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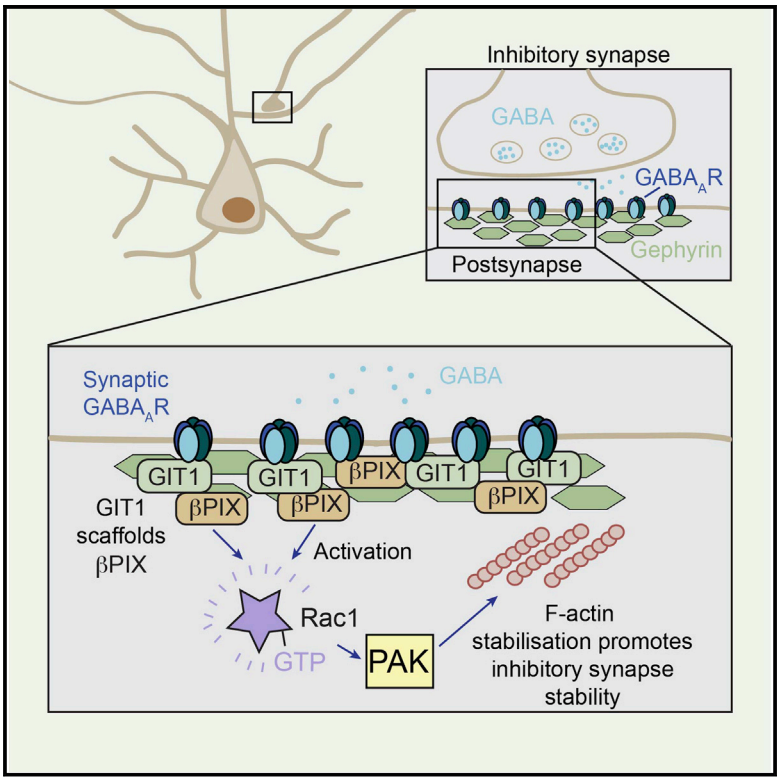
GIT1 and βPIX Are Essential for $GABA_A$ Receptor Synaptic Stability and Inhibitory Neurotransmission

Graphical Abstract



Authors

Katharine R. Smith, Elizabeth C. Davenport, ..., Zhen Yan, Josef T. Kittler

Correspondence

j.kittler@ucl.ac.uk

In Brief

Clustering of $GABA_A$ receptors at inhibitory synapses is important for maintaining the correct balance of excitation and inhibition in the brain. Smith et al. reveal a signaling mechanism at inhibitory synapses involving the scaffold GIT1, which anchors βPIX to the inhibitory synaptic site and activates Rac1 and PAK, thereby stabilizing F-actin. This signaling pathway underlies the stabilization of synaptic $GABA_A$ receptors and therefore contributes to efficient inhibitory synaptic transmission in the brain.

Highlights

GIT1 and βPIX are present at inhibitory synapses and complex with $GABA_A$Rs

GIT1 and βPIX are important for $GABA_A$R clustering and inhibitory transmission

Rac1 and PAK activity is required for stabilization of $GABA_A$Rs at synapses

A GIT1/βPIX/Rac1/PAK pathway is required for inhibitory synaptic transmission

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GIT1 and βPIX Are Essential for $GABA_A$ Receptor Synaptic Stability and Inhibitory Neurotransmission

Katharine R. Smith,[1,3] Elizabeth C. Davenport,[1] Jing Wei,[2] Xiangning Li,[2] Manavendra Pathania,[1] Victoria Vaccaro,[1] Zhen Yan,[2] and Josef T. Kittler[1,*]

[1]Department of Neuroscience, Physiology and Pharmacology, University College London, Gower Street, London WC1E 6BT, UK
[2]Department of Physiology and Biophysics, State University of New York at Buffalo, School of Medicine and Biomedical Sciences, Buffalo, NY 14214, USA
[3]Present address: Department of Physiology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA
*Correspondence: j.kittler@ucl.ac.uk
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SUMMARY

Effective inhibitory synaptic transmission requires efficient stabilization of $GABA_A$ receptors ($GABA_A$Rs) at synapses, which is essential for maintaining the correct excitatory-inhibitory balance in the brain. However, the signaling mechanisms that locally regulate synaptic $GABA_A$R membrane dynamics remain poorly understood. Using a combination of molecular, imaging, and electrophysiological approaches, we delineate a GIT1/βPIX/Rac1/PAK signaling pathway that modulates F-actin and is important for maintaining surface $GABA_A$R levels, inhibitory synapse integrity, and synapse strength. We show that GIT1 and βPIX are required for synaptic $GABA_A$R surface stability through the activity of the GTPase Rac1 and downstream effector PAK. Manipulating this pathway using RNAi, dominant-negative and pharmacological approaches leads to a disruption of $GABA_A$R clustering and decrease in the strength of synaptic inhibition. Thus, the GIT1/βPIX/Rac1/PAK pathway plays a crucial role in regulating $GABA_A$R synaptic stability and hence inhibitory synaptic transmission with important implications for inhibitory plasticity and information processing in the brain.

INTRODUCTION

$GABA_A$ receptors ($GABA_A$Rs) are essential mediators of inhibitory neurotransmission in the central nervous system and are critical for maintaining the correct balance of excitation and inhibition in the brain (Smith and Kittler, 2010). GABAergic synapses undergo extensive synaptic plasticity that alters the strength and efficacy of synaptic inhibition (Luscher et al., 2011a). Inhibitory synapse strength can be rapidly controlled by changing the number of $GABA_A$Rs in the postsynaptic domain, which is achieved by receptor insertion into and removal from the plasma membrane at extrasynaptic sites and by dynamic movements of $GABA_A$Rs to and from the synapse via lateral diffusion in the plasma membrane (Arancibia-Cárcamo et al., 2009; Bannai et al., 2009; Muir et al., 2010; Smith et al., 2012; Twelvetrees et al., 2010). However, the molecular mechanisms and regulatory signaling pathways that locally control $GABA_A$R surface levels and synaptic stability remain unclear.

The stabilization of synaptic $GABA_A$Rs opposite GABAergic presynaptic terminals is crucial for efficient synaptic inhibition, circuit excitability, and animal behavior (Blundell et al., 2009; Crestani et al., 1999; Papadopoulos et al., 2007). $GABA_A$R clustering is mediated by a complex inhibitory postsynaptic density, the major constituent of which is the hexameric scaffold, gephyrin (Fritschy et al., 2008). However, in the absence of gephyrin, subsets of inhibitory synapses remain (Essrich et al., 1998; Kneussel et al., 1999), suggesting the existence of other inhibitory synaptic scaffold molecules. The inhibitory postsynaptic specialization also contains key adhesion molecules such as neuroligin 2 and Slitrk3 (Takahashi et al., 2012; Varoqueaux et al., 2004), in addition to cytoskeletal-associated proteins, which together contribute to controlling the formation and stabilization of GABAergic synapses. Interestingly, several filamentous actin (F-actin) regulatory proteins have been associated with the inhibitory postsynaptic density and gephyrin (Luscher et al., 2011a), suggesting a potential role for the actin cytoskeleton at inhibitory synapses. However, little is known regarding the regulatory signaling scaffolds that can act locally to coordinate cytoskeletal dynamics to tune $GABA_A$R synaptic stability and synaptic inhibition.

The Rho family of small GTPases—Rho, Rac, and Cdc42—and their regulators play essential roles in modulating actin dynamics and are increasingly implicated in synaptic pathology and neurological dysfunction. The activation state of small GTPases is determined by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), which together control GTP-GDP exchange and thereby promote GTPase activation and inactivation, respectively (Nobes and Hall, 1999). Local regulation of GTPase signaling can be further controlled by subcellular compartmentalization of GEFs and GAPs determined by protein scaffolds (Kiraly et al., 2010). Currently the key GTPases, regulatory GEFs and signaling scaffolds acting to regulate $GABA_A$R trafficking and inhibitory transmission are poorly understood.

In this study, we have identified a GIT1/βPIX/Rac1/PAK signaling complex that is important for maintaining $GABA_AR$ surface clusters and synaptic inhibition in neurons. Using a combination of imaging, biochemical, and electrophysiological approaches, we show that the signaling scaffold protein GIT1 (G protein-coupled receptor kinase-interacting protein 1), which interacts with the Rac1 GEF βPIX, forms complexes with $GABA_ARs$ and is essential for normal $GABA_AR$ clustering. Furthermore, we demonstrate that downstream Rac1 activity is also crucial to maintain inhibitory synapse stability and works in concert with the key Rac1 effector and actin-regulator, PAK. We find that Rac1 activity stabilizes $GABA_ARs$ at inhibitory synapses while disrupting GIT1, Rac1 or PAK all lead to impaired inhibitory transmission. Thus GIT1 and βPIX, in complex with $GABA_ARs$, play a key role in locally coordinating Rac1 and downstream effector activity to regulate $GABA_AR$ surface stability and inhibitory synapse strength.

RESULTS

The GIT1/βPIX Complex Is Localized at Inhibitory Synapses and Forms Complexes with Synaptic $GABA_ARs$

The signaling scaffolds that regulate postsynaptic $GABA_AR$ stability in neurons remain largely unknown. GIT1 is a signaling scaffold that can recruit βPIX, a GEF for the small GTPase Rac1, to locally control the activation of Rac1 (Zhang et al., 2005). GIT1 has been localized to synaptic sites in neurons but its potential association with $GABA_ARs$ and role in regulating signaling in the inhibitory postsynaptic domain remains unstudied. We hypothesized that GIT1 and βPIX might control Rac1 signaling at inhibitory synapses and are important for $GABA_AR$ clustering and inhibitory synaptic transmission. We initially determined if GIT1 and βPIX were localized at inhibitory synapses using immunocytochemistry and confocal laser scanning microscopy (CLSM) of hippocampal neurons. Neurons labeled with antibodies to GIT1, VGAT (Vesicular GABA transporter) to label inhibitory presynaptic terminals, and the γ2 $GABA_AR$ subunit to label synaptic $GABA_AR$ clusters showed GIT1 was distributed along dendrites and exhibited colocalization with synaptic $GABA_AR$ clusters (Figure 1A; Figure S1A). Approximately 55% of GIT1 localized at inhibitory synapses, whereas ~78% of synaptic $GABA_AR$ clusters colocalized with GIT1. In addition, GIT1 is known to be at excitatory synapses (Zhang et al., 2003), which we confirm by showing that the excitatory synapse marker, homer, demonstrates ~60% colocalization with GIT1 puncta (Figure 1B). GIT1 and βPIX are known to form supramolecular signaling platforms and are consistently found in a tight signaling complex in many cell types (Premont et al., 2004; Schlenker and Rittinger, 2009). We therefore imaged neurons labeled with GIT1, βPIX, and γ2 $GABA_AR$ antibodies, showing that ~79% of inhibitory synapses colocalized with βPIX and thereby verifying the localization of both GIT1 and βPIX at inhibitory synapses (Figure 1C). Interestingly, GIT1 localizes with synaptic $GABA_ARs$ (γ2, β3, α2 subunits, Figures S1B and S1C) but showed little overlap with extrasynaptic $GABA_ARs$ (δ subunits, Figures S1D and S1E).

We also demonstrated that $GABA_AR$ β3 and γ2 subunit antibodies readily coimmunoprecipitate GIT1 or βPIX from rat brain lysate as analyzed by western blotting (Figures 1D–1F), confirming that GIT1 and βPIX can form native complexes with synaptic $GABA_ARs$ in vivo. GIT1 did not interact in a complex with extrasynaptic δ subunits (Figure S1F). We also found exogenous FLAG-GIT1 to interact with the intracellular domain of the $GABA_AR$-β3 subunit, by COS7 cell pull-down assays (Figure S1G), supporting GIT1's interaction with $GABA_ARs$. Coimmunoprecipitation of GIT1 with the inhibitory postsynaptic scaffold gephyrin confirmed this postsynaptic localization (Figure 1G), although our data suggest that this interaction is indirect, as demonstrated by lack of coimmunoprecipitations in transfected COS7 cells (Figure S1H). As an alternative approach for further validating $GABA_AR$ and βPIX complexes in neuronal dendrites, we performed proximity ligation assays (PLAs), which provide valuable information about native protein interactions in situ (Ko et al., 2012). Using PLAs, we demonstrate that βPIX complexes with $GABA_ARs$ in neuronal dendrites (Figures 1H and 1I). Furthermore, we show that GIT1 and gephyrin also interact in situ via PLAs (Figures S1J and S1K), providing additional evidence to support an inhibitory postsynaptic and close association for these proteins in dendrites.

GIT1, βPIX, and F-Actin Regulation Are Important for Maintaining Surface $GABA_AR$ Levels

To investigate the role of GIT1 at inhibitory synapses, we utilized RNAi to knock down its protein expression in neurons. RNAi caused a significant reduction of GIT1 expression levels in addition to causing a small reduction in dendrite length as previously shown (Figures S2A–S2D; Menon et al., 2010). To determine the consequences of GIT1 knockdown on inhibitory synapse and surface $GABA_AR$ cluster area, we performed immunocytochemistry and CLSM of neurons expressing GIT1 or control RNAi constructs, using an extracellular γ2 subunit antibody to label surface $GABA_ARs$ and antibodies to VGAT to identify inhibitory synapses. GIT1 knockdown neurons exhibited a significant decrease in surface $GABA_AR$ and VGAT cluster area compared to control (Figures 2A–2C), suggesting a possible role for GIT1 in maintaining the integrity of inhibitory pre- and postsynaptic domains in neuronal dendrites. Importantly, this effect could be rescued by coexpression of RNAi-resistant human GIT1 (hGIT1; Figures S2E and S2F). GIT1 knockdown also caused a large decrease in gephyrin cluster area (Figures 2D and 2E), suggesting that GIT1 is important for maintaining both $GABA_AR$ clusters and the gephyrin scaffold in neurons. This was further confirmed by surface biotinylation assays, which revealed that surface $GABA_AR$ levels were reduced in GIT1 knockdown neurons compared with control (Figures 2F and 2G).

Considering its role in other cell types, we hypothesized that GIT1 may be important for localizing F-actin regulatory pathways to inhibitory synapses. Therefore, we sought to determine whether surface $GABA_ARs$ were sensitive to short-term disruption of the actin cytoskeleton by treating neurons with latrunculin-A, an inhibitor of actin polymerization (Renner et al., 2009). We found that a 30 min application of 3 μM latrunculin-A to neurons caused a significant decrease in surface $GABA_ARs$ (Figures 2H and 2I), with no effect on extrasynaptic $GABA_AR$ populations (Figures S2G and S2H), suggesting that actin polymerization does indeed play an important role in the maintenance of

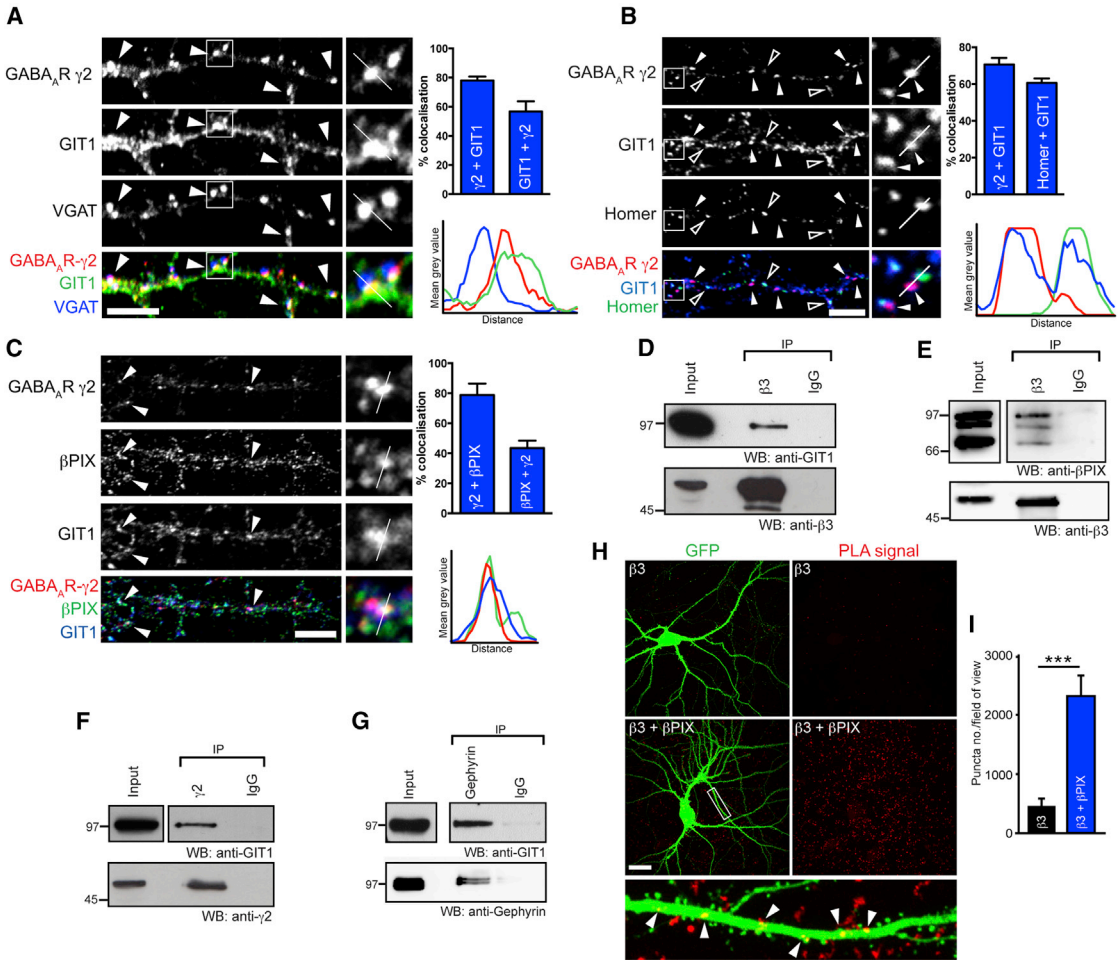

Figure 1. GABA$_A$Rs Form Complexes with GIT1 and βPIX in Neurons

(A–C) CLSM of neurons labeled with antibodies to GABA$_A$R-γ2 (red) and (A) VGAT (blue) and GIT1 (green), (B) GIT1 (blue) and homer (green) or (C) βPIX (green) and GIT1 (blue). Arrowheads, colocalization; scale bar represents 5 μm. Bar graphs summarize colocalization quantification (n = 5–10 cells). Example line scans through clusters show localization of GIT1 to inhibitory synapses (A and C) and excitatory synapses (B). Values are mean ± SEM.

(D–G) Western blots of coimmunoprecipitation assays of GABA$_A$Rs (β3 and γ2 subunits) and gephyrin, with GIT1 and βPIX from rat brain homogenate (WB, western blot; IP, immunoprecipitation).

(H and I) Proximity ligation assay of neurons with antibodies to GABA$_A$R-β3 and βPIX in situ, (n = 3). Scale bar represents 20 μm. Values are mean ± SEM.

See also Figure S1.

inhibitory synapses. We then asked whether the inhibitory synapse effects we observed upon knockdown of GIT1 were due to altered F-actin regulation. We therefore treated control or GIT1 RNAi-expressing neurons with the F-actin-stabilizing drug, jasplakinolide (Hering et al., 2003), prior to surface biotinylation and western blot analysis (Figure 2J). As predicted, GIT1 RNAi caused a significant loss of surface GABA$_A$Rs compared to control, which was restored by treatment with jasplakinolide (Figures 2J and 2K). This suggests that the decrease in surface GABA$_A$Rs observed in GIT1-deficient neurons is caused by impaired F-actin regulation, and points toward a mechanism involving actin-regulatory proteins.

GIT1's primary binding partner βPIX is one such actin-regulatory protein and is a strong candidate to collaborate with GIT1 in mediating actin regulation at inhibitory synapses. To test this hypothesis, we utilized RNAi to βPIX, which caused a significant reduction of βPIX expression levels (Figures S3A and S3B). βPIX knockdown in neurons caused a similar effect to that of GIT1 RNAi, reducing both surface GABA$_A$Rs and VGAT cluster area (Figures 3A–3C). Surface biotinylation assays revealed the same phenotype, with βPIX knockdown neurons exhibiting reduced surface GABA$_A$R levels compared with control cells (Figures 3D and 3E). We found GIT1 or βPIX knockdown had no effect on AMPA receptor clustering or extrasynaptic δ- containing GABA$_A$Rs (Figures S3C–S3F), suggesting that this protein complex is important for synaptic GABA$_A$R clustering only. We then sought to determine whether GIT1 and βPIX are important in controlling actin regulation at inhibitory synapses. We treated control neurons with phalloidin to label F-actin and found that ~80% of inhibitory synapses were positive for

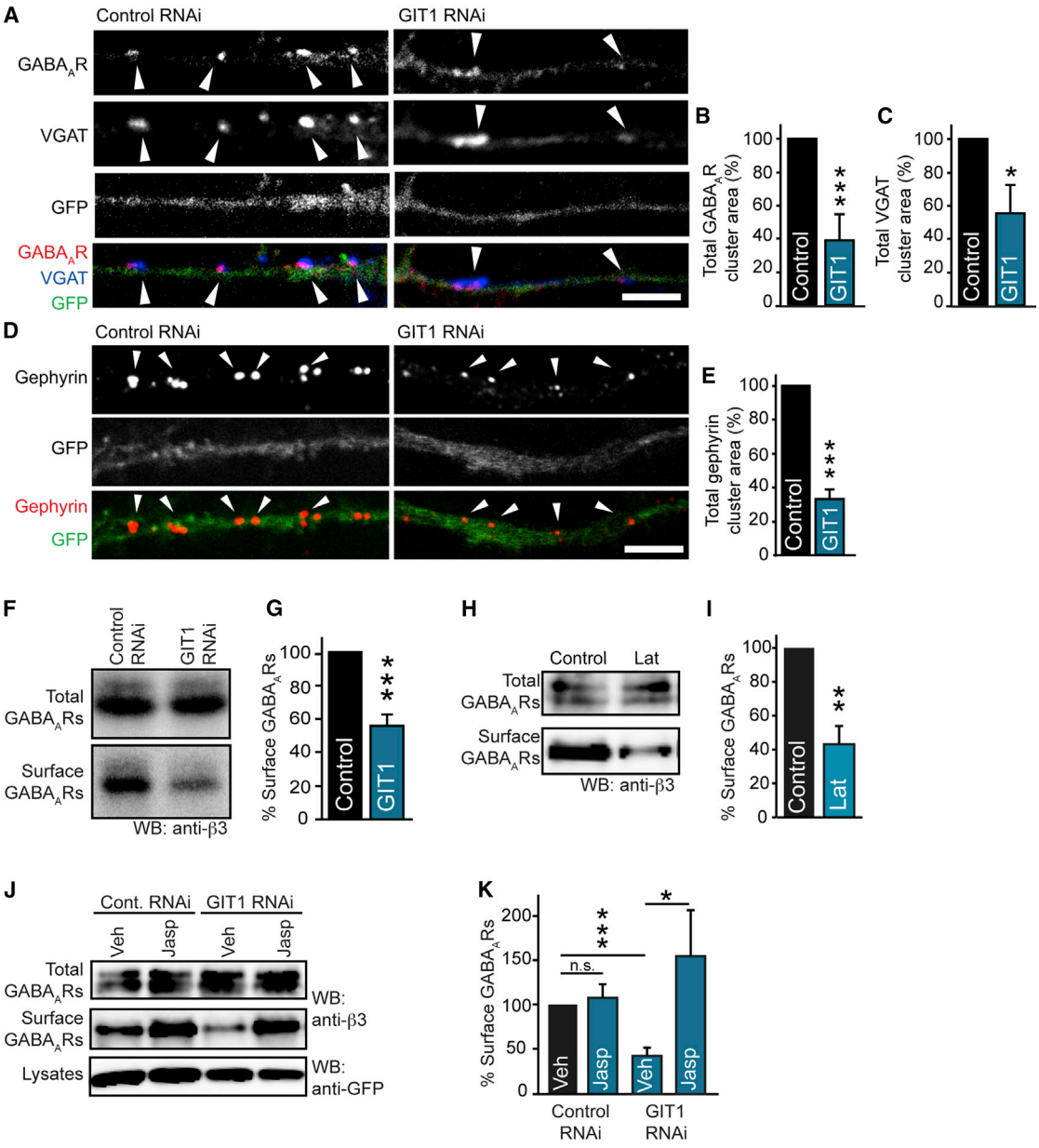

Figure 2. GIT1 Knockdown Alters GABA$_A$R Surface Stability

(A) Confocal images of GIT1 or control RNAi-transfected neurons (green) labeled with antibodies to GABA$_A$R-γ2 (red) and to VGAT (blue). Arrowheads, GABA$_A$R clusters. Scale bar represents 5 μm.

(B and C) Bar graphs of GABA$_A$R and VGAT cluster area showing a reduction to 39.3% ± 13.6% and 56.7% ± 13.3% of control (***p = 0.0008 and *p = 0.03, n = 3, ten cells). Values are mean ± SEM.

(D) Confocal images of control and GIT1 RNAi-transfected neurons (green) immunostained with antibodies to gephyrin (red). Arrowheads, gephyrin clusters. Scale bar represents 5 μm.

(E) Bar-graph of gephyrin cluster area showing a 67.0% ± 5.6% decrease compared with control (***p = 0.0003, n = 5, 20 cells). Values are mean ± SEM.

(F and G) Expression of GIT1 RNAi reduces surface expression of GABA$_A$Rs to 55.9% ± 6.0% of control as assayed by surface biotinylation and western blotting with anti-GABA$_A$R-β3 (***p = 2.6 × 10^{-5}, n = 5). Values are mean ± SEM.

(H) Surface biotinylations of neurons treated with 3 μM latrunculin-A for 30 min and analyzed by western blotting with anti-GABA$_A$R-β3.

(I) Bar graph showing reduction in surface GABA$_A$Rs on treatment with latrunculin-A (**p = 0.004, n = 3). Values are mean ± SEM.

(J) Surface biotinylations of control or GIT1 RNAi neurons treated with 2 μM jasplakinolide or vehicle and analyzed by western blotting with GABA$_A$R-β3 antibodies. Lysates were probed with GFP antibodies to show transfection (lower panel).

(K) Summary bar graphs showing a 57.2% ± 5.8% reduction in surface GABA$_A$Rs in neurons transfected with GIT1 RNAi (***p = 0.0002, n = 5); however, treatment with jasplakinolide prevented this reduction (*p = 0.047, n = 5). Values are mean ± SEM.

See also Figure S2.

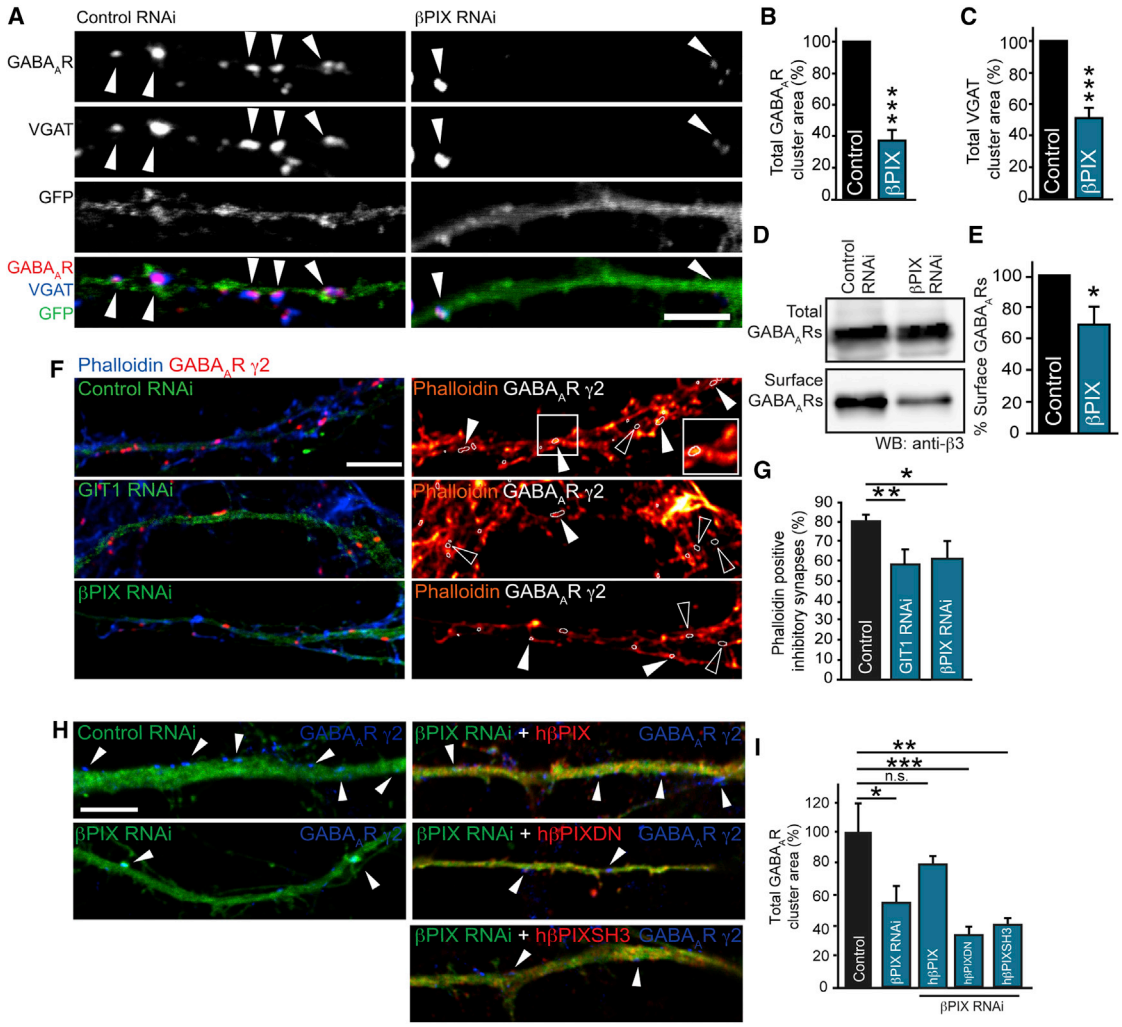

Figure 3. The Rac1 Activator βPIX Is Essential for GABA$_A$R Surface Stability

(A) Confocal images of control and βPIX RNAi-transfected neurons (green), labeled with antibodies to GABA$_A$R-γ2 (red) and to VGAT (blue). Arrowheads, GABA$_A$R clusters. Scale bar represents 5 μm.

(B and C) Bar graphs of GABA$_A$R and VGAT cluster area showing a reduction to 37.5% ± 5.9% and 49.2% ± 5.4% of control on knockdown of βPIX (***p = 0.0009 in B, ***p = 0.0006 in C, n = 5, 18 cells). Values are mean ± SEM.

(D and E) βPIX RNAi reduces surface expression of GABA$_A$Rs to 65.3% ± 13.0% of control as assayed by surface biotinylation of neurons and western blotting with anti-GABA$_A$R-β3 (*p = 0.04, n = 4). Values are mean ± SEM.

(F and G) Neurons transfected with control, GIT1 and βPIX RNAi were labeled with antibodies to the GABA$_A$R-γ2 (red) and Alexa-647-conjugated Phalloidin to label F-actin. Scale bar represents 5 μm. (G) The percentage of synaptic phalloidin-positive GABA$_A$R clusters were reduced in GIT1 and βPIX knockdown neurons compared with control (*p = 0.028, **p = 0.005, n = 3, seven to nine cells). Values are mean ± SEM.

(H and I) Coexpression of RNAi resistant βPIX (hβPIX) with βPIX RNAi rescues the effect of the βPIX knockdown surface GABA$_A$R clusters (n = 4, 18–22 cells, one-way ANOVA ***p = 0.0001). However, dominant-negative (hβPIXDN) or SH3 domain mutants (hβPIXSH3) are unable to rescue these effects. Values are mean ± SEM. Scale bar represents 5 μm.

See also Figure S3.

F-actin. GIT1 and βPIX knockdown caused a significant decrease in the percentage of inhibitory synapses containing F-actin (Figures 3F and 3G), suggesting that GIT1 and βPIX have an important role in controlling F-actin at inhibitory synapses. The effect of βPIX knockdown on surface GABA$_A$R clusters was rescued by RNAi resistant human βPIX (hβPIX) showing the specificity of the RNAi. In contrast, versions of hβPIX that no longer have GEF activity for Rac1 (hβPIX-DN), or that contain a mutation of the βPIX SH3 domain (which is critical for coupling to a downstream effector, PAK [Hoelz et al., 2006]), were unable to rescue βPIX knockdown induced GABA$_A$R declustering (Figures 3H and 3I). This suggests that the ability of βPIX to activate Rac1 and interact with PAK is essential for its role in maintaining inhibitory synapses and supports the idea that there exists a key actin regulatory mechanism controlling inhibitory synapse maintenance.

Rac1 Activity Maintains Surface $GABA_AR$ Levels

GIT1 can anchor βPIX, which directly interacts with the small GTPase Rac1 and mediates its activation (ten Klooster et al., 2006). Because we could not rescue the effects of βPIX knockdown with a version of βPIX with impaired GEF activity toward Rac1, we hypothesized that Rac1 might be responsible for the changes in $GABA_AR$ clustering in GIT1 or βPIX knockdown neurons. We verified that Rac1 was localized to synaptic $GABA_AR$ clusters in neurons using immunocytochemistry with antibodies to Rac1, the γ2 $GABA_AR$ subunit and VGAT, followed by CLSM (Figure 4A). We found that ~45% of Rac1 colocalized with synaptic $GABA_AR$ clusters along the dendritic shaft (Figure 4B), suggesting that Rac1-positive signaling complexes may influence synaptic $GABA_AR$ trafficking or function.

To test whether Rac1 activity has a role in maintaining synaptic $GABA_AR$ clustering, we utilized a dominant-negative Rac1 mutant (Nobes and Hall, 1999) (Rac1-DN) to block Rac1 activation in neurons. Immunostaining and CLSM of hippocampal neurons transfected with GFP or Rac1-DN revealed a decrease in surface $GABA_AR$ cluster area and VGAT area in neurons expressing Rac1-DN compared with control neurons (Figures 4C–4E). In addition, surface biotinylation assays revealed that blocking Rac1 activation with the Rac1-DN caused a decrease in $GABA_AR$ surface levels (Figures 4F and 4G).

As an alternative approach, we determined if acute short-term inhibition of Rac1 would cause similar effects on $GABA_AR$ surface levels. To achieve this, we incubated neurons for 1 hr with the Rac1 inhibitor EHT 1864 (EHT; Shutes et al., 2007), followed by immunocytochemistry and CLSM. Similar to the results with Rac1-DN, analysis of these neurons showed that acute inhibition of Rac1 activity reduced the area of $GABA_AR$ and gephyrin clusters (Figures 4H–4J). Moreover, surface biotinylation assays with neurons treated with EHT caused a decrease in surface $GABA_ARs$ (Figures 4K and 4L), indicating that Rac1 contributes to GABAergic synapse stability in neurons.

PAK Activation Is Important for $GABA_AR$ Surface Stability

We then wanted to explore the mechanism downstream of active Rac1 leading to the stabilization of $GABA_AR$ clusters. PAK is a major effector of Rac1 that modulates F-actin to stabilize essential cellular structures (Kreis and Barnier, 2009). We hypothesized that Rac1 might mediate its action at inhibitory synapses by activating PAK, which is supported by our observation that an SH3-domain mutant of βPIX (which can no longer couple to PAK) is unable to rescue the effects of βPIX RNAi on $GABA_AR$ clusters (Figures 3H and 3I).

Active PAK is autophosphorylated; therefore, we tested whether we could detect phospho-PAK at inhibitory synaptic sites by performing immunocytochemistry and CLSM of neurons labeled with antibodies to $GABA_AR$ γ2 subunit, the inhibitory presynaptic marker GAD6 and phospho-PAK (Figure 5A). Active PAK was found clustered along dendrites and colocalized with both synaptic $GABA_AR$ clusters and GAD6, confirming its presence at inhibitory synapses. To determine whether PAK activity is important for maintaining $GABA_AR$ and gephyrin cluster stability, we incubated hippocampal neurons with IPA (Deacon et al., 2008), a specific PAK inhibitor and assessed $GABA_AR$ γ2, gephyrin and VGAT cluster area. Treatment with IPA caused a substantial decrease in $GABA_AR$ and gephyrin cluster area, with little effect on VGAT cluster area (Figures 5B–5E). Surface biotinylation assays showed a decrease in surface $GABA_AR$ expression in neurons treated with IPA compared with vehicle-treated neurons (Figures 5F and 5G), showing that PAK activity is required for maintaining surface $GABA_ARs$ in neurons. To further verify a role for PAK in controlling $GABA_AR$ surface stability, we utilized the autoinhibitory domain (AID) of PAK fused to GFP, which has widely been used to inhibit PAK activity in culture by blocking its autophosphorylation (Kreis and Barnier, 2009). Surface biotinylation of neurons transfected with GFP or GFP-PAK-AID revealed that GFP-PAK-AID expression caused reduced surface $GABA_AR$ expression compared with control (Figures 5H and 5I). To confirm that PAK acts downstream of GIT1 and βPIX at inhibitory synapses, we performed rescue experiments with neurons cotransfected with GIT1 or βPIX RNAi and a constitutively active (CA) mutant of PAK. Cotransfection with PAK-CA effectively prevented the depletion of $GABA_AR$ clusters observed with GIT1 or βPIX RNAi alone (Figures 5J and 5K), suggesting that PAK acts downstream of GIT1 and βPIX to control inhibitory synapse maintenance in neurons. Together, these data suggest that a GIT1/βPIX/Rac1/PAK signaling pathway plays an important role in stabilizing $GABA_AR$ and gephyrin clusters and the maintenance of inhibitory synapses.

Inhibitory Synaptic Transmission Is Dependent on the Activity of GIT1/βPIX, Rac1, and PAK

Our results suggest that components of a signaling pathway involving GIT1, βPIX, Rac1, and PAK are critical for stabilizing surface and synaptic $GABA_ARs$ and maintaining GABAergic synapse integrity in neurons. We then asked whether this pathway directly affects GABAergic transmission in neurons. Whole-cell patch-clamp recordings were performed to measure inhibitory synaptic transmission in neurons expressing the GIT1 RNAi, βPIX RNAi or GFP-PAK AID constructs. Analysis of spontaneous IPSC (sIPSC) traces from these cells revealed that GIT1 or βPIX knockdown, or inhibition of PAK all caused a considerable decrease in sIPSC amplitude compared to control neurons (Figures S4A–S4E). These reduced amplitudes can be seen in representative sIPSC traces (Figure S4A) and the leftward shift to smaller amplitudes in cumulative probability plots (Figures S4B and S4D). Analysis of these data showed there was no significant change in the sIPSC frequency (Figures S4C and S4E).

To further explore the impact of inhibiting the GIT1 signaling pathway, we measured the impact on miniature IPSCs (mIPSCs). We recorded mIPSCs from neurons transfected with control or GIT1 RNAi (Figures 6A–6E). Analysis of traces from these neurons showed that knockdown of GIT1 caused a significant decrease in both mIPSC amplitude and frequency, which could be rescued with RNAi resistant hGIT1, again showing the specificity of the RNAi knockdown. We also recorded mIPSCs from neurons transfected with βPIX RNAi and PAK-AID, analysis of which demonstrated a similar reduction in mIPSC amplitude and frequency as that of GIT1 RNAi-expressing neurons (Figures 6A, and 6C–6E). The effects of the βPIX RNAi were successfully rescued by coexpression with hβPIX, and also by PAK-CA, suggesting that PAK indeed mediates the effects of βPIX on

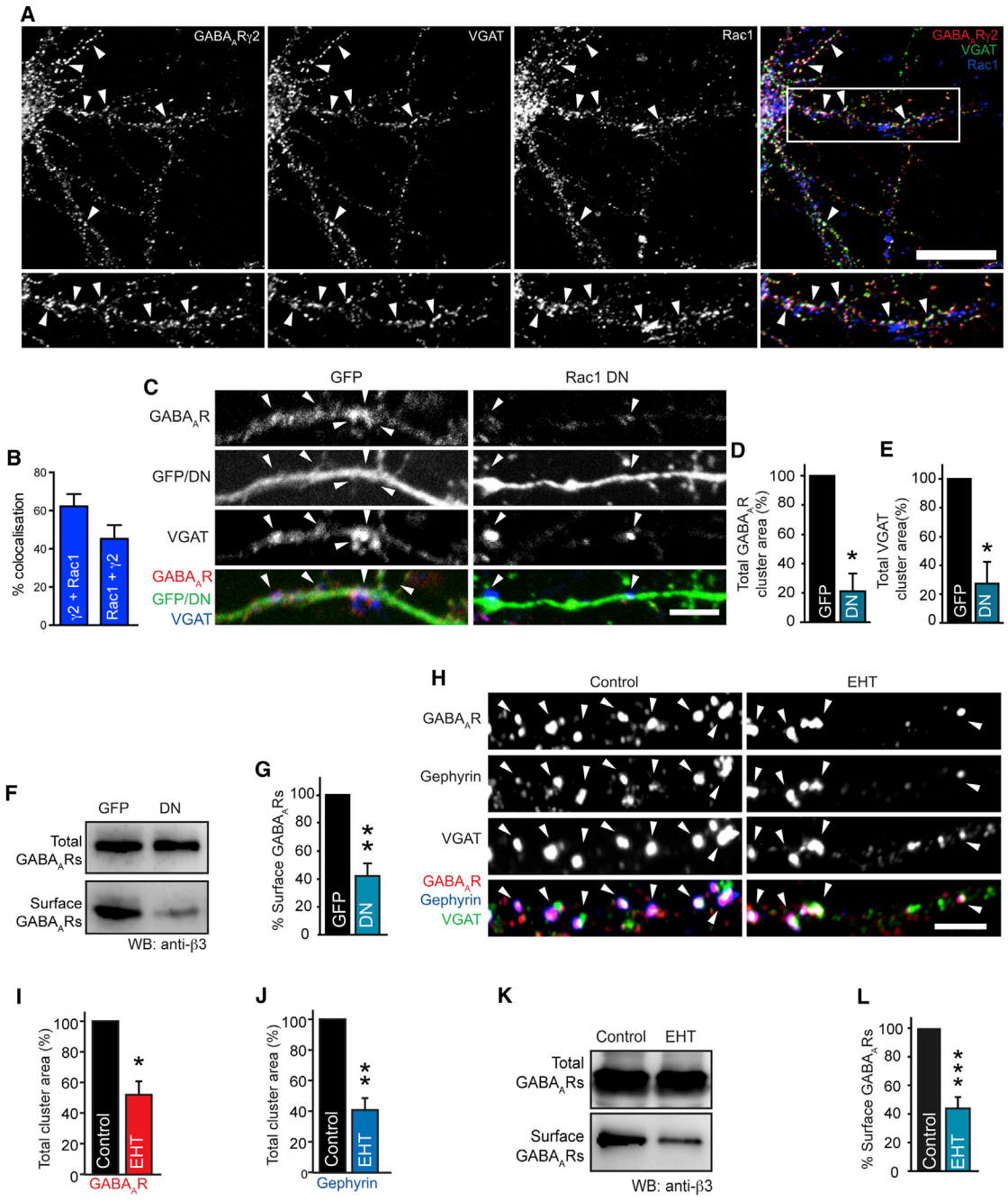

Figure 4. Rac1 Is Localized at Inhibitory Synapses and Maintains Surface GABA$_A$R Stability

(A) Neurons were labeled with antibodies to Rac1 (blue), GABA$_A$R-γ2 (red), and VGAT (green). Scale bar represents 10 μm. Rac1 colocalizes (white) with synaptic GABA$_A$R clusters and VGAT in neurons (arrowheads).

(B) Bar graph of colocalization analysis of GABA$_A$R-γ2 and Rac1 in neurons. Values are mean ± SEM.

(C) Confocal images of GFP or Rac1 dominant-negative (DN) -expressing neurons (green) immunostained with antibodies to the GABA$_A$R-γ2 (red) and VGAT (blue). Scale bar represents 5 μm.

(D and E) Summary bar graphs showing expression of Rac1 DN causes a decrease in surface GABA$_A$R and VGAT cluster area to 20.92% ± 12.2% and 27.28% ± 13.5% of control (*p = 0.02 in D, *p = 0.03 in E, n = 3, eight cells). Values are mean ± SEM.

(F and G) Neurons expressing GFP or Rac1 DN were surface biotinylated and analyzed by western blotting with anti-GABA$_A$R-β3, revealing a significant decrease in surface GABA$_A$Rs to 43.8% ± 9.0% of control (**p = 0.005, n = 4). Values are mean ± SEM.

(H) Confocal images of neurons were treated with EHT and labeled with antibodies to GABA$_A$R-γ2 (red) and VGAT (green) and gephyrin (blue). Scale bar represents 5 μm.

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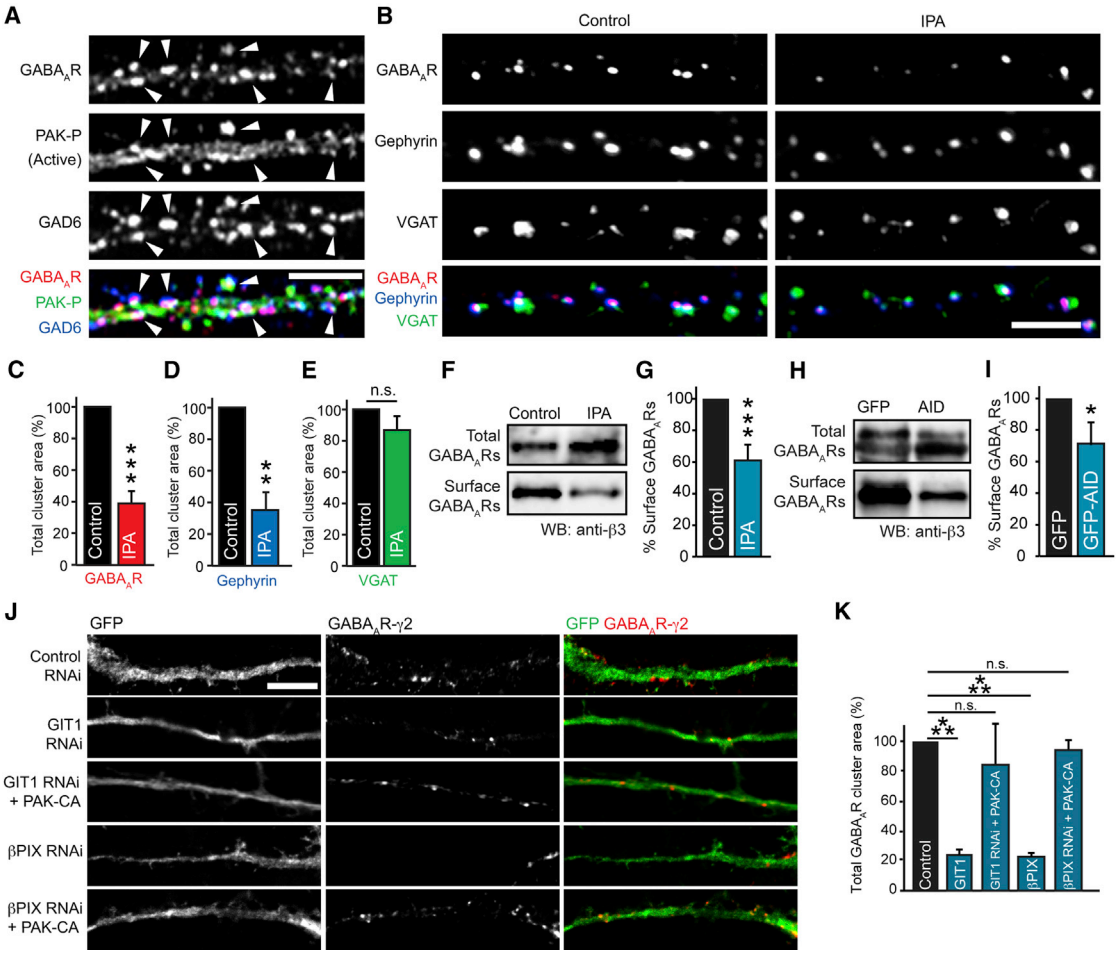

Figure 5. PAK Activation Is Important for GABA$_A$R Surface Stability

(A) Neurons labeled with antibodies to phospho-PAK (PAK-P; green), GABA$_A$R-γ2 (red) and the inhibitory presynaptic marker, GAD6 (blue). Scale bar represents 5 μm. Arrowheads, colocalization.

(B) Neurons labeled with antibodies to the GABA$_A$R-γ2 (red), gephyrin (blue), and VGAT (green) treated with IPA or vehicle. Scale bar represents 5 μm.

(C–E) Summary bar graphs showing that inhibition of PAK with IPA causes a 60.8% ± 7.2% decrease in GABA$_A$R cluster area, a 65.0% ± 9.3% decrease in gephyrin cluster area (***p = 0.0009, **p = 0.003, n = 3, 10–12 cells), and no change in VGAT cluster area. Values are mean ± SEM.

(F and G) Surface biotinylation of neurons treated with IPA or vehicle, followed by western blotting with anti-GABA$_A$R-β3, demonstrates a 41.0% ± 8.0% decrease in surface GABA$_A$Rs (***p = 0.0003, n = 7). Values are mean ± SEM.

(H and I) Surface biotinylation of neurons expressing GFP or GFP-PAK-AID reveals a 28.8% ± 12.0% decrease in surface GABA$_A$Rs (*p = 0.03, n = 6). Values are mean ± SEM.

(J) Expression of constitutively active PAK (PAK-CA) rescues the effects of GIT1/βPIX knockdown on surface GABA$_A$Rs. Representative images of transfected neurons (green) labeled with antibodies to the GABA$_A$R-γ2 (red). Scale bar represents 5 μm.

(K) Summary bar graphs of average GABA$_A$R cluster area showing significant decreases in surface GABA$_A$Rs in GIT1 and βPIX knockdown neurons, but not knockdown neurons cotransfected with PAK-CA (***p = 0.0003, n = 3, 9–15 cells). Values are mean ± SEM.

GABAergic synaptic transmission. Importantly, there was no significant difference between the decay time constants of the events recorded from neurons expressing GIT1 RNAi, βPIX RNAi, or PAK-AID compared with control neurons, indicating that the receptor properties are unaltered (Figure S4F). In addition, βPIX RNAi expression had no significant effect on miniature excitatory postsynaptic currents (Figures S4G–S4I), suggesting that inhibition of this pathway under these conditions has specific effects on inhibitory synaptic transmission.

To determine the impact of inhibiting this pathway on GABAergic transmission in an intact network, we also performed patch-clamp recordings from pyramidal neurons in cortical

(I and J) Analysis of cluster area reveals decreased GABA$_A$R (to 49.9% ± 8.2% of control, **p = 0.006, n = 4, 17 cells) and gephyrin (to 39.7% ± 7.7% of control, **p = 0.001, n = 4, 17 cells) cluster area. Values are mean ± SEM.

(K and L) Surface biotinylation of neurons treated with 100 μM EHT or vehicle for 1 hr and analyzed by western blotting with anti-GABA$_A$R-β3 demonstrates a 62.1% ± 6.7% decrease in surface GABA$_A$Rs (***p = 9.0 × 10^{-5}, n = 7). Values are mean ± SEM.

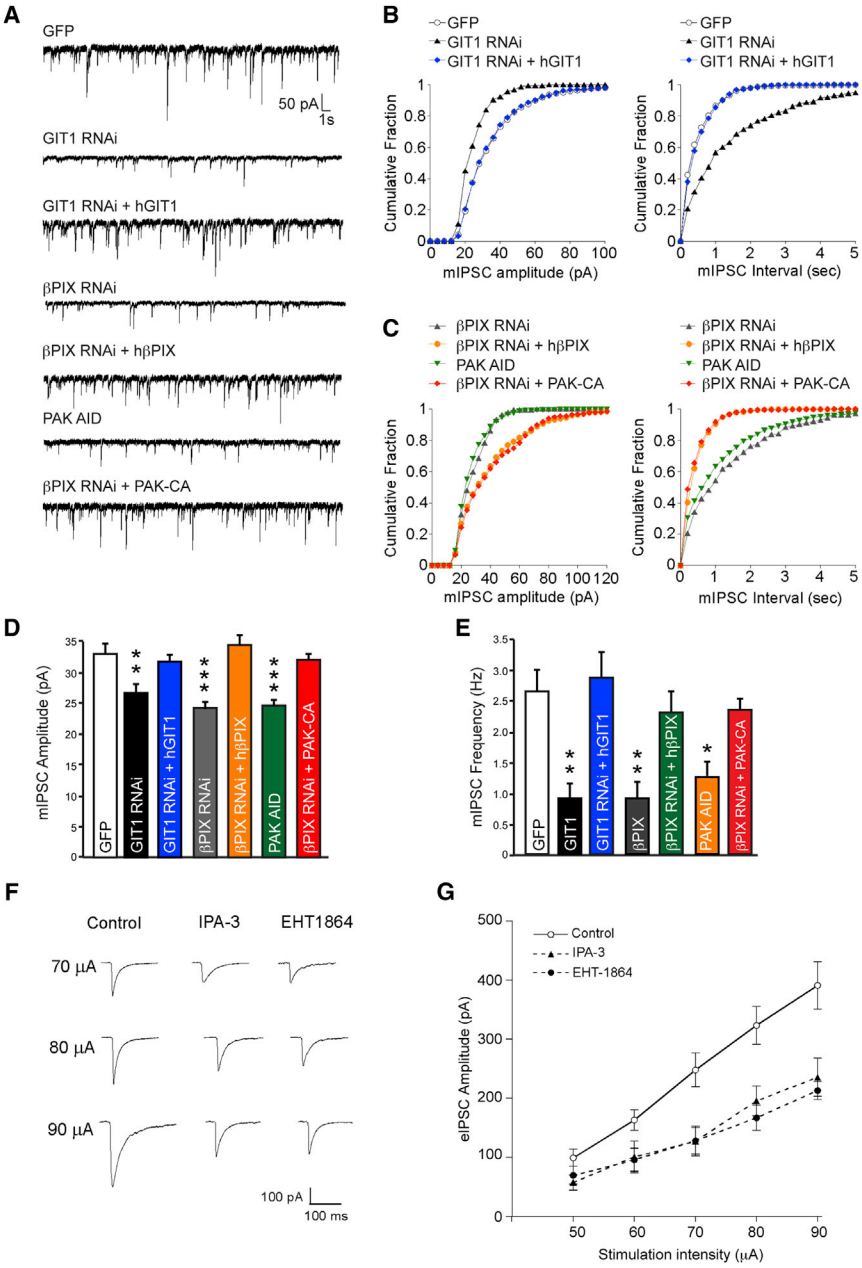

Figure 6. A GIT1/βPIX/Rac1/PAK Signaling Pathway Is Crucial for GABAergic Synaptic Transmission

(A) Representative traces of mIPSCs recorded from transfected neurons.

(B) Cumulative distribution plots of mIPSC shows that neurons transfected with GIT1 RNAi have smaller event amplitudes and larger interevent intervals in comparison to control and GIT1 RNAi + hGIT1-transfected neurons.

(C) Cumulative distribution plots of mIPSC of event amplitude and mIPSC interval for neurons expressing βPIX RNAi, PAK1-DN, βPIX RNAi + hβPIX or βPIX RNAi + PAK-CA.

(D and E) Summary bar graphs of average amplitude and frequency (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, GFP control, n = 27 cells; GIT1 RNAi, n = 12 cells; GIT1 RNAi + hGIT1, n = 16 cells; βPIX RNAi, n = 17 cells; GFP-PAK-AID, n = 19 cells; βPIX RNAi + PAK-CA, n = 15 cells). Values are mean ± SEM.

(F) Representative traces of eIPSCs in brain slices with or without treatment with either EHT or IPA to inhibit Rac1 or PAK, respectively.

(G) Input-output curves of $GABA_AR$-IPSCs evoked by a series of stimulus intensities in slices incubated with EHT or IPA (control, n = 15 cells; EHT, n = 9; IPA, n = 8 cells). Values are mean ± SEM. See also Figure S4.

DISCUSSION

Clustering of $GABA_ARs$ at inhibitory synapses is imperative for correct synaptic inhibition in the brain and is tightly controlled by components of the inhibitory postsynaptic density. Reduced $GABA_AR$ synaptic clustering equates to reduced inhibition in neuronal circuits and subsequent disruption of excitatory/inhibitory (E/I) balance, producing defects in information processing at the network level. Our results describe a signaling complex localized to the inhibitory postsynaptic domain that is crucial for correct inhibitory neurotransmission and the maintenance of GABAergic synaptic transmission. We show surface $GABA_AR$ clusters are maintained by a GIT1/βPIX/Rac1/PAK signaling complex that modulates F-actin, thereby stabilizing the inhibitory postsynaptic density and synaptic $GABA_ARs$ (Figure S4J).

We demonstrate that GIT1 interacts with synaptic $GABA_AR$ subunits and is localized at inhibitory postsynaptic sites, suggesting that it is intimately involved with the inhibitory postsynapse and its function. Indeed, the interaction between GIT1 and $GABA_ARs$ places it in the exact location to scaffold βPIX at the inhibitory synapse where it could locally activate Rac1 signaling (Zhang et al., 2003). Consistent with this, we also show that βPIX is located at inhibitory synapses and interacts in a complex with $GABA_ARs$. GEFs such as βPIX are essential

slices. Slices were incubated with either IPA or EHT to inhibit PAK or Rac1, respectively (Figure 6F). Analysis of the strength of inhibition with input/output curves of evoked IPSCs (eIPSCs) generated by a series of stimulus intensities showed that inhibition of the Rac1-PAK pathway in brain slices caused a substantial depression of the input/output eIPSC curves (40%–50% reduction for IPA and 30%–50% reduction for EHT; Figures 6F and 6G). This indicates that Rac1 and PAK are indeed critical to maintain GABAergic synaptic transmission in the brain. Together, these electrophysiological experiments reveal that inhibiting the GIT1/βPIX/Rac1/PAK pathway in neurons not only reduces surface $GABA_AR$ cluster and gephyrin cluster area, but also leads to reduced inhibitory synaptic transmission.

signaling coordinators that localize and regulate small GTPase signaling at specific sites within the cell (Kiraly et al., 2010). Therefore, our data showing the presence of βPIX and Rac1 at the inhibitory synaptic site, combined with the effects of βPIX knockdown and Rac1 inhibition on $GABA_AR$ clustering and inhibitory synaptic transmission, strongly suggest that a Rac1 signaling pathway is important for maintaining synaptic inhibition. Our results also point to a scaffolding role for GIT1 at the inhibitory synapse, potentially as an additional scaffolding protein to increase the stability of gephyrin and $GABA_AR$ surface clusters. GIT1 and βPIX are also shown to be important for excitatory synapse function, acting via a Rac1/PAK/actin pathway (Zhang et al., 2005), in an activity-dependent manner (Saneyoshi et al., 2008). Our findings are supportive of similar scaffolding mechanisms at inhibitory synapses, and suggest that the GIT1/βPIX signaling pathway may represent a key coordinator of actin pathways at synapses. Indeed, this is in agreement with the role of this GIT1/βPIX signaling module at regions of cell-cell contact in multiple cell types (Hoefen and Berk, 2006). Further study will now be required to define how the GIT1/βPIX complex may coordinate potential crosstalk between excitatory and inhibitory synapses.

The number of $GABA_ARs$ at the neuronal surface and synaptic sites directly correlates with the strength of inhibitory synaptic transmission; therefore, the modulation of $GABA_AR$ synaptic accumulation is a key mechanism for plasticity of inhibitory synapses. Here, we show that GIT1 or βPIX knockdown causes reduced $GABA_AR$ clustering and a decrease in the number of $GABA_ARs$ at the neuronal surface, and this effect is mimicked by inhibition of Rac1 or PAK in neurons. Indeed, disruption of $GABA_AR$ clustering by βPIX knockdown cannot be rescued by βPIX mutants lacking GEF activity for Rac1 or the ability to couple to PAK, implicating PAK as a downstream effector. Importantly, the effects of RNAi mediated knockdown of either GIT1 or βPIX on $GABA_AR$ clustering is rescued by active PAK. These biochemical and imaging data are supported by electrophysiological data, which show that knockdown of GIT1 or βPIX, or inhibition of Rac1 or PAK, causes reduced GABAergic currents in neurons, suggesting the reduction in $GABA_AR$ clusters does indeed correlate with reduced inhibition. In addition to these postsynaptic effects, we also observe reductions in VGAT clustering and mIPSC frequency, suggestive of additional presynaptic effects of GIT1/βPIX knockdown. In our imaging and electrophysiological experiments, we analyze GIT1/βPIX knockdown in the postsynaptic neuron, suggesting the presynaptic effects we observe are due to destabilization of the presynaptic GABAergic synaptic bouton concurrent with the loss of postsynaptic receptors, scaffolds, and adhesion molecules. Previously, disruption of gephyrin has been shown to reduce $GABA_AR$ clusters postsynaptically, accompanied by a loss of presynaptic GABAergic innervation (Marchionni et al., 2009; Yu et al., 2007). Similarly, loss of the γ2 subunit (as we demonstrate here occurs upon disruption of GIT1/βPIX) causes loss of both postsynaptic clustering and presynaptic innervation (Li et al., 2005). It is becoming clear that crosstalk between the pre- and postsynaptic sites of inhibitory synapses is essential for their plasticity, demonstrated by the observation that inhibitory pre- and postsynaptic structures are highly mobile and can move as a single entity (Dobie and Craig, 2011). Because our RNAi experiments are targeting the postsynaptic cell, our results are consistent with alteration of the GIT1/βPIX complex disrupting inhibitory postsynaptic domains, which also causes subsequent disruption of presynaptic innervation.

The GIT1/βPIX/Rac1/PAK pathway we have presented here documents a signaling pathway that links $GABA_AR$ stability to the actin cytoskeleton via a GTPase signaling cascade. By treating neurons with latrunculin-A for 30 min, we show that an intact actin cytoskeleton is important for $GABA_AR$ stability. This is in contrast with earlier findings showing no effect of latrunculin-A on $GABA_AR$ clustering (Allison et al., 2000); however, this study differed in both age of neurons and length of treatment (24 hr). We conclude that, although actin is not structurally required at inhibitory synapses as in dendritic spine heads, it is emerging that actin is required for the integrity of the inhibitory postsynaptic site (Charrier et al., 2006) and for postsynaptic scaffold mobility in general (Kerr and Blanpied, 2012). Gephyrin also interacts with collybistin (Kins et al., 2000), a GEF for the small GTPase Cdc42. The role of collybistin in region-specific inhibitory synapse formation has been investigated (Papadopoulos and Soykan, 2011), although it is still unclear whether Cdc42 activity is required for gephyrin clustering with several studies suggesting that collybistin functions independently of its GEF activity (Reddy-Alla et al., 2010). Thus, other Rho GTPase signaling and scaffolding mechanisms are likely to be present at inhibitory synapses. In agreement with this, we show that βPIX and Rac1 activity is required for inhibitory synapse function, by maintaining $GABA_AR$ surface levels at synapses, gephyrin clustering, and GABAergic currents.

GIT1 knockout (KO) mice have been investigated in the context of neuronal function in two independent reports. Reduced dendritic spine density and dendrite length in the hippocampal CA1 region have been reported in one GIT1 KO model, in addition to impaired performance in learning tasks (Menon et al., 2010). In a second study, investigators also observed memory and learning impairments, with increased hyperactivity and reduced inhibitory presynaptic input in CA1 (Won et al., 2011). Here, we demonstrate the effects of acute GIT1 knockdown and the short-term effects of inhibiting the GIT/βPIX/Rac1/PAK pathway on $GABA_AR$ clustering and synaptic inhibition and find reduced clustering of essential inhibitory synapse components including gephyrin. We therefore attribute the differences in our findings and those of Won et al., to the use of global KO strategies, which cause GIT1 knockdown in all cell types throughout development, which is in contrast to the short-term RNAi targeting and pharmacological treatment that we employ here.

Reduced inhibition due to depletion of $GABA_ARs$ from the neuronal cell surface can alter the E/I balance of neuronal circuits, causing disrupted information processing, which may lead to altered animal behavior (Blundell et al., 2009; Crestani et al., 1999; Papadopoulos et al., 2007; Tretter et al., 2009; Yizhar et al., 2011). Deficits in GABAergic neurotransmission leading to an altered E/I balance have also been implicated in multiple neuropsychiatric disorders including depression (Luscher et al., 2011b), bipolar disorder (Craddock et al., 2010), schizophrenia (Charych et al., 2009), and attention deficit hyperactivity disorder (ADHD; Won et al., 2011). Therefore, identifying the

molecular mechanisms that are essential for maintaining inhibitory synaptic transmission in the brain is also critical to understanding the development of these neuropsychiatric disorders, where it may become necessary to readdress pathological alterations in E/I balance. Our findings showing that a Rac1 signaling pathway is important for regulating inhibitory synaptic transmission may shed light on the molecular mechanisms underlying mental illness. Indeed, many of the proteins involved in the GTPase signaling pathway we describe here have been directly linked to mental disorders (Allen et al., 1998; Boda et al., 2004; Govek et al., 2004; Won et al., 2011). Altered PAK signaling due to mutations in the PAK3 gene has been linked to patients with mental retardation, and PAK signaling is additionally implicated in models of fragile-X syndrome and schizophrenia (Chen et al., 2010; Hayashi et al., 2007; Hayashi-Takagi et al., 2014), making it an important molecule in the synaptic pathology of psychiatric disorders. In addition, GIT1 was recently shown to harbor a single nucleotide polymorphism causing reduced GIT1 expression that is strongly linked to ADHD in humans (Won et al., 2011). Our results suggest how a postsynaptic GIT1 signaling complex plays a key role in controlling synaptic inhibition, by stabilizing $GABA_A$Rs at the inhibitory synaptic site, and may be an important locus for altered animal behavior and psychiatric and cognitive deficits.

We have characterized a GIT1/βPIX/Rac1/PAK signaling pathway that controls $GABA_A$R clustering from a molecular and physiological viewpoint. We found that GIT1, βPIX, Rac1, and PAK are all essential to maintain surface $GABA_A$R clusters and inhibitory currents in neurons, therefore making this signaling pathway an important regulator of inhibitory signaling in the brain.

EXPERIMENTAL PROCEDURES

Details regarding antibodies, immunocytochemistry and analysis, coimmunoprecipitations, biotinylations, and cDNA cloning are included in the Supplemental Experimental Procedures.

Neuronal Cell Culture

All experimental procedures were carried out in accordance with institutional animal welfare guidelines and the UK Animals (Scientific Procedures) Act 1986. Cultures of cortical and hippocampal neurons were prepared from E18 Sprague-Dawley rat embryos as described previously (Pathania et al., 2014; Twelvetrees et al., 2010); see also the Supplemental Experimental Procedures.

Electrophysiology

Standard voltage-clamp techniques were used for whole-cell recordings of spontaneous IPSC in cultured neurons (Twelvetrees et al., 2010). Electrodes were filled with the following internal solution (in mM): 100 CsCl, 30 N-methyl-D-glucamine, 10 HEPES, 1 $MgCl_2$, 5 EGTA, 2 QX314, 12 phosphocreatine, 5 MgATP, 0.5 Na_2GTP, 0.2 leupeptin (pH 7.2–7.3), and 270–280 mOsm. The external solution consisted of the following (in mM): 130 NaCl, 26 $NaHCO_3$, 3 KCl, 5 $MgCl_2$, 1.25 NaH_2PO_4, 1 $CaCl_2$, and 10 glucose (pH 7.4), 300 mOsm. Neurons were held at −70 mV. CNQX (20 μM) and APV (50 μM) were added to block AMPA and NMDA receptors. Mini IPSC recordings were performed in the presence of 0.01 mM TTX. Data were analyzed with Kaleidagraph (Albeck Software) and Mini Analysis program (Synaptosoft).

To measure GABAergic transmission in cortical slices, we used standard whole-cell recording techniques (Yuen et al., 2011). Pyramidal neurons at layer V of prefrontal cortex were used for recordings. Slices were incubated at room temperature (20°C –22°C) in artificial CSF (ACSF) bubbled with 95% O_2, 5% CO_2, and then slices were treated with various agents for 1 hr before recordings.

Proximity Ligation Assay

The in situ proximity ligation assay (PLA) was used according to the manufacturer's instructions (Olink Bioscience). Neurons were fixed in 4% PFA/30% sucrose, blocked (10% horse serum, 0.5% BSA, and 0.2% Triton X-100, 10 min at room temperature), and incubated with primary antibodies. For control PLA, a single antibody was applied. Cells were washed in 1 × PBS and then incubated with secondary antibodies conjugated to oligonucleotides. Ligation and amplification reactions were conducted at 37°C, before mounting and visualization with CLSM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.061.

AUTHOR CONTRIBUTIONS

K.R.S. performed and analyzed most of the biochemistry and confocal imaging experiments, led the project, and wrote the paper. E.C.D. performed molecular biology and performed and analyzed some of the biochemistry and confocal imaging experiments. M.P. performed and analyzed the PLA. V.V. performed molecular biology. J.W. and X.L. performed and analyzed electrophysiology experiments. Z.Y. supervised and advised on electrophysiology experiments. J.T.K. supervised the project and wrote the paper. E.C.D. and J.W. contributed equally to this work.

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Cell Reports, Volume *9*

Supplemental Information

GIT1 and βPIX Are Essential for $GABA_A$ Receptor Synaptic Stability and Inhibitory Neurotransmission

Katharine R. Smith, Elizabeth C. Davenport, Jing Wei, Xiangning Li, Manavendra Pathania, Victoria Vaccaro, Zhen Yan, and Josef T. Kittler

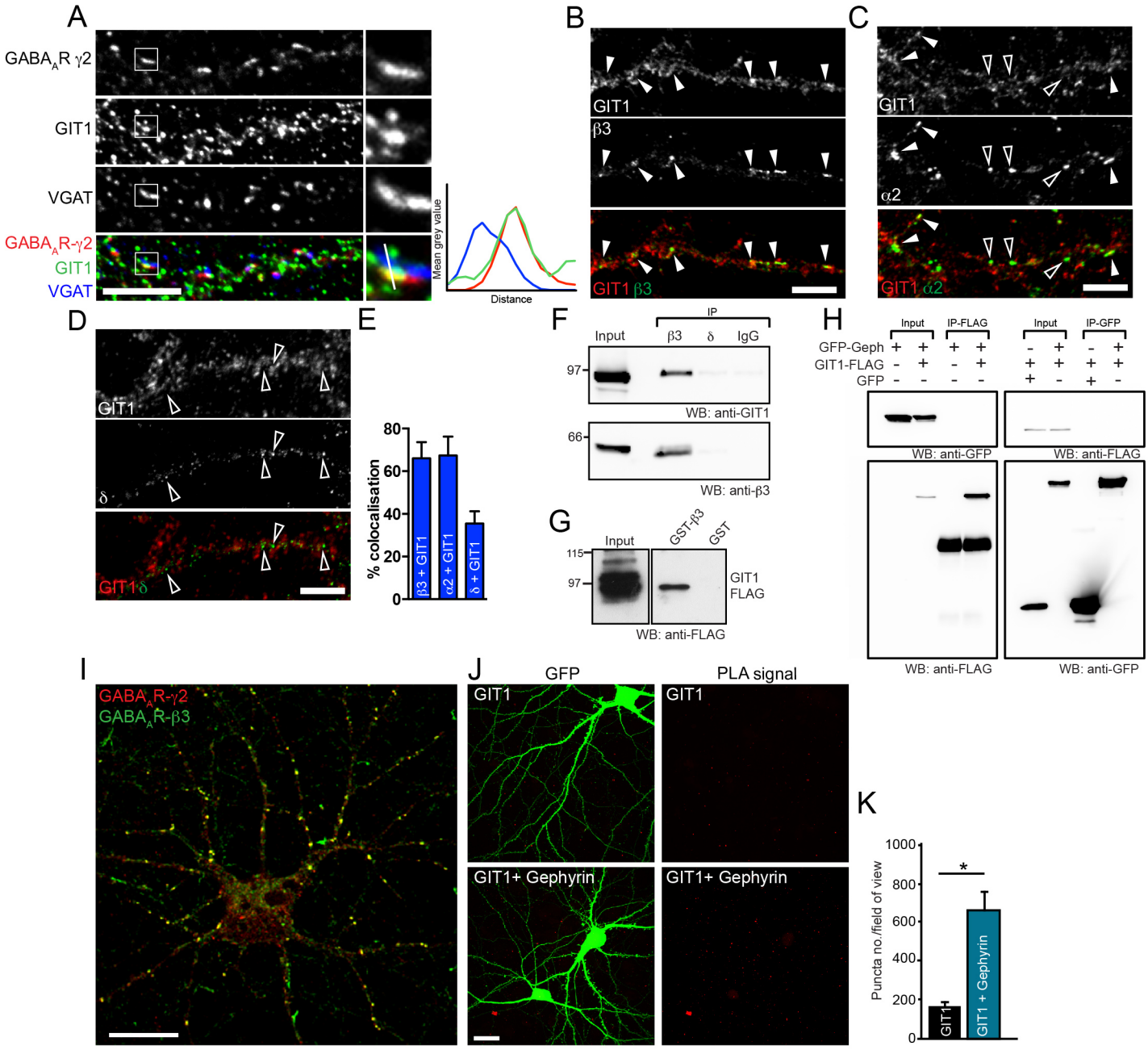

Figure S1, Related to Figure 1; GIT1 localisation in neurons

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

(B-D) GIT1 localisation with other GABA$_A$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$_A$R β3 or δ subunit. The β3 subunit can coimmunoprecipitate GIT1, but the δ subunit cannot.

(G) Western blot of pulldown 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$_A$Rs 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.

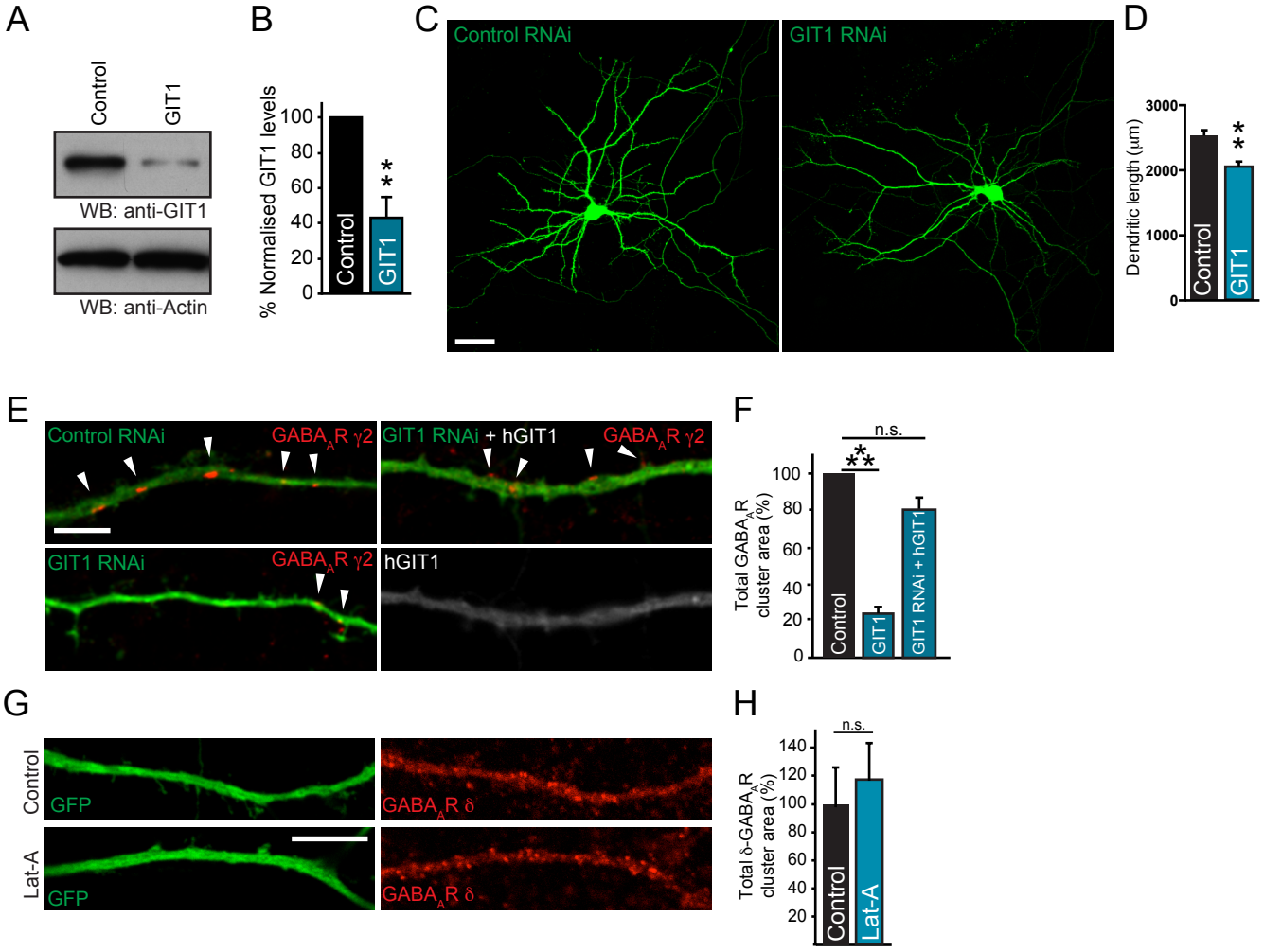

Figure S2, Related to Figure 2; Characterisation of GIT1 RNAi

(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 ± 11.4 % of control, **p =0.002, n=4 experiments, values are mean ± SEM).

(C,D) Analysis of dendrite length in neurons transfected with control or GIT1 RNAi. (C) Confocal images of representative neurons, scale bar=40μm. (D) Bar graph of dendritic length, **p=0.006, n= 15 cells. Values are mean ± SEM.

(E,F) Coexpression of human GIT1 (hGIT1) with GIT1 RNAi rescues the effect of the GIT1 knock-down surface GABA$_A$R clusters (n=3 experiments,12-15 cells,***p=3x10^{-5}, n.s. p=0.089). Values are mean ± SEM.

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

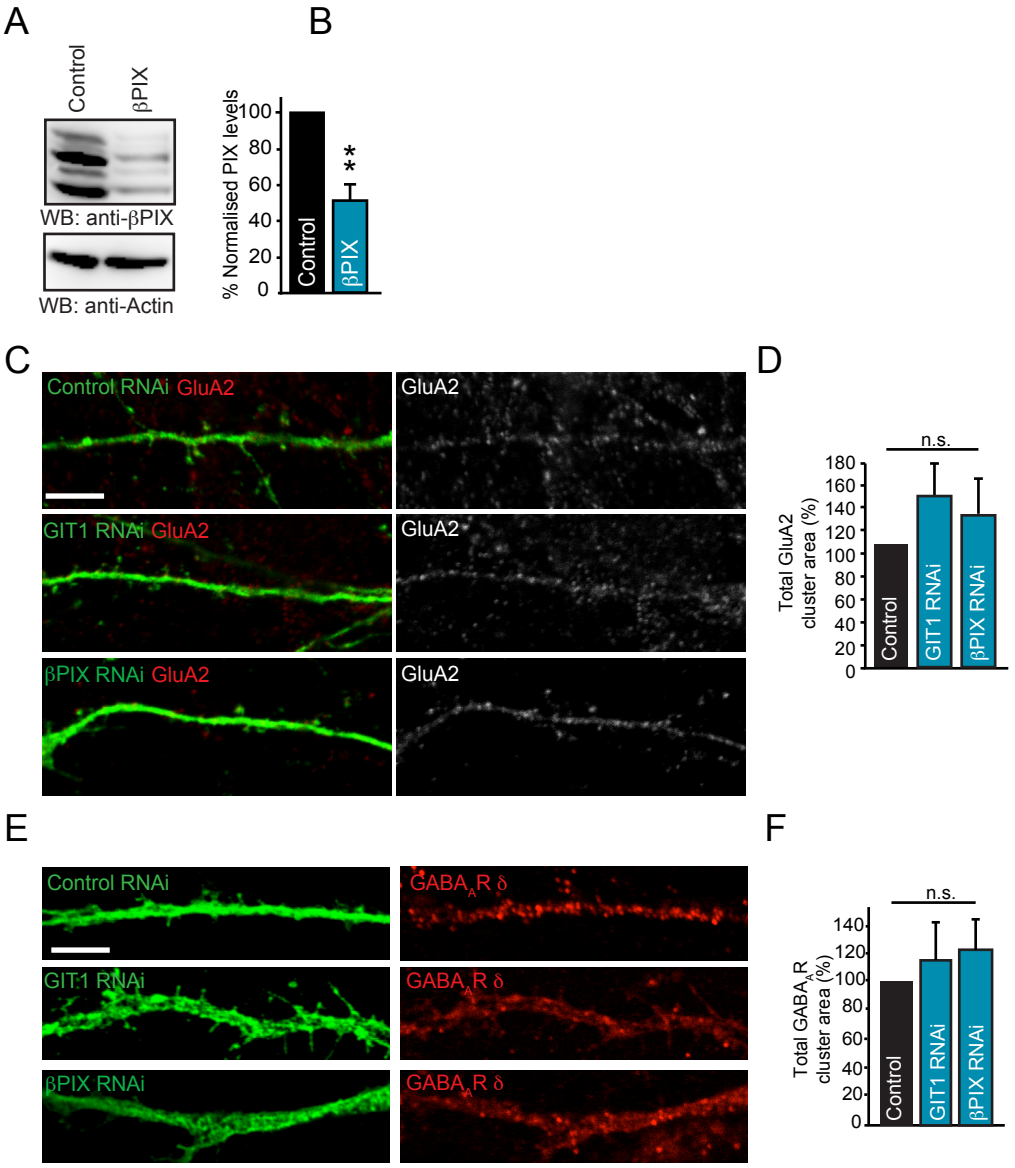

Figure S3, Related to Figure 3; Characterisation of βPIX RNAi

(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 ± 11.4 % of control, **p=0.002, n=5). Values are mean ± 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 ± SEM.

(E,F) Neurons expressing control, GIT1 or βPIX RNAi and labelled with antibodies to $GABA_AR$-δ subunit showed no change in surface δ-subunit containing receptors (no significant difference, n=3, 9-13 cells). Values are mean ± SEM.

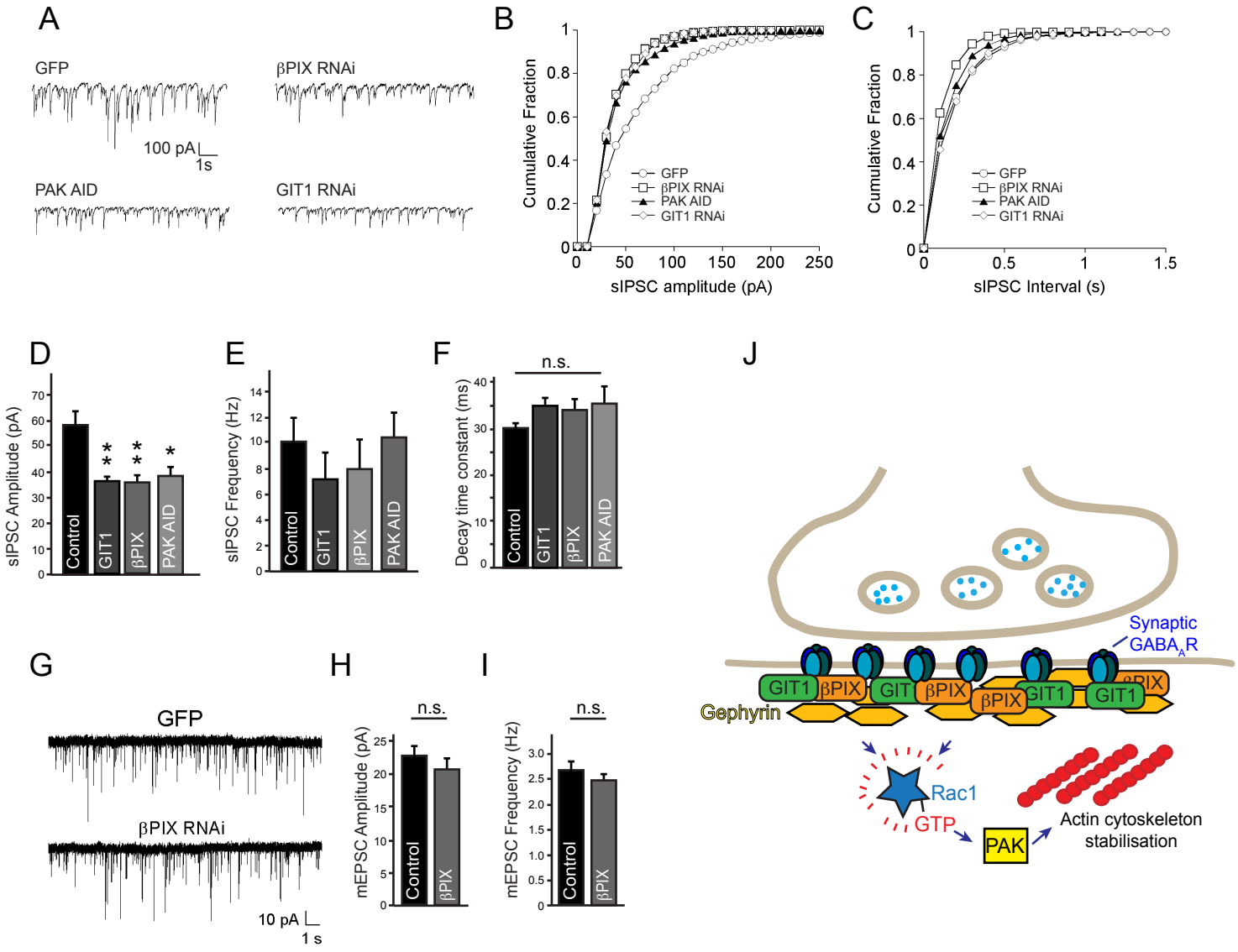

Figure S4, Related to Figure 6; Effects of disrupting the GIT1/βPIX signalling pathway on inhibitory neurotransmission

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

(A) Representative traces showing a reduction in sIPSC amplitude in neurons expressing GIT1 RNAi, βPIX 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, βPIX 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 ± 5.4 pA, n=11, GIT1 RNAi neurons: 36.2 ± 1.8 pA, n=10, p=0.003, βPIX RNAi neurons: 36.0 ± 3.0 pA, n=11, p=0.004, PAK-AID neurons: 38.9 ± 3.1 pA, n=11, p=0.009. Values are mean ± SEM.

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

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

(J) Schematic showing the GIT1/βPIX/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-γ2 (serum,(Kittler et al., 2001)(IF 1:100)), rabbit anti-Myc (Santacruz) (IP 2 1:200), mouse monoclonal to β3 (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-βPIX (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), δ-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, βPIX 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-βPIX were from Addgene (Addgene plasmids 15223, 12208 and 15234). Human βPIX 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 βPIX 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 μM, 1 hr), Jasplakinolide (Millipore, 2μM, 2 hours), IPA-3 (30 μM, 1 hour), Latrunculin-A (Tocris, 3 μM, 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 (25μM) and D-APV (25μM). Patch electrodes were filled with the following internal solution (in mM): 100 CsCl, 30 N-methyl-D-glucamine, 4 NaCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA, 2 QX314, 12 phosphocreatine, 5 MgATP, 0.2 Na3GTP, 0.2 leupeptin. A Multiclamp 700A amplifier was used for these recordings. Tight seals (2-10 GΩ) 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 Ca^{2+}/Mg^{2+}) and quenched with quench buffer (PBS Ca^{2+}/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 µg/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 µl 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 $GABA_A$Rs were identified by using anti-β3 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 μm) 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 0.8µm. 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 μ m x 0.056 μ m x 0.25 μ 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 ± SEM. Error bars represent SEM.

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