Abstract

G protein-gated inwardly rectifying potassium (GIRK) channels play an important role in regulating neuronal excitability. Sorting nexin 27b (SNX27b), which reduces surface expression of GIRK channels through a PDZ domain interaction, contains a putative Ras-association (RA) domain with unknown function. Deleting the RA domain in SNX27b (SNX27b-ΔRA) prevents the down-regulation of GIRK2c/GIRK3 channels. Similarly, a point mutation (K305A) in the RA domain disrupts regulation of GIRK2c/GIRK3 channels and reduces H-Ras binding in vitro. Finally, the dominant-negative H-Ras (S17N) occludes the SNX27b-dependent decrease in surface expression of GIRK2c/GIRK3 channels. Thus, the presence of a functional RA domain and the interaction with Ras-like G proteins comprise a novel mechanism for modulating SNX27b control of GIRK channel surface expression and cellular excitability.

Introduction

G protein-gated inwardly rectifying potassium (GIRK or Kir3) channels have been implicated in the pathophysiology of diseases such as epilepsy, Down’s syndrome, and drug addiction [1]. Like other inwardly rectifying K⁺ channels, GIRK channels conduct outward potassium current near the resting membrane potential, thereby hyperpolarizing the cell and decreasing membrane excitability. Stimulation of G protein-coupled receptors (GPCRs) that couple to pertussis toxin-sensitive G proteins (Gαi/o family) activates GIRK channels through G protein Gβγ subunits [2–5]. Four mammalian GIRK subunits are expressed in the brain, GIRK1, GIRK2, GIRK3 and to a lesser extent GIRK4. These subunits assemble into heterotetramers of primarily GIRK1/2, GIRK1/3 or GIRK2/3, and homotetramers of GIRK2 [1]. Neurotransmitters, such as GABA, serotonin and adenosine, stimulate their cognate GPCRs and drive the G protein-dependent activation of GIRK channels, leading to inhibition of neuronal activity [6]. Despite their importance in regulating neuronal excitability, little is known about intracellular trafficking pathways and proteins that control expression of GIRK channels.

We recently demonstrated that sorting nexin 27 (SNX27) regulates GIRK channels through a PDZ domain interaction [7,8]. SNX27 is a member of a large family of sorting nexins (SNXs) that are classified by the presence of lipid-binding phox-homology (PX) domain [9]. Sorting nexins have been identified across different phyta, with 33 different mammalian SNXs [9]. This rapidly expanding family of cytoplasmic proteins is important for regulating protein trafficking [9]. For example, SNX9 participates in the formation of the narrow neck of endocytic vesicles before scission occurs [10], SNX13 controls trafficking of the G protein Gαi subunit [11], SNX3 is involved in neural development [12], and SNX17 is involved in trafficking of the amyloid precursor protein (APP) implicated in Alzheimer’s disease [13]. SNX27 associates with a variety of signaling proteins, including GIRK channels [7,8], cytohesin-associated scaffolding protein (CASP) [14], 5-HT4a receptors [15], β2 adrenergic receptors [16], NMDA receptors [17], diacylglycerol kinase zeta (DGKζ) [18] and amyloid precursor protein (APP) [19]. SNX27 protein has been detected in synaptosomes purified from mouse cortex [20], rat hippocampal neurons [7] and lymphocytes [14], and exists in two splice forms, SNX27a and SNX27b [21]. Interestingly, SNX27b may provide a link between drugs of abuse and changes in neuronal excitability [21]. Thus, SNX27 plays an important role in signal transduction and protein trafficking.

SNX27 has three functional domains; PDZ (PSD95/Disc large/Zona occludens), PX (Phox phagocytic oxidase domain) and RA (Ras-association) domains. The PX domain binds specifically to PI(3)P and targets SNX27 to the early endosomes (EE) [8]. The PDZ domain mediates a direct protein-protein interaction with a
Class I PDZ binding motif located in the C-terminal domain of target proteins [7,8,15–19]. For GIRK channels, the PX and PDZ domains are essential for targeting GIRK3-containing channels to the early endosomes and redirecting away from the plasma membrane, thereby reducing surface expression and producing smaller GIRK currents [7,6]. However, the role of the RA domain in the SNX27b-dependent regulation of GIRK channel function and neuronal excitability is largely unknown. In general, the RA domain is hypothesized to be involved in facilitating the exchange of GTP for GDP on small Ras-like G proteins [22]. Recently, the carboxyl terminal fragment of SNX13, SNX27 and SNX31, which contains the RA domain, was reported to associate with activated H-Ras in an in vitro binding assay [19]. The functional consequence of this interaction remains poorly understood.

In the present study, we investigated the function of the RA domain in the SNX27b-dependent regulation of GIRK channels. We provide new evidence that a functional RA domain is essential for SNX27b control of trafficking and down-regulation of GIRK3-containing channels. These findings provide the first clues into a pathway for regulating SNX27b function in the brain, establishing a possible new link between Ras signaling and neuronal excitability.

**Results and Discussion**

We have previously observed that overexpression of SNX27b leads to accumulation of GIRK3-containing channels in early endosomes and subsequent reduction in surface expression of GIRK channels (Figure 1A) [8]. To investigate the function of the SNX27b RA domain, we first constructed a mutant of SNX27b that lacks the RA domain (SNX27b-ARA) (Figure 1B). We examined the functional effect of SNX27b by measuring the amplitude of GABA<sub>B</sub> receptor-activated GIRK currents in HEK293T cells expressing GIRK2c/GIRK3 channels along with GABA<sub>A</sub> and GABA<sub>B</sub> receptors. Coexpression of SNX27b with GIRK2c/GIRK3 channels significantly reduced the amplitude of GABA<sub>B</sub> receptor-activated currents (I<sub>BAclocfen</sub>) measured with a saturating concentration (100 μM) of the GABA<sub>B</sub>R agonist baclofen (~10 pA/pF vs. ~41 pA/pF for control, Figure 1C, 1D). In contrast to SNX27b, expression of SNX27b-ARA did not significantly change I<sub>BAclocfen</sub> (~55 pA/pF) with GIRK2c/GIRK3 channels (Figure 1C, D). Similarly, SNX27b-ARA did not significantly alter I<sub>BAclocfen</sub> for HEK293T cells expressing GIRK1/3 channels (Figure 1D), in contrast to previous studies where wild-type SNX27b consistently reduced GIRK1/3 currents [7,8,23]. Thus, removal of the RA domain appeared to interfere with the ability of SNX27b to regulate GIRK3-containing channels in HEK293T cells.

To determine whether SNX27b-ARA continued to associate with GIRK2c/3 channels, we next examined the localization of GIRK2c/3 channels in the presence of SNX27b-ARA. We used bimolecular fluorescence complementation (BiFC), a novel method for monitoring the expression pattern of GIRK2c/3 heterotrimers (Figure 2A) [24]. BiFC involves genetically fusing non-fluorescent, complementary fragments of YFP to two different proteins. When the two proteins interact, as would be the case for two GIRK subunits (e.g. GIRK2c and GIRK3), the two non-fluorescent fragments associate to reconstitute a functional fluorescent protein [24]. CYFP was fused to the N-terminal domain of GIRK2c (G<sup>CYFP</sup>GIRK2c) and NYFP was fused to the N-terminal domain of GIRK3 (G<sup>NYFP</sup>GIRK3) (Figure 2A), allowing the unencumbered C-terminal domains of the channels to freely associate with SNX27b. Coexpression of G<sup>CYFP</sup>GIRK2c and G<sup>NYFP</sup>GIRK3 led to fluorescently labeled proteins that expressed diffusely in the cytoplasm and functionally coupled with GABA<sub>B</sub> receptors on the plasma membrane (Figure 2A, B Control), similar to a previous study [25]. Expression of SNX27b with G<sup>CYFP</sup>GIRK2c/N<sup>NYFP</sup>GIRK3 induced puncta of fluorescence in the cytoplasm, typical of endosomal localization (Figure 2Bii) [7,6]. Expression of SNX27b-ARA on the other hand, resulted in a diffuse pattern of G<sup>CYFP</sup>GIRK2c/N<sup>NYFP</sup>GIRK3 fluorescence (Figure 2Bii), similar to that observed in control cells. Thus, deleting the RA domain appeared to disrupt the interaction of SNX27b-ARA with GIRK2c/GIRK3 channels, altering the ability of SNX27b to control the trafficking and surface expression of GIRK channels. This result was unexpected since SNX27b-ARA contains intact PDZ and PX domains, which would be expected to associate with the PDZ motif of GIRK2c/3 and endosomal PI(3)P, respectively. Introducing a mutation (Y31L) in the PDZ domain of SNX27b known to disrupt PDZ binding [7] led to a similar diffuse pattern of G<sup>CYFP</sup>GIRK2c/N<sup>NYFP</sup>GIRK3 fluorescence (Figure 2Biii), suggesting a potential defect in PDZ binding when the RA domain is deleted. To rule out the possibility that deleting the RA domain simply altered the expression of SNX27b itself, we examined the localization of YFP-tagged SNX27b-ARA (SNX27b-ARA-YFP). Discrete puncta of green fluorescence typical of endosomal localization were observed in HEK293T cells expressing either SNX27b-YFP or SNX27b-ARA-YFP (Figure 2C), indicating that SNX27b-ARA is expressed and targeted properly.

To investigate the possible mechanism underlying the inability of SNX27b-ARA to regulate GIRK channel expression, we examined the effect of selective point mutations in the RA domain. Previously, RA domains have been shown to interact with Ras-like proteins [19,26]. Examination of a high resolution structure complex of the Ras and the Ras-interacting domain (RID) of Raf or Ra, which is homologous to the RA domain of SNX27b, revealed several basic amino acids (R20, K32, K52) in the RID that electrostatically attract and form hydrogen bonds with acidic residues in Ras-Switch II (e.g. D33) [27–30] (Figure 3B). We aligned the sequence of SNX27b RA domain with the RID domains of Raf/GDS and Raf proteins (Figure 3A) and identified four basic amino acids in the putative RID of SNX27b that might be involved in binding Ras (R276, R288, K291, K305). We created three mutants, R276A, R288A/K291A and K305A SNX27, which neutralized these basic amino acids, and examined their effects on the expression pattern of G<sup>CYFP</sup>GIRK2c/N<sup>NYFP</sup>GIRK3 channels using BiFC (Figure 3C). Similar to SNX27b-ARA, each point mutation appeared to impair the ability of SNX27b to induce punctate fluorescence for G<sup>CYFP</sup>GIRK2c/N<sup>NYFP</sup>GIRK3 channels (Figure 3C). Focusing on SNX27b-K305A, immunostaining revealed partial co-localization with the early endosomal marker, early endosome antigen 1 (EEA1), (Figure 3D), similar to wild-type SNX27b [8]. Thus, the PX domain which targets SNX27b to the early endosome appears to be unaffected by the K305A mutation [19]. We then examined the effect of the K305A mutation in SNX27b on GIRK2c/3 channels expressed in HEK293T cells. Similar to SNX27b-ARA, SNX27b-K305A did not induce I<sub>BAclocfen</sub> for GIRK2c/GIRK3 channels (Figure 3E).

To examine whether the K305A mutation in the RA domain interfered with the association of SNX27b with H-Ras, we performed a pull-down experiment using recombinant proteins expressed and purified from E. coli (Figure 4A, 4B) [19]. A GST fusion protein containing the C-terminal region of SNX27b (Asp272-Tyr526) that includes the RA domain (GST-R<sub>AWT</sub>) pulled down a His<sub>6</sub>-tagged constitutively active form H-Ras, His<sub>6</sub>-H-Ras<sub>G12V</sub>CA (Figure 4C). By contrast, GST-R<sub>AK305A</sub> showed weak or no association with His<sub>6</sub>-H-Ras<sub>G12V</sub>CA (Figure 4C).
Figure 1. Deletion of RA domain in SNX27b impairs functional regulation of GIRK channels. A, Cartoon shows model of GIRK channels regulation by SNX27b. GIRK channels recycle through endosomal compartments. SNX27b associating with GIRK2c/3 channels in the early endosome (EE) reduces plasma membrane expression (PM) by targeting some channels to the late endosome (LE). B, SNX27 contains three functional domains; PDZ, PX and RA. GIRK2c and GIRK3 contain a C-terminal PDZ binding motif (-E(S/N)ESKV). C, Examples of baclofen-induced (100 μM) and Ba^{2+}-sensitive (1 mM Ba^{2+}) currents in HEK293T cells transfected with cDNA for GABAB1a/B2 receptors, GIRK2c/GIRK3 and either control vector, SNX27b or SNX27b-ΔRA. Agonist-independent basal currents are revealed by inhibition with 1 mM Ba^{2+}. D, Average baclofen-induced current densities (IpA) for control (−41.3 ± 5.2 pA·pF⁻¹, n = 8), SNX27b (−11.0 ± 3.6 pA·pF⁻¹, n = 8) and SNX27b-ΔRA (−55.9 ± 8.2 pA·pF⁻¹, n = 13) with GIRK2c/GIRK3. E, Average IpA for control (−15.7 ± 3.6 pA·pF⁻¹, n = 6) and SNX27b-ΔRA (−18.6 ± 4.9 pA·pF⁻¹, n = 5) with GIRK1/GIRK3 (**P<0.05, one way ANOVA followed by Bonferroni post hoc test; n.s. – not significant). doi:10.1371/journal.pone.0059800.g001
A point mutation consistently reduced by \( \sim 75\% \) the association of the RA domain with H-RasG12VCA (Figure 4D; \( n = 4 \)). Taken together, these findings raise the possibility that the interaction of H-Ras with the RA domain is an important step in the SNX27-dependent regulation of GIRK channels.

To explore this hypothesis further, we examined the effect of a dominant-negative (S17N) form of H-Ras (H-RasS17NDN). We hypothesized that H-RasS17NDN would compete for endogenous Ras and possibly impair the ability of SNX27b to regulate GIRK2c/3 channels. In HEK293T cells coexpressing GIRK2c/3 with H-RasS17NDN, \( I_{\text{baclofen}} \) was indistinguishable from that in HEK293T cells coexpressing GIRK2c/3, H-RasS17NDN and SNX27b (Figure 5B, 5C), suggesting SNX27b regulation of GIRK channels required activated Ras. Interestingly, expression of H-RasS17NDN alone not only reduced \( I_{\text{baclofen}} \) (Figure 5B, 5C) but also reduced \( I_{\text{Barium}} \) compared to control cells (Figure 5B).
suggesting a direct effect of the dominant negative Ras on channel expression perhaps mediated by endogenous SNX27. Indeed, Lauffer et al [16] and Cai et al [17] have demonstrated endogenous expression of SNX27b in HEK293 cells and that knockdown of SNX27 leads to reduced surface expression of β adrenergic receptors [16]. Consistent with this, we attempted to measure endogenous SNX27b in the HEK293T cells and detected low levels of protein (data not shown). Thus, SNX27 levels may be tightly regulated where too little or too much SNX27 can interfere with GIRK trafficking to the plasma membrane.

To learn more about the role of Ras proteins, we examined the effect of expressing the constitutively active H-RasG12VCA with GIRK2c/3 in the absence and presence of SNX27. Surprisingly, H-RasG12VCA alone reduced IBaclofen for GIRK2c/3 currents and coexpression with SNX27b did not reduce the current further (Figure S1A). However, H-RasG12VCA did not affect the basal IBarium for GIRK2c/3 channels, in contrast to the dominant negative effect of H-RasS17NDN (Figure S1B). Furthermore, SNX27b reduced IBarium in the presence of H-RasG12VCA. Taken together, these differences between the dominant-negative and constitutively active H-Ras forms are consistent with a model where the dominant negative Ras effect on GIRK2c/3 involves endogenous SNX27 and the constitutively active form affects other signaling pathways (e.g. GPCR function). We therefore developed an alternative strategy to probe the possible effect of the dominant negative H-Ras by modifying the GIRK channels. Previously, we identified two variants of GIRK2/3 channels that were not regulated by SNX27b; GIRK2a, which lacks a PDZ-binding motif, and GIRK3-RR, which contains two Arginine substitutions in the C-terminal PDZ motif that selectively disrupt PDZ binding [7] (Figure 5A). Coexpression of SNX27b with these GIRK2a/GIRK-RR variants did not lead to smaller receptor-activated currents [7]. In contrast to wild-type channels, expression of H-RasS17NDN did not significantly change IBaclofen or IBarium for

Figure 3. K305A point mutation in SNX27b RA domain disrupts functional regulation of GIRK2c/GIRK3 channels. A, Alignment of two different RA domains: a RID (Ras interacting domain) from RalGDS and a RA domain of RGL, with RA domain of SNX27. Residues implicated in Ras binding in Raf and RalGDS domains [28–30] are highlighted in red. B, High-resolution structure shows R20, K32 and K52 at the binding interface of RID and Ras [26]. C, RA domain mutations impair the ability of SNX27b to induce formation of GIRK2c/3 puncta using BiFC. HEK293T cells were co-transfected with cDNA for CYGIRK2c/NYGIRK3 and either wild-type SNX27b (i), SNX27b-R276A (ii), SNX27b-R288A/K291A (iii) or SNX27b-K305A (iv). Green fluorescence indicates molecular complementation of CYGIRK2c/NYGIRK3. Scale bar: 10 μm. D, Colocalization of wild-type SNX27b (SNX27b-YFP; green) and SNX27b-K305A (anti-SNX27; green) with an early endosomal marker (anti-EEA1, green). Scale bar: 5 μm. E, Average IBaclofen currents for control (−53.4±9.4 pA·pF⁻¹, n = 5), SNX27b (−8.05±3.44 pA·pF⁻¹, n = 6) and SNX27b-K305A (−52.9±8.8 pA·pF⁻¹, n = 10) with GIRK2c/GIRK3 channels.

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GIRK2a/GIRK3-RR channels (Figure 5D,E). Taken together, these results suggest that Ras S17NDN interferes with both endogenously and exogenously expressed SNX27, though additional experiments are needed to probe the role of endogenous SNX27b more explicitly using a knockout strategy.

In summary, the RA domain of SNX27b has an unexpected but important role in modulating SNX27b control of GIRK2c/GIRK3 trafficking. Our findings contribute to an emerging idea that endomembrane-associated Ras-like G proteins are capable of signaling through selective interactions with RA domain-containing proteins, in addition to their well-known plasma membrane signaling pathway [31]. The molecular details on the putative interaction between the RA domain and H-Ras and how it regulates PDZ binding remain unclear. One possibility is the complex of H-Ras with SNX27b recruits sorting machinery required to recycle GIRK channels from the endosome to lysosome or back to the membrane; the destination being dependent on the level of SNX27b present in the cell. Alternatively, the RA domain may regulate PDZ binding through some unknown mechanism. Interestingly, we demonstrated previously that the β2-β3 loop of the SNX27-PDZ domain is critical for GIRK3 binding [7], perhaps offering a mechanism for regulating PDZ binding. Nonetheless, the discovery that the RA domain is important for SNX27b function could provide a novel mode for regulating SNX27b regulation of these proteins.

Methods

Molecular Biology

For bimolecular fluorescence complementation (BiFC), the N-terminal half (amino acids 1 to 155) of the yellow fluorescent protein (YFP) or the C-terminal half (amino acids 156 to 231) of YFP was cloned into XhoI-XbaI sites of...
Human Embryonic Kidney 293 (HEK293) cells were grown in Dulbecco’s modified Eagle’s medium, DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1 m sodium pyruvate, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 10% (v/v) Fetal Bovine Serum (FBS) at 30 or 37 °C in 5% CO2 humidified atmosphere. HEK293 cells were transiently and 10% (v/v) Fetal Bovine Serum (FBS) at 30 or 37 °C transfected with cDNA using Transfectin (BioRad) or Lipofectamine 2000 (Life Technologies). Whole-cell patch-clamp recordings were performed 48 h post transfection using borosilicate glass electrodes from Missouri S&T cDNA Resource Center (http://www.cdna.org) and subcloned into the pHi8-2T vector (GE Biosciences). Point mutations were introduced by site-directed mutagenesis kit (QuikChange II XL, Agilent) and confirmed by automatic DNA sequencing. SNX27-RA construct has an internal deletion encompassing the RA domain (Asp272-Tyr526) was fused to GST by sub cloning into EcoRI and BamHI sites of pGEX-2T vector (GE Biosciences). Point mutations were performed as described by Ghai et al. [19]. Briefly, purified GST (control) or a GST fused to SNX27-Asp272-Tyr526 (SNX27-RA) was mixed with His6-H-RasG12VCA for 1 h at room temperature in pull-down buffer (50 mM Tris, 200 mM NaCl, 1 mM DTT, and 1% IGEPAL-CA630, pH 8.0). Protein-protein complexes were pelleted by centrifugation using glutathione sepharose beads, separated on SDS-PAGE gel, transferred to nitrocellulose and exposed to anti-His6 antibodies for western detection of His6-H-RasG12VCA.

Statistical and Protein Analyses
Data are presented as mean ± SEM. Statistical differences were examined using unpaired Student’s t-test for two groups or one-way ANOVA with Bonferroni post hoc test for multiple groups. P<0.05 was considered statistically significant. Protein sequences were first aligned using ClustalV algorithm in LaserGene MegAlign software ver.9.0.4 (DNASTAR Inc., WI, USA) and manually adjusted.

Supporting Information
Figure S1 SNX27b-dependent down-regulation of basal GIRK2c/GIRK3 channels is maintained in the presence of constitutively active H-Ras (H-RasG12VCA). A. Bar graph shows I_{Ba,B} for control (GIRK2c/GIRK3 alone; –66.4 ± 7.2 pA/pF, n = 6) and GIRK2c/GIRK3 plus H-RasG12VCA (–12.9 ± 1.8 pA/pF, n = 7), GIRK2c/GIRK3 plus H-RasG12VCA and SNX27b (–9.75 ± 1.5 pA/pF, n = 7), and GIRK2c/GIRK3 plus SNX27b alone (–12.4 ± 3.5 pA/pF, n = 9). B. Bar graph shows I_{Ba,B} for control (GIRK2c/GIRK3 alone; –24.6 ± 3.0 pA/pF, n = 6) and GIRK2c/GIRK3 plus H-RasG12VCA (–23.5 ± 7.1 pA/pF, n = 5), GIRK2c/GIRK3 plus H-RasG12VCA and SNX27b (–57.9 ± 0.9 pA/pF, n = 7), and GIRK2c/GIRK3 plus SNX27b alone (–48.4 ± 1.5 pA/pF, n = 8).

*p<0.05, one way ANOVA followed by Bonferroni post hoc test; n.s. – not significant. (TIF)

Author Contributions
Conceived and designed the experiments: PAS BB LB. Performed the experiments: BB JJT NMT MYN LB FC KB. Analyzed the data: BB LB KB PAS PC. Contributed reagents/materials/analysis tools: BB LB KB PAS PC. Wrote the paper: PAS BB LB.
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