

Report

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Presynaptic Deletion of GIT Proteins Results in Increased Synaptic Strength at a Mammalian Central Synapse

Highlights

- Presynaptic deletion of GITs increases AP-evoked release with no change in RRP size
- Loss of GIT proteins does not affect voltage gated calcium currents
- GIT1 has a specific role in regulating release probability, distinct from GIT2
- GITs regulate synaptic strength by regulation of exocytosis efficiency

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In Brief

Montesinos et al. report for the first time on GIT protein function in the mammalian presynaptic terminal. They demonstrate that GIT1 and GIT2 regulate synaptic strength by increasing exocytosis efficiency and identify GIT1 as a specific regulator of presynaptic release probability.



Presynaptic Deletion of GIT Proteins Results in Increased Synaptic Strength at a Mammalian Central Synapse

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SUMMARY

A cytomatrix of proteins at the presynaptic active zone (CAZ) controls the strength and speed of neurotransmitter release at synapses in response to action potentials. However, the functional role of many CAZ proteins and their respective isoforms remains unresolved. Here, we demonstrate that presynaptic deletion of the two G protein-coupled receptor kinase-interacting proteins (GITs), GIT1 and GIT2, at the mouse calyx of Held leads to a large increase in AP-evoked release with no change in the readily releasable pool size. Selective presynaptic GIT1 ablation identified a GIT1-specific role in regulating release probability that was largely responsible for increased synaptic strength. Increased synaptic strength was not due to changes in voltage-gated calcium channel currents or activation kinetics. Quantitative electron microscopy revealed unaltered ultrastructural parameters. Thus, our data uncover distinct roles for GIT1 and GIT2 in regulating neurotransmitter release strength, with GIT1 as a specific regulator of presynaptic release probability.

INTRODUCTION

The release probability (P_r) of synaptic vesicles (SVs) in response to action potentials (APs) from a pool of fusion competent SVs, the readily releasable pool (RRP), regulates synapse strength (Regehr, 2012). Critical to the regulation of SV release at the active zone (AZ) is an electron dense network of proteins, the cytomatrix at the active zone (CAZ), which regulates SV release through distinct pathways (Gundelfinger and Fejtova, 2012). A family of functionally conserved molecules, the G protein-coupled receptor (GPCR) kinase-interacting proteins (GITs), are located in the CAZ and function as part of an oligomeric complex with PIX proteins, which serve as a scaffold for numerous signaling partners (Hoefen and Berk, 2006).

In mammals, two genes, *Git1* and *Git2*, encode GITs that share highly conserved domain structures and amino acid sequences (Hoefen and Berk, 2006). In the CNS, GITs are widely co-expressed (Schmalzigaug et al., 2007) and localized within both pre- and postsynaptic compartments (Ko et al., 2003). In *D. melanogaster* and *C. elegans*, a single conserved GIT ortholog is ubiquitously expressed (Bahri et al., 2009; Lucanic and Cheng, 2008). *Git2* knockout (KO) mice are viable but have hearing impairments (White et al., 2013) and anxiety-like behaviors (Schmalzigaug et al., 2009b). Unlike *Git2* KO mice, most *Git1* KO mice die at birth, and adult survivors have impaired fear memory (Schmalzigaug et al., 2009a), operant conditioning (Menon et al., 2010), and spatial and object learning (Won et al., 2011). In addition, GIT1 has been implicated in Huntington's disease (Goehler et al., 2004) and attention deficit hyperactivity disorder (Won et al., 2011), although this is controversial (Klein et al., 2015).

Although GIT1 function has been characterized in the postsynaptic compartment (Segura et al., 2007; Zhang et al., 2003), due to the inability to selectively ablate presynaptic GIT expression, little is known about GIT function at the mammalian presynapse. The calyx of Held/medial nucleus of the trapezoid body (MNTB) is a giant glutamatergic axosomatic synapse in the auditory brainstem, which tightly regulates RRP release dynamics to support the early stages of auditory processing (Borst and Soria van Hoeve, 2012). Due to its unparalleled experimental accessibility, presynaptic mechanisms of synaptic transmission can be analyzed independent of postsynaptic contributions. Thus, this is an ideal synapse to address presynaptic GIT functions in synaptic transmission regulation. Here, we used *Git1* conditional knockout (CKO) (*Git1*^{-/-}, in presence of Cre recombinase), *Git2* KO (*Git2*^{-/-}), and *Git1* CKO/*Git2* KO (*Git1*^{-/-}/*Git2*^{-/-}) transgenic mice, together with our ability to conditionally ablate GIT1 expression in the calyx of Held, to analyze the role of GITs in synaptic transmission. We show that loss of both GIT1 and GIT2 results in increased AP-evoked release but with no change in RRP size, SV number or distribution at the AZ, voltage-gated calcium channel currents, or calyx morphology. However, individual ablation of *Git1* increased P_r , while ablation of *Git2* did not. Our data demonstrate that GIT1 and GIT2 proteins regulate synaptic strength through distinct presynaptic mechanisms that increase exocytosis efficiency, with a distinct role for GIT1 regulation of P_r .

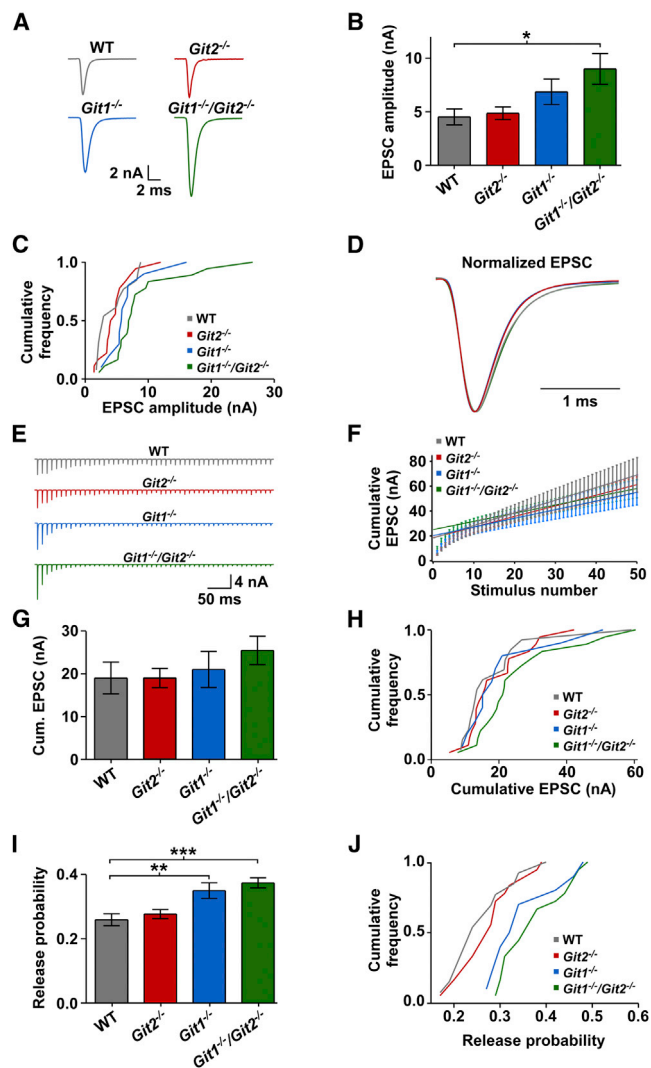


Figure 1. GIT Proteins Regulate AP-Evoked Release and P_r

(A) Representative traces of single AP-evoked EPSCs from WT, *Git2*^{-/-}, *Git1*^{-/-}, or *Git1*^{-/-}/*Git2*^{-/-} calyces.
 (B) Summary data showing average EPSC amplitudes.
 (C) Cumulative frequency histogram of EPSC amplitudes.
 (D) Average normalized EPSC amplitudes.
 (E) Example traces in response to a 100-Hz stimulus train from WT, *Git2*^{-/-}, *Git1*^{-/-}, or *Git1*^{-/-}/*Git2*^{-/-} calyces.
 (F) Cumulative plots of EPSC amplitudes with back-extrapolated linear fits.
 (G) Average values for the calculated RRP size.
 (H) Cumulative frequency histogram of RRP size.
 (I) P_r obtained by dividing the amplitude of the first EPSC from the 100-Hz train by the calculated RRP size.
 (J) Cumulative frequency histogram of P_r .

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ one-way ANOVA with a post hoc Dunnett's test.

RESULTS

GIT Protein Expression at the Calyx of Held

Since the GITs' expression pattern at the calyx of Held was unknown, we carried out immunohistochemical (IHC) staining on the P18-21 calyx of Held. Our results indicated that GIT1

and GIT2 could be detected in the calyx. Line scan analysis indicated that GIT1 and GIT2 colocalized with vesicular glutamate transporter 1 (VGLUT1) (Figures S1A and S1B). Our results confirm prior studies where GIT1 was found at hippocampal presynapse (Ko et al., 2003; Podufall et al., 2014) and demonstrate that GIT2 is also expressed presynaptically.

GIT1 and GIT2 Regulate AP-Evoked Release

In order to selectively ablate GIT1 expression or ablate all GIT expression at the calyx, we injected a rAd that independently co-expressed Cre and EGFP, using a stereotactic injection approach into the cochlear nucleus (CN) of P0-1 *Git1* CKO or *Git1* CKO/*Git2* KO mice (Supplemental Experimental Procedures). This permitted specific analysis of GIT1 presynaptic function independent of its postsynaptic role, which was not possible in previous studies using *Git1* KO animals (Menon et al., 2010; Schmalzigaug et al., 2009a). In addition, ablating GIT1 presynaptically and GIT2 expression globally, we were able to examine how complete loss of GITs affected presynaptic function.

To analyze how the individual and combined GIT proteins regulated AP-evoked release, we performed midline stimulation of calyx axons while the resultant AMPA excitatory postsynaptic currents (EPSCs) from principal cells of the MNTB innervated by transduced and non-transduced P18-21 calyces were recorded in whole-cell voltage-clamp mode (Supplemental Experimental Procedures). To determine how GITs affect basal synaptic transmission, we used a stimulation frequency of 0.05 Hz. Loss of both GIT1 and GIT2 together led to a 2-fold increase in the AP-evoked EPSCs compared to wild-type (WT) (9.01 ± 1.44 nA, $n = 18$ versus 4.52 ± 0.75 nA, $n = 13$; $p < 0.05$). Loss of GIT2 had no effect, while loss of GIT1 showed a trend toward increased AP-evoked release, but did not reach statistical significance (Figures 1A–1C, Table S1). Expression of Cre alone had no effect on AP-evoked release (Figures S2A–S2D, Table S1). Analysis of normalized AP-evoked EPSC waveforms revealed that GIT loss did not affect AP-evoked release kinetics (Figure 1D). Analysis of mEPSC revealed no changes in mEPSC frequency or kinetics (Figure S3, Table S1). These results demonstrate that GITs regulate synaptic strength at mammalian CNS synapses.

Loss of GIT1, but Not GIT2, Increases Initial Release Probability

Increased AP-evoked release could be explained in two ways: (1) an increase in RRP size or (2) an increase in initial P_r . To determine the RRP size, we carried out afferent fiber stimulation at 100 Hz and plotted the data using the back extrapolation method (Schneppenburger et al., 1999) and by using the methodology of Elmquist and Quastel (EQ) (Figure S4) (Elmquist and Quastel, 1965). Subsequent analysis revealed that individual loss of GIT1 or GIT2 alone, or the combined loss of GIT1 and GIT2, did not lead to any statistically significant increase in the RRP size compared to WT (Figures 1E–1H, Figures S4A–S4C, Table S1). Expression of Cre alone had no effect on RRP size (Figures S2E–S2H, Table S1). Therefore, our functional measure of RRP

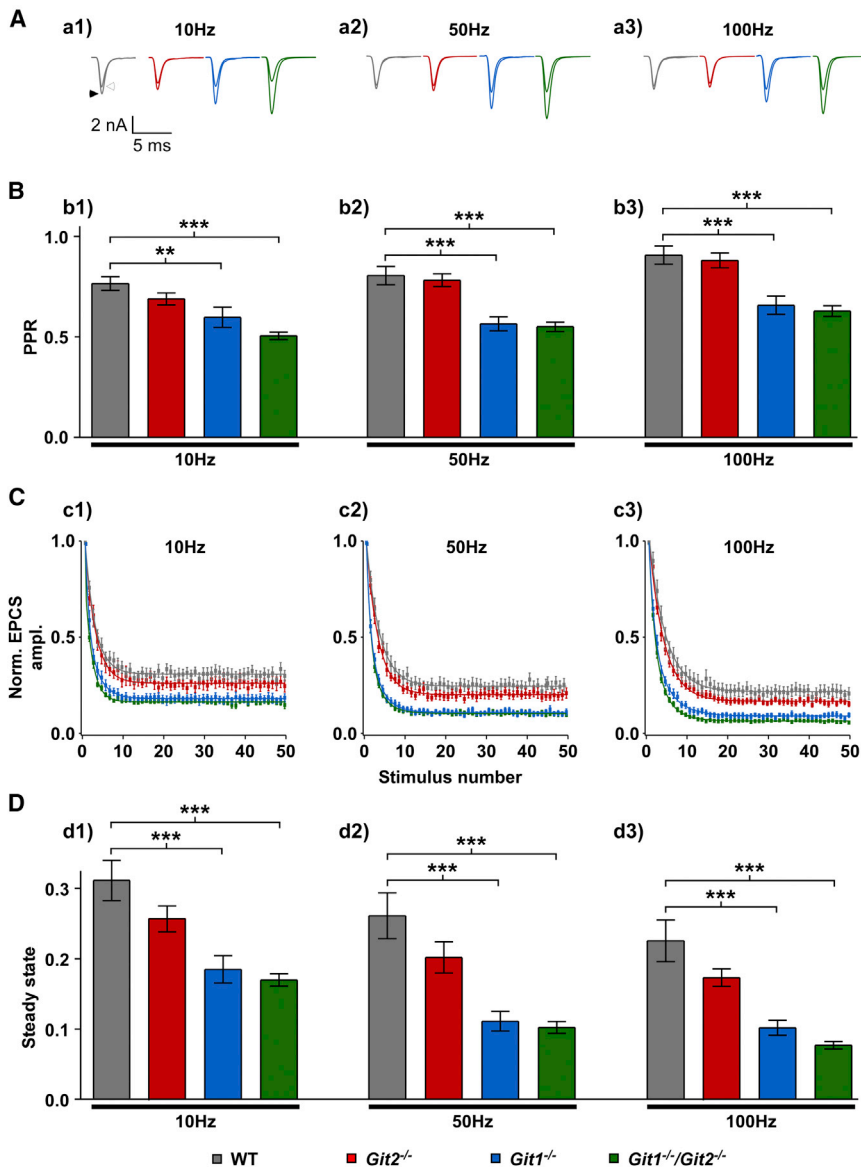


Figure 2. Synaptic Plasticity Is Altered by the Loss of GIT Expression

(A) Example traces of the EPSC obtained by fiber stimulation after applying two consecutive pulses at the frequencies indicated (a1, 10 Hz; a2, 50 Hz; a3, 100 Hz). Black and white arrowheads indicate the first and second EPSC, respectively.

(B) PPR was calculated by dividing the amplitude of the second EPSC by the amplitude of the first EPSC at 10 Hz (b1), 50 Hz (b2), and 100 Hz (b3).

(C) Summary plot of normalized EPSC amplitude to the first EPSC during train stimuli using 10 Hz (c1), 50 Hz (c2), and 100 Hz (c3) against the stimulus number.

(D) Steady-state depression level measured and plotted as a function of the stimulation frequency (d1, 10 Hz; d2, 50 Hz; d3, 100 Hz).

** $p < 0.01$, *** $p < 0.001$ one-way ANOVA with a post hoc Dunnett's test.

S4E). Expression of Cre alone did not change P_r compared to WT (Figures S2I and S2J, Table S1).

To further confirm GIT1 regulates initial P_r , we applied a paired pulse stimulation protocol and calculated the paired pulse ratio (PPR) at 10, 50, and 100 Hz (Regehr, 2012). Specifically, decreases in the PPR are hallmarks of increases in P_r due to presynaptic regulation. Figure 2 shows that at all frequencies tested there was a significant reduction in the PPR in both the *Git1*^{-/-} or *Git1*^{-/-}/*Git2*^{-/-} calyces, but not the *Git2*^{-/-} (Figures 2A and 2B, Table S1). Expression of Cre alone had no effect on PPRs (Figures S2K and S2L, Table S1). These results show that deletion of GITs increased synaptic strength independent of increased RRP size. We conclude that GIT1 is the dominant isoform that regulates initial P_r at the calyx leading to changes in synaptic strength. In contrast, GIT2 contributes to synaptic strength increases independent of initial P_r regulation.

Loss of GIT1 and Combined Loss of GIT1 and GIT2 Increases Short-Term Depression

Since the initial P_r is increased without changes in the RRP size, this should lead to an increase in the rate of depression in response to a train stimulation. We analyzed the RRP release kinetics at 10, 50, and 100 Hz. We found an increase in the rate of onset of depression and lower steady-state levels with the loss of GIT1 alone, and this amount of depression was not increased with loss of both GIT1 and GIT2 together (Figures 2C and 2D, Table S1). Furthermore, unlike the WT or *Git2*^{-/-} responses, *Git1*^{-/-} and *Git1*^{-/-}/*Git2*^{-/-} responses were best fit with a double exponential (Table S2). Expression of Cre resulted in no changes compared to WT (Figures S2M and S2N, Table S1). Since loss

size in the absence of GITs indicates RRP size changes are not responsible for increased AP-evoked release.

Since the RRP size was unchanged, the increased AP-evoked release was likely to be caused by increased P_r . To calculate the initial P_r , we divided the first EPSC amplitude from each train by the RRP size as calculated by the two different methods. Our results demonstrated that loss of GIT1 and GIT2 together led to a significant increase in initial P_r compared to WT (0.37 ± 0.02 , $n = 18$ versus 0.26 ± 0.02 , $n = 13$; $p < 0.001$) (Figures 1I and 1J). The same results are obtained by the EQ method (0.34 ± 0.02 , $n = 18$ versus 0.18 ± 0.03 , $n = 12$; $p < 0.001$) (Figures S4D and S4E). Interestingly, GIT1 loss alone also led to similar increases in initial P_r compared to WT regardless of the method used (0.35 ± 0.02 , $n = 19$ versus 0.26 ± 0.02 , $n = 13$; $p < 0.01$) (EQ method, 0.31 ± 0.02 , $n = 10$ versus 0.18 ± 0.003 , $n = 12$; $p < 0.001$), while GIT2 did not (Figures 1I and 1K, Figures S4D and

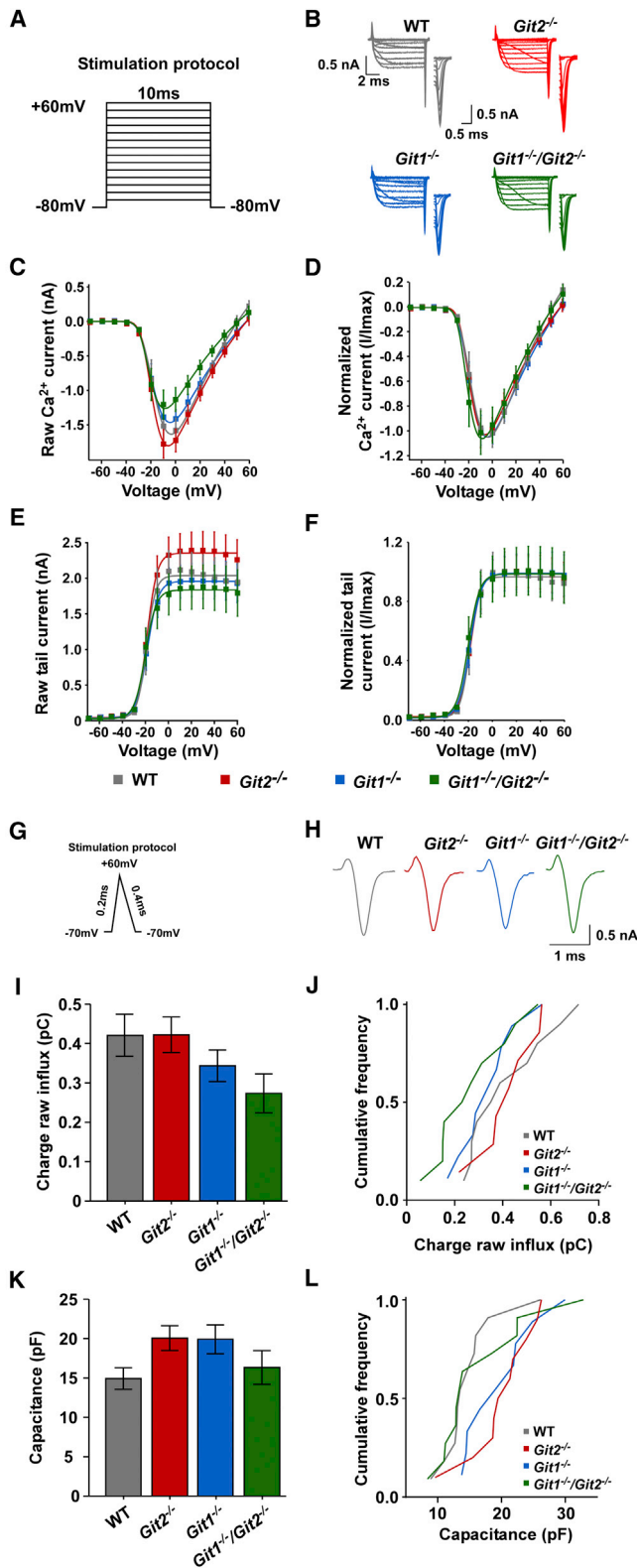


Figure 3. Loss of GIT Proteins Results in No Changes of Ca^{2+} Current and Activation Kinetics

(A) Stimulus protocol used for the experiment from (B) to (F).

of GIT1 alone led to an increase in frequency-dependent depression, and with no further increases with the loss of GIT1 and GIT2 together, our data demonstrate that GIT1 regulation of P_r is the major determinant impacting frequency dependent synaptic plasticity.

Loss of GIT Proteins Does Not Affect Voltage-Gated Calcium Currents

Changes in presynaptic calcium currents (I_{Ca}) have dramatic effects on AP-evoked release (Regehr, 2012). To determine if increased I_{Ca} might explain the initial P_r increase with loss of GIT1 or additional synaptic strength increase with loss of both GIT1 and GIT2, we measured the I_{Ca} in the WT, $\text{Git}2^{-/-}$, $\text{Git}1^{-/-}$, and $\text{Git}1^{-/-}/\text{Git}2^{-/-}$ calyces. Analysis of the I_{Ca} current-voltage (I/V) relationship demonstrated no change in the I_{Ca} steady-state amplitude in the $\text{Git}1^{-/-}$ or $\text{Git}2^{-/-}$ calyces (Figure 3, Table S1). However, there was a slight reduction in the $\text{Git}1^{-/-}/\text{Git}2^{-/-}$ calyces, but this did not reach significance (Figures 3A–3C, Table S1). Normalization of I_{Ca} to the I_{Ca} maximum revealed no changes in the activation kinetics (Figure 3D). In addition, there was no change in I_{Ca} tail current peak amplitudes and activation kinetics (Figures 3E and 3F, Table S1). Finally, C_{slow} values revealed no difference in calyx size between the WT and $\text{Git}1^{-/-}$, $\text{Git}2^{-/-}$, or $\text{Git}1^{-/-}/\text{Git}2^{-/-}$ calyces (Figure 3K).

Since our fiber stimulation experiments used APs to trigger SV release, we tested whether I_{Ca} influx triggered by a pseudo-AP waveform would be affected. To do so, we recorded the I_{Ca} evoked by pseudo-AP waveforms (Yang and Wang, 2006) (Figures 3G and 3H). Since the integral of I_{Ca} in response to an AP is the key determinant of the resultant EPSC, we measured the I_{Ca} charge in response to the pseudo-AP waveform and found no reduction in the I_{Ca} charge in $\text{Git}1^{-/-}$, $\text{Git}2^{-/-}$, or $\text{Git}1^{-/-}/\text{Git}2^{-/-}$ calyces compared to WT (Figure 3I). Since the loss of GITs did not affect I_{Ca} amplitude, charge, or activation kinetics, this indicates that the loss of GIT proteins leads to an increased exocytosis efficiency, similar to what happens during calyx development (Taschenberger et al., 2002). Finally, we conclude that GIT1 regulation of P_r is independent of VGCC regulation.

Loss GIT Proteins Does Not Change SV Distribution at the Active Zone

Since GITs interact with CAZ proteins implicated in AZ organization (Kim et al., 2003; Ko et al., 2003), it is possible that the

(B) Representative I_{Ca} traces for I/V curve analysis from -80 mV to $+60$ mV from WT, $\text{Git}2^{-/-}$, $\text{Git}1^{-/-}$, and $\text{Git}1^{-/-}/\text{Git}2^{-/-}$ calyces. Insets show in detail the tail currents.

(C) I/V plot representing the average steady state I_{Ca} amplitudes plotted against voltage.

(D) Normalized average raw I_{Ca} amplitudes to I_{max} .

(E) Analysis of voltage-dependent activation of VGCCs.

(F) Normalized tail currents to I_{max} .

(G) Stimulus protocol used for the experiment from (H) to (L).

(H) Representative I_{Ca} obtained from WT, $\text{Git}2^{-/-}$, $\text{Git}1^{-/-}$, and $\text{Git}1^{-/-}/\text{Git}2^{-/-}$ calyces.

(I) Average values of I_{Ca} charge.

(J) Cumulative frequency of I_{Ca} charge.

(K) Average values of capacitance (C_{slow}).

(L) Cumulative frequency of C_{slow} .

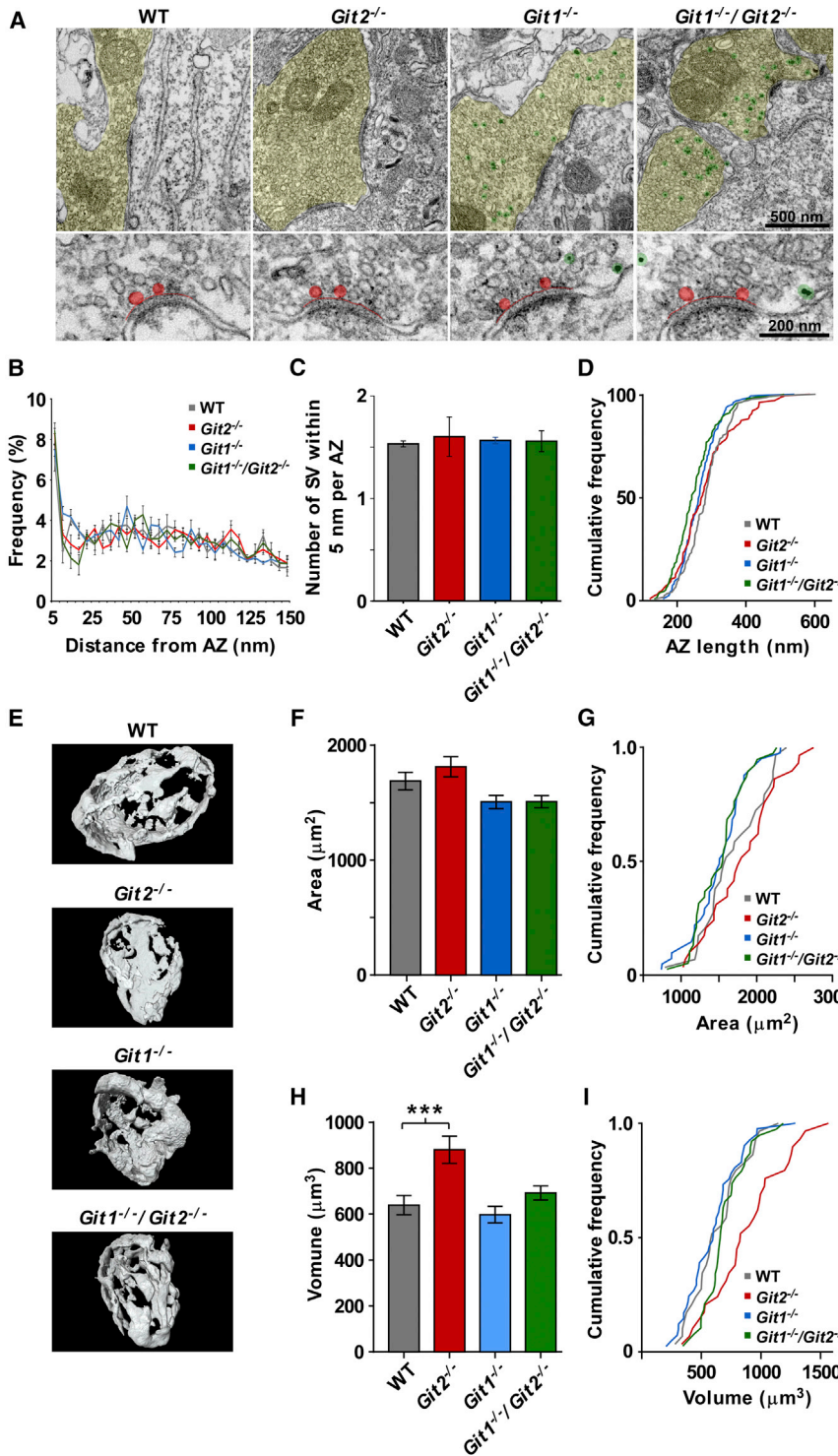


Figure 4. Ablation of GIT Expression Does Not Affect the Number of Docked SV or SV Distribution

(A) EM sample images taken from WT, *Git2*^{-/-}, *Git1*^{-/-}, and *Git1*^{-/-}/*Git2*^{-/-} calyces. Top panels: calyx of Held is demarcated in yellow, and the nanogold GFP labeling in green (scale bar = 500 nm). Bottom panels: detail of the AZ analyzed. SVs closest to the AZ are shown in red shade, and AZ length is denoted by a red line (scale bar = 200 nm). (B) Summary of normalized distribution of SV distance from AZs. (C) Average number of SVs localized within 5 nm relative to the AZs. (D) Cumulative frequency histogram of the AZ length. (E) Example of 3D reconstructions from WT, *Git2*^{-/-}, *Git1*^{-/-}, and *Git1*^{-/-}/*Git2*^{-/-} calyces. (F and G) Average values of surface area (F) and the corresponding cumulative frequency distribution (G). (H and I) Summary data showing average values of volume (H) and the corresponding cumulative frequency distribution (I).

that SV distribution and number of docked SVs were not changed in any of the genotypes tested (Figures 4A–4C, Table S1), and there was only minor reduction in the AZ length in the *Git1*^{-/-}/*Git2*^{-/-} compared to WT (262.1 \pm 5.82 nm, n = 120 versus 293.6 \pm 6.2 nm, n = 125; p < 0.01) (Figure 4D, Table S1). Since docked SVs are correlated with RRP size (Schikorski and Stevens, 2001), these results are consistent with our previous observation that loss of GITs does not affect RRP size (Figure 1). In conclusion, our results indicate that loss of GITs does not affect synaptic substructure, SV docking, or SV distribution relative to the AZ and is not the cause for increased P_r in the *Git1*^{-/-} calyces or synaptic strength increases in the *Git1*^{-/-}/*Git2*^{-/-} synapses.

Although in murine neurons, interference with GIT1 function changes dendrite morphogenesis and morphology (Zhang et al., 2003), it remains unknown if GIT proteins affect presynaptic morphology. Despite the fact that C_{slow} values indicate that loss of GITs did not affect calyx size

increased exocytosis efficiency we observed in the *Git1*^{-/-} and *Git1*^{-/-}/*Git2*^{-/-} calyces was due to altered presynaptic substructure. To test this hypothesis, we acquired and analyzed electron microscopy (EM) images from WT, *Git2*^{-/-}, *Git1*^{-/-}, and *Git1*^{-/-}/*Git2*^{-/-} calyces to assess if SV docking, SV distribution, or AZ length were altered. Analysis of EM images revealed

(Figure 3K), C_{slow} values do not report the actual calyx size, but the capacitance of the calyx and the variable length of axon that remains attached (Borst and Sakmann, 1998). Therefore, to accurately quantitate if calyx morphology was altered by the loss of GITs, we carried out 3D reconstructions from confocal z stack images of WT, *Git2*^{-/-}, *Git1*^{-/-}, or *Git1*^{-/-}/*Git2*^{-/-}

calyces and determined the surface area and volume from the P18-21 calyces (Figures 4E–4I). Analysis revealed no surface area reduction in the *Git2*^{-/-}, *Git1*^{-/-}, or *Git1*^{-/-}/*Git2*^{-/-} calyces compared to WT (Figures 4F and 4G, Table S1). However, despite no change in surface area, there was an increase in the volume of *Git2*^{-/-} calyces compared to WT ($880.50 \pm 59.17 \mu\text{m}^3$, $n = 29$ versus $639.60 \pm 41.46 \mu\text{m}^3$, $n = 29$; $p < 0.05$) (Figures 4H and 4I, Table S1). Nevertheless, based on the absence of surface area and volume changes in *Git1*^{-/-} or *Git1*^{-/-}/*Git2*^{-/-}, we conclude that the increased exocytosis efficiency due to increases of P_r , with loss of GIT1, and further synaptic strength increase in the absence of GIT1 and GIT2 are not due to altered calyx morphology.

DISCUSSION

In our present study, we tested whether GIT proteins have a role in presynaptic regulation of mammalian synaptic function. Using our ability to selectively study GIT presynaptic function, we conclude that GIT1 and GIT2 proteins regulate synaptic strength by increasing the exocytosis efficiency through distinct mechanisms: GIT1 by negatively regulating P_r , while GIT2 acts through an unknown mechanism.

Although GIT1 and GIT2 share the same domain structure, they appear to have some distinct functions at the molecular level (Hoefen and Berk, 2006) and behaviorally (Schmalzigaug et al., 2009a, 2009b). Only loss of GIT1 alone led to an increase in P_r , while no further increases in P_r were seen with loss of both GIT1 and GIT2. These GIT isoform differences of P_r regulation might be explained by high levels of GIT1 expression relative to GIT2 in the calyx, GIT1 mRNA levels are ~10-fold higher than GIT2 mRNA levels (Körber et al., 2014) or by GIT2 having an independent mechanism for regulating synaptic strength. Nonetheless, it is clear that GIT2 contributes to regulation of presynaptic function as the combined loss of GIT1 and GIT2 is required to significantly increase AP-evoked EPSCs. At the calyx, P_r is reduced during development (Iwasaki and Takahashi, 2001; Taschenberger and von Gersdorff, 2000). Although increases in GIT1 and GIT2 expression levels after hearing onset may contribute to P_r reduction, GIT mRNA levels in GBCs are unchanged before and after hearing (Körber et al., 2014).

The RRP consists of two SVs populations, those that are readily releasable and those that are reluctant to be released in response to an AP. The regulation of the number that are readily releasable directly effects initial P_r (Neher, 2010). CAZ proteins have emerged as potential regulators of P_r through different mechanisms: (1) positional priming, which regulates SV to VGCC coupling (Neher, 2010), and (2) post priming, which regulates downstream signaling cascades after positional priming that lower the energy barrier for fusion (Lee et al., 2013). At the calyx, positional priming is tightly regulated to ensure continuous SV availability for encoding auditory information (Chen et al., 2015; Fedchyshyn and Wang, 2005; Nakamura et al., 2015). However, understanding of the molecular mechanisms that regulate positional priming is still in the early stages. At the calyx, Munc13-1 has been suggested to positively regulate positional priming by bringing SVs closer to the

VGCCs (Chen et al., 2013). At hippocampal synapses, the actin cytoskeleton is implied to be a structural barrier that prevents close association between SVs and VGCCs, thereby serving as negative regulators of positional priming (Cingolani and Goda, 2008). Studies at the calyx on Septin 5 (Yang et al., 2010) and those that acutely disrupt the actin cytoskeleton (Lee et al., 2012) demonstrate that these structural barriers can serve as negative regulators of SV release in response to an AP and affect P_r . Since GIT1 is located at the AZ at the same distance as RIM proteins with respect to Bassoon (Podufall et al., 2014), it is possible that GIT/PIX complexes might regulate actin dynamics at the AZ, which affects positional priming of SVs. Evidence supporting this hypothesis comes from a study in which knockdown of Piccolo, a regulator of F-actin dynamics and a GIT binding protein, results in facilitation of SV exocytosis and increases the rates of depression (Waites et al., 2011). Future studies will be needed to address this possibility.

Our data cannot rule out a possible role for GITs in post priming. Previous studies revealed that modulation of lipid signaling at the AZ can change P_r (Basu et al., 2007; Lee et al., 2013). GITs deactivate Arf6, which in turn can regulate the production of lipids PA and PIP₂ (Hoefen and Berk, 2006). Work in neuroendocrine cells suggests that GITs negatively regulate Arf6 pathways to inhibit exocytosis (Meyer et al., 2006). However, the granule docking site in neuroendocrine cells is different from the AZ in neurons, and they have different release rates. Thus, the mechanisms of GIT function found in neuroendocrine cells will need to be verified in neurons.

Our findings on the functional role of GIT proteins at the calyx define a different phenotype than reported in a *Drosophila* transgenic model where dGIT function was analyzed at the NMJ (Podufall et al., 2014). These differences might be due to: (1) sequence differences between the mammalian GIT and *Drosophila* dGIT proteins (43% similar) (Bahri et al., 2009); (2) complete deletion of GIT proteins in our study, and hypomorphs in *Drosophila*; (3) differences in AZ organization at the fly NMJ compared to a mammalian AZ (Zhai and Bellen, 2004). Differing results with GITs across species is not unreasonable, as disruption of the ELKS/CAST homolog *bruchpilot* (Kaeser et al., 2009) differentially effects synaptic transmission in *Drosophila* and mammals. Interestingly, disruption of dGIT function in high external Ca²⁺ did lead to increased presence of synaptopHluorin on the plasma membrane at 10 and 50 Hz. This result could be interpreted as the loss of GIT resulting in an increase in P_r at the *Drosophila* NMJ, which would be consistent with our findings in the calyx. Finally, dGIT regulates endocytosis in *Drosophila*, but it remains to be tested in mammals.

In summary, we have demonstrated for the first time that GITs are important presynaptic regulators of synaptic strength. This regulation of synaptic strength by GITs is likely to contribute to the disruption of the neuronal circuit output that leads to hearing loss phenotypes, impairment in fear memory, and spatial and object learning. Future studies will resolve the mechanisms by which GITs regulate RRP dynamics and their roles in the early stages of auditory processing and neurological diseases.

EXPERIMENTAL PROCEDURES

Afferent fiber stimulation and presynaptic recordings were performed as previously described on P16–21 mice (Chen et al., 2013, 2015). Confocal images were acquired with a Zeiss LSM 780 and analyzed using Amira 5.6 and Fiji imaging analysis software. For EM experiments, Tecnai G2 Spirit BioTwin TEM was used, and images were taken with a Veleta CCD camera (Olympus) operated by TIA software (FEI) and analyzed using Fiji. All procedures were performed in accordance with the animal welfare guidelines of MPFI Institutional Animal Care and Use Committee.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2015.10.042>.

AUTHOR CONTRIBUTIONS

M.S.M., W.D., K.G., B.D., D.G.-G., N.K., and S.M.Y. designed experiments, carried out experiments, and analyzed data. R. Satterfield produced rAD vectors. M.S.M. and S.M.Y. wrote the manuscript. R. Schmalzigaug and R.T.P. provided mouse lines and edited the manuscript.

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