgATP, 0.2 Na<sub>3</sub>GTP, 0.2 leupeptin. A Multiclamp 700A amplifier was used for these recordings. Tight seals (2-10 G $\Omega$ ) from visualised pyramidal neurons were obtained by applying negative pressure.

The membrane was disrupted with additional suction, and the whole-cell configuration was obtained. A bipolar stimulating electrode (FHC, Bowdoinham, ME) was positioned ~100µm from the recording neuron. Membrane potential was held at -70mV. To generate the input-output responses, a series of different stimulation intensities (50-90µA) with the same duration of pulses (0.1ms) was used to elicit synaptic currents. Data analyses were performed with Clampfit (Axon instruments) and Kaleidagraph (Albeck Software).

### **Coimmunoprecipitation assays from rat brain homogenate**

Coimmunoprecipitation experiments from brain were performed as previously described (Twelvetrees et al., 2010). Briefly, adult rat brain was homogenised in pull-down buffer (50 mM HEPES pH 7.5, 0.5 % triton X-100, 150 mM NaCl, 1 mM EDTA, 1mM PMSF with antipain, pepstatin and leupeptin at 10 µg/ml) and solubilised for 2 hours. Solubilised material was ultracentrifuged at 66,000 g for 40 minutes at 4°C and the supernatant (solubilised protein) was incubated with 2 µg of antibody overnight at 4°C. To precipitate complexes, 20 µl protein-A or -G beads were added for 1 hour at 4°C. Beads were then washed extensively and bound complexes were analysed by SDS-PAGE and western blotting.

### **GST pulldown assays from transfected COS7 cells**

GST pulldown assays were performed with bacterially expressed GST-β3 intracellular domain and lysates from COS7 cells expressing FLAG-GIT1, and have previously been described (Smith et al., 2012).

### **Biotinylation assays**



Surface biotinylation assays have been fully described previously (Smith et al., 2010; Twelvetrees et al., 2010). Briefly, DIV 8-10 cortical neurons were incubated on ice with biotin solution (Sulpho-NHS-biotin(PIERCE) at 0.5 mg/ml in PBS containing  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) and quenched with quench buffer (PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$  containing 1 mg/ml BSA). Neurons were solubilised for 1 hour in RIPA buffer (50 mM Tris pH 7.5, 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, and 1 mM PMSF with antipain, pepstatin and leupeptin 10  $\mu\text{g}/\text{ml}$ ) and the lysates were then centrifuged to pellet cell debris. 15% of the supernatant was taken to use as a total protein sample and the remainder was incubated for 2h with 25  $\mu\text{l}$  Ultralink immobilized NeutrAvidin (PIERCE) 50% slurry at 4 °C to precipitate biotin labeled membrane proteins. Beads were washed three times in RIPA buffer and analysed by SDS-PAGE and western blotting. Biotinylated surface  $\text{GABA}_A\text{Rs}$  were identified by using anti- $\beta 3$  primary antibody and detection of enhanced chemilluminescence from HRP-coupled anti-rabbit secondary antibodies followed by detection with an ImageQuant LAS4000 mini imaging system and analysis with ImageQuant software (GE Healthcare).

### **Immunofluorescence and confocal microscopy**

Neurons for surface staining were fixed with PFA (4% paraformaldehyde /4% sucrose/ PBS pH 7) for 6 minutes and blocked with block solution (PBS, 10 % horse serum, 0.5 % BSA) for ten minutes at RT. Neurons were incubated for 1 hour with primary antibody followed by washing and permeabilisation with block solution containing 0.2% Triton X-100. Neurons were then incubated with a further round of primary antibody for any intracellular labelling and subsequently washed and incubated with appropriate Alexa-fluorophore conjugated secondary antibodies for 1 hour (Molecular Probes 1:1000). After extensive washing, coverslips were mounted on microscope slides using ProLong Gold

antifade reagent (Invitrogen) and sealed with nail varnish. Neurons from sister cultures were used and at least 2 sections ( $25\ \mu\text{m}$ ) of dendrites from at least 3 cells per condition from at least 3 different experiments were imaged. All images within a data set were obtained under the same conditions using a Zeiss 700 confocal microscope with a 63X oil objective (1.4 NA). Images were digitally captured using ZEN software with excitation at 488nm for GFP and Alexa-Fluor 488, 555nm for Alexa-Fluor 543 and Alexa-Fluor 568 and 633nm for Alexa-Fluor 647 and Cy5 conjugated secondary antibodies. Pinholes were set to 1 Airy unit creating an optical slice of  $0.8\ \mu\text{m}$ . Using Metamorph software (Universal Imaging Corporation), a suitable threshold was selected for each data set and applied to all images and clusters above this threshold were measured. Quantification of colocalisation was performed with 5-10 cells per experiment as described previously (Srivastava et al., 2012). ImageJ was used to generate deconvolved confocal images (NIH). Image stacks of 18 slices were acquired with voxel dimensions of  $0.056\ \mu\text{m} \times 0.056\ \mu\text{m} \times 0.25\ \mu\text{m}$ . The point spread function (PSF) for each channel was calculated using the Born and Wolf model within the PSF Generator plugin (Kirshner et al., 2013). Images were deconvolved using the Deconvolution Lab plugin (Vonesch and Unser, 2008) and the Richardson-Lucy algorithm with 10 iterations.

### **Statistical analysis**

All experiments were performed at least 3 times from different neuronal preparations. Unless otherwise stated, n numbers refer to the number of experiments performed from different preparations and number of cells analysed are stated per condition. *P*-values were calculated from two-tailed t-test unless otherwise stated. Values are given as mean  $\pm$  SEM. Error bars represent SEM.

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