$G\beta\gamma$ Binds to the Extreme C Terminus of SNAP25 to Mediate the Action of $G_{i/o}$ -Coupled G Protein–Coupled Receptors

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Received September 1, 2015; accepted October 30, 2015

ABSTRACT

$G_{i/o}$ -coupled G protein–coupled receptors can exert an inhibitory effect on vesicle release through several G protein–driven mechanisms, more than one of which may be concurrently present in individual presynaptic terminals. The synaptosomal-associated protein of 25 kDa (SNAP25) is a key downstream effector of $G\beta\gamma$ subunits. It has previously been shown that proteolytic cleavage of SNAP25 by botulinum toxin A reduces the ability of $G\beta\gamma$ to compete with the calcium sensor synaptotagmin 1 (Syt1) for binding to SNAP25 in a calcium-dependent manner. These truncated SNAP25 proteins sustain a low level of exocytosis but are unable to support serotonin-mediated inhibition of exocytosis in lamprey spinal neurons. Here, we generate a SNAP25 extreme C-terminal mutant that is deficient

in its ability to bind $G\beta\gamma$ while retaining normal calcium-dependent Syt1 binding to soluble *N*-ethylmaleimide attachment protein receptor (SNARE) and vesicle release. The SNAP25 Δ 3 mutant, in which residue G204 is replaced by a stop codon, features a partial reduction in $G\beta_{1\gamma_2}$ binding in vitro as well as a partial reduction in the ability of the lamprey 5-hydroxytryptamine_{1b}-type serotonin receptor to reduce excitatory postsynaptic current amplitudes, an effect previously shown to be mediated through the interaction of $G\beta\gamma$ with SNAP25. Syt1 calcium-dependent binding to SNAP25 Δ 3 was reduced by a small extent compared with the wild type. We conclude that the extreme C terminus of SNAP25 is a critical region for the $G\beta\gamma$ -SNARE interaction.

Introduction

Regulation of neurotransmitter and hormone release is an essential component of homeostasis and plasticity in many systems. Inhibitory G protein–coupled receptors (GPCRs) protect exocytotic machinery from overstimulation by inhibiting exocytosis and the release of vesicle contents into the extracellular space. They do so by several mechanisms. One well-studied mechanism is the direct binding of G protein $\beta\gamma$ subunits to voltage-gated calcium channels, leading to voltage-dependent inhibition of calcium entry (Ikeda, 1996). The ability of $G_{i/o}$ -coupled GPCRs to inhibit exocytosis downstream of voltage-gated calcium channels is well documented in a number of different cell types (Blackmer et al., 2001; Delaney et al., 2007; Yoon et al., 2008; Iremonger and Bains, 2009; Zhao et al., 2010; Hamid et al., 2014). We have previously demonstrated that inhibition can also occur through the direct interaction of $G\beta\gamma$ with the soluble *N*-ethylmaleimide attachment protein receptor (SNARE) protein SNAP25 (Blackmer et al., 2005; Gerachshenko et al., 2005;

Yoon et al., 2007). $G\beta\gamma$ competes in a calcium-dependent manner with the fusogenic calcium sensor synaptotagmin 1 (Syt1) for binding sites on SNARE (Blackmer et al., 2005; Yoon et al., 2007). Upon calcium binding, Syt1 binds to the SNARE complex and demixes and disorders lipid membranes to promote fusion of the vesicle membrane with the cell membrane (Zhang et al., 2002; Bai et al., 2004; Lai et al., 2011). Syt1 calcium-dependent binding to SNARE complexes requires three negatively charged residues on the SN2 helix of SNAP25 located proximally to the C terminus (Zhang et al., 2002). Both the N terminus (Wells et al., 2012) and the C terminus of SNAP25 (Gerachshenko et al., 2005; Yoon et al., 2007) contain key residues for the interaction with $G\beta\gamma$. Alanine mutagenesis of eight residues on SNAP25 reduces its ability to bind $G\beta\gamma$ without disrupting its ability to bind Syt1 (Wells et al., 2012). Injection of an exogenous mutant SNAP25 containing these eight residues mutated to Ala with a botulinum toxin E (BoNT/E) resistance site into presynaptic neurons, along with BoNT/E light-chain protease, restores fusion while abrogating the ability of serotonin [5-hydroxytryptamine (5-HT)] to inhibit vesicle release in lamprey reticulospinal axons (Wells et al., 2012). Interestingly, data were recently shown supporting the notion that a distinct “microarchitecture” is prevalent at presynaptic 5-HT_{1b} receptors, predisposing them to this mode of $G\beta\gamma$ -driven inhibition, whereas other microarchitectures both within the same synapses and within

This work was supported by the National Institutes of Health National Eye Institute [R01- EY010291]; and the National Institutes of Health National Institute of Mental Health [MH101679- 01A1]. ZZ was supported by the T32 Training in Pharmacological Sciences, T32 GM07628.

All authors declare that no conflicts of interest exist for them within the contents of this article.

dx.doi.org/10.1124/mol.115.101600.

ABBREVIATIONS: BoNT/E, botulinum toxin E; GPCR, G protein–coupled receptor; GST, glutathione S-transferase; 5-HT, 5-hydroxytryptamine; SNARE, soluble *N*-ethylmaleimide attachment protein receptor; Syt1, synaptotagmin 1; t-SNARE, target soluble *N*-ethylmaleimide attachment protein receptor; WT, wild type.

other types of synapses function through other mechanisms, such as the $G\beta\gamma$ -mediated inhibition of calcium influx through voltage-gated calcium channels at the $GABA_B$ receptor (Hamid et al., 2014). From this, our current understanding of presynaptic inhibition is that presynaptic $G_{i/o}$ -coupled GPCRs function through a variety of mechanisms, including the direct binding of $G\beta\gamma$ to SNAP25.

While the molecular requirements of the $G\beta\gamma$ -SNAP25 interaction are reasonably well understood, much less is known about the physiology and pathophysiology of the interaction. It is not currently known which $G_{i/o}$ -coupled GPCRs work through this mechanism, or whether it is used in only certain cellular contexts. Further, it is not clear whether a specific disease state is dependent upon dysregulation of the $G\beta\gamma$ -SNARE interaction. Presynaptic $G_{i/o}$ -coupled GPCRs have been shown to be relevant drug targets for anxiety and schizophrenia (Swanson et al., 2005; Patil et al., 2007), but the mechanisms for these effects are not known. The $G\beta\gamma$ -SNARE interaction has been shown to be functionally relevant for a number of presynaptic $G_{i/o}$ -coupled GPCRs (Glitsch, 2006; Delaney et al., 2007; Heinke et al., 2011; Zhang et al., 2011; Betke et al., 2012). To explore these and other potential areas of therapeutic relevance further, a transgenic model deficient in the $G\beta\gamma$ -SNARE interaction is required. The generation of such a model presents a number of challenges. A knockout-based strategy would be unsuitable. There are five $G\beta$ subunits and 12 $G\gamma$ subunits (Betke et al., 2012) in the human genome, indicating a high degree of redundancy, making knockout or mutagenesis of $G\beta\gamma$ subunits unfeasible (Betke et al., 2012). Although studies have been conducted pertaining to the distribution of $G\beta$ and $G\gamma$ subunits in the brain (Betke et al., 2014), it is not currently known whether a specific combination of subunits is responsible for the $G\beta\gamma$ -SNARE interaction. The possibility of multiple effectors for any given $G\beta\gamma$ would also be a confounding factor in such a knockout. A knockout of SNAP25 would also be unsuitable, as SNAP25 knockouts are neonatally lethal (Washbourne et al., 2002). Finally, the mutations proposed in Wells et al. (2012) are also unsuitable for introduction into a transgenic animal, as the large number of mutations (eight) spread throughout the eight exons (Oyler et al., 1989) makes homologous recombination challenging. Insertion of the eight mutations as a minigene would also be unsuitable, as the full-length SNAP25 transcript is differentially spliced into two splice variants with differing roles, SNAP25a and SNAP25b. Thus, to obtain a mutation that was suitable for introduction as a transgene, further exploration is required. Here, we have identified an extreme C-terminal mutation suitable for introduction into the native mouse SNAP25 that reduces $G\beta\gamma$ binding while retaining most Syt1 binding and supporting vesicle fusion.

Materials and Methods

Plasmids. The open reading frames for mouse SNAP25b and the C2AB domain of synaptotagmin 1 were subcloned into the glutathione *S*-transferase (GST) fusion vector pGEX-6p-1 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for expression in the Rosetta DE3 strain of *Escherichia coli* (Merck Millipore, Darmstadt, Germany). Mutagenesis of SNAP25 was accomplished via the method of overlapping primers. Sequencing of all plasmids was performed using BigDye Terminator (Applied Biosystems,

Foster City, CA) dyes and resolved on an ABI 3730 DNA Analyzer (Applied Biosystems).

Antibodies. The antibody for mouse anti-Syt1 C2AB (clone 41.1) was obtained from Synaptic Systems (Goettingen, Germany). The goat anti-GST antibody containing conjugated DyLight 800 and the goat anti-rabbit IgG antibody containing IRDye700DX were both from Rockland Immunochemicals (Gilbertsville, PA).

SNAP25 and Synaptotagmin 1 Protein Purification. Recombinant bacterially expressed GST-fusion proteins were expressed in *E. coli* strain Rosetta DE3 (Merck Millipore). SNAP25 protein expression was induced with 100 μ M isopropyl β -D-1-thiogalactopyranoside for 16 hours at 25°C. Syt1 (residues 96–422) protein expression was induced with 400 μ M isopropyl β -D-1-thiogalactopyranoside for 8 hours at 30°C. Bacterial cultures were pelleted and washed once with phosphate-buffered saline before resuspension in 25 mM HEPES-KOH (pH 8.0); 150 mM KCl; 5 mM 2-mercaptoethanol; standard concentrations of the protease inhibitors leupeptin, aprotinin, and pepstatin; 200 μ M phenylmethylsulfonyl fluoride; and 1 mM EDTA. Resuspended cells were lysed with a sonic dismembrator at 4°C for 5 minutes. Lysates were cleared via ultracentrifugation at 26,000g for 20 minutes in a TI-70 rotor (Beckman Coulter, Fullerton, CA). For GST-Syt1, lysates were treated with 0.1 mg/ml DNase and RNase prior to purification to remove residual nucleic acids. SNAP25 fusion proteins were then purified from cleared lysates by affinity chromatography on Pierce Glutathione Agarose (Pierce, Rockford, IL). Lysates were exposed to resin for 4 hours before being washed once with resuspension buffer containing 1% Triton X-100 (Dow Chemical, Midland, MI). After centrifugation at 3000g, resins were then washed once with elution buffer [25 mM HEPES-KOH (pH 8.0), 150 mM KCl, 5 mM 2-mercaptoethanol, 0.5% *n*-octylglucoside, 1 mM EDTA, and 10% glycerol]. SNAP25 and Syt1 C2AB proteins were eluted from GST fusion proteins immobilized on resin via proteolytic cleavage with a GST-tagged fusion of rhinovirus 3C protease. Protein concentrations were determined with a Bradford assay kit (Thermo Fisher Scientific, Waltham, MA), and purity was assessed by SDS-polyacrylamide gel electrophoresis.

$G\beta\gamma$ Purification. $G\beta_1\gamma_1$ was purified from bovine retina according to previously published methods (Mazzoni et al., 1991). $G\beta_1\gamma_2$ dimers were expressed in Sf9 cells and purified using the method of Kozasa and Gilman (1995) with the following exceptions: frozen Sf9 cell pellets were lysed by gentle sonication pulse, 10 seconds on and 20 seconds off for 3 minutes at 30% intensity on ice. $G\beta_1\gamma_2$ dimers were affinity-purified from detergent-solubilized crude cell membrane using Talon cobalt resin (Clontech, Mountain View, CA) followed by three rounds of dialysis in the following buffer: 20mM HEPES, 100 mM NaCl, 10 mM β -mercaptoethanol, 0.8% *n*-octylglucoside, and 10% glycerol, pH 8.0.

Biotinylation. Purified recombinant SNAP25 or GST was diluted to 1 mg/ml in 25 mM HEPES-KOH (pH 8.0), 150 mM KCl, 0.5% *n*-octylglucoside, 1 mM EDTA, and 10% glycerol. A stock solution of EZ-Link NHS-SS-Biotin (Pierce, Rockford, IL) was made by dissolving 6 mg in 1 ml of H_2O . Biotinylation reagents were added slowly to SNAP25 proteins to a 20:1 molar excess. Reactions were allowed to proceed for 30 minutes at 25°C before removal of excess reagent via two rounds of dialysis in 2 l of 25 mM HEPES-KOH (pH 8.0), 150 mM KCl, 0.5% *n*-octylglucoside, 1 mM EDTA, and 10% glycerol. Biotinylation was verified via the Pierce Biotin Quantification Kit.

Alphascreen Binding Assays. Alphascreen luminescence measurements were performed in an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA) at 27°C. Biotinylated SNAP25 was diluted into a final concentration of 20 nM in assay buffer [20 mM HEPES (pH 7.0), 10 mM NaCl, 40 mM KCl, 5% glycerol, and 0.01% Triton X-100]. A concentration-response curve of purified 6xHis- $G\beta_1\gamma_2$ ranging from 1 μ M to 1 nM was made using assay buffer. After incubation while shaking for 5 minutes, Alphascreen Histidine Detection Kit (nickel chelate) acceptor beads were added to a final concentration of 20 μ g/ml in assay buffer. The assay plate was shaken for 30 minutes. At that point, Alphascreen Streptavidin Donor Beads

were added to a final concentration of 20 $\mu\text{g}/\text{ml}$ in low-light conditions. The final volume in the assay plate (384-well white PerkinElmer OptiPlate) was 25 μl . Plates were incubated for 1 hour at 27°C before being read in the EnSpire. Biotinylated GST (20 nM) in place of SNAP25 with the four highest concentrations of $G\beta_1\gamma_2$ was used as a negative control for nonspecific binding in each assay. EC_{50} concentrations of $G\beta_1\gamma_2$ were determined by sigmoidal dose-response curve fitting with variable slope.

GST Pulldown Assay. Five micrograms of GST-SNAP25 protein bound to glutathione-agarose resin was incubated with a 400 μM concentration of purified recombinant Syt1 C2AB domains for 1 hour at 4°C and washed 3 $\times$ with assay buffer [20 mM HEPES (pH 7.2), 80 mM KCl, 20 mM NaCl, and 0.2% *n*-octylglucoside] in a 1.5-ml Eppendorf tube (Eppendorf, Hauppauge, NY). Assay buffers would contain either 2 mM EGTA or 1 mM CaCl_2 . To reduce nonspecific binding, immobilized protein complexes were then transferred to a second 1.5-ml Eppendorf tube. Syt1-SNAP25 complexes were eluted with 20 μl of standard Laemmli sample buffer followed by separation via SDS-polyacrylamide gel electrophoresis. The presence of Syt1 C2AB was detected via Western blot with a mouse anti-Syt1 antibody. Western blots were imaged using the LI-COR Odyssey imager (LI-COR Biosciences, Lincoln, NE) with labeled antibodies: anti-GST (goat) antibody DyLight 800 conjugated and rabbit IgG (H&L) antibody IRDye700DX conjugated.

Electrophysiology and Microinjections. All studies were conducted using isolated spinal cords from sea lampreys (*Petromyzon marinus*). Sea lampreys were anesthetized with tricaine methanesulfonate (100 mg/l; Sigma-Aldrich, St. Louis, MO) and sacrificed by decapitation. Spinal cords were then dissected free of the tissue in an ice-cold Ringer's saline solution of the following composition: 100 mM NaCl, 2.1 mM KCl, 2.6 mM CaCl_2 , 1.8 mM MgCl_2 , 4 mM glucose, and 5 mM HEPES (pH 7.6). All animal experiments conformed to institutional guidelines (University of Illinois at Chicago Animal Care Committee).

For electrophysiological experiments, paired cell recordings were collected between reticulospinal axons and neurons of the spinal ventral horn. Recordings were obtained from axons of reticulospinal neurons with conventional sharp microelectrodes containing 1 M KCl, 5 mM HEPES-KOH (pH 7.2), and a mixture of SNAP25 and BoNT/E (65 $\mu\text{g}/\text{ml}$). The electrode had impedances from 20 to 50 M Ω . Recordings were obtained from postsynaptic neurons using whole-cell patch clamp under voltage-clamp conditions. Patch electrodes were filled with 102.5 mM CsMeSO_3 , 1 mM NaCl, 1 mM MgCl_2 , 5 mM EGTA, and 5 mM HEPES- CsOH (pH 7.2).

The light chain of BoNT/E (65 $\mu\text{g}/\text{ml}$; List Biologic Laboratories Inc., Campbell, CA) was stored at -20°C in 20 mM HEPES- NaOH (pH 7.4), 50 mM NaCl, and 1 mg/ml bovine serum albumin. Buffered solutions of BoNT/E were diluted as 5 μl with 20 μl of 2 M KMeSO_4 and 5 mM HEPES along with 20 μl of solution containing recombinant SNAP25 mutant proteins. SNAP25 proteins were stored at -20°C in a buffer containing 25 mM HEPES-KOH (pH 8.0), 150 mM KCl, 5 mM 2-mercaptoethanol, 0.5% *n*-octylglucoside, 1 mM EDTA, and 10% glycerol. SNAP25 solutions mixed with BoNT/E were diluted 1:5 with 2 M KMeSO_4 and 5 mM HEPES. BoNT/E and SNAP25 mutants were pressure microinjected through presynaptic microelectrodes using the Picospritzer II (Parker Hannifin, Hollis, NH). All presynaptic recordings were made within 100 μm of the synaptic contact between the paired neurons.

Statistics. All statistical tests and all concentration-response curve fitting (sigmoidal dose-response with variable slope) were performed using GraphPad Prism v.4.03 for Windows (GraphPad Software, La Jolla, California; www.graphpad.com).

Results

To explore the binding of a number of different SNAP25 mutants to $G\beta\gamma$, we developed an Alphascreen assay

(PerkinElmer) with higher throughput and greater dynamic range. In this assay, biotinylated recombinant mouse SNAP25 (biotinylated nonspecifically upon primary amine residues with EZ-Link NHS-SS-biotin) interacts with His-tagged $G\beta_1\gamma_2$ subunits purified from Sf9 cells inoculated with baculovirus. When the $G\beta\gamma$ -SNAP25 complex forms, the complex is anchored to an Alphascreen streptavidin-conjugated donor bead via the biotinylation on SNAP25 and an Alphascreen Ni-nitrilotriacetic acid acceptor bead via the His tag on $G\beta\gamma$. When the donor bead is illuminated with 680 nm of coherent light, dye molecules attached to it generate singlet oxygen, which can travel a short distance in solution and strike an adjacent acceptor bead. The acceptor bead generates 520–620 nm of light in response to singlet oxygen (Fig. 1A). High specificity for the $G\beta\gamma$ -SNARE interaction was observed, with minimal signal being generated in the absence of protein, but a large signal when 20 nM SNAP25 and 170 nM $G\beta_1\gamma_2$ are present in solution. As a control for nonspecific binding, 20 nM GST, a protein that does not interact with $G\beta\gamma$ (Yoon et al., 2007), was added to the solution. SNAP25 (20 nM) did not generate a signal in the presence of His-tagged 170 nM $G\alpha_i$ -GDP as a second nonspecific binding control (Fig. 1B).

The SNAP25 8A mutant (Wells et al., 2012) has eight $G\beta\gamma$ -binding residues on SNAP25 mutated to Ala. Two of those residues, R198 and K201, are at the C terminus of SNAP25 and within the final exon of the mRNA. Mutation of these two residues to Ala (termed SNAP25 2A) produced a 1.9-fold reduction in affinity for $G\beta\gamma$, whereas no change was observed in the ability of proteins containing these mutations to bind

Fig. 1. The Alphascreen $G\beta\gamma$ –SNAP25 protein-protein interaction assay. (A) Diagram of assay principle. Biotinylated SNAP25 interacts with His-tagged $G\beta_1\gamma_2$ subunits in vitro. The $G\beta\gamma$ -SNAP25 complexes are captured on Alphascreen Ni-nitrilotriacetic acid (Ni-NTA) acceptor beads via the His tag on $G\beta\gamma$, while simultaneously being captured on Alphascreen streptavidin donor beads via the biotinylation on SNAP25. If 680-nm light strikes a donor bead, singlet oxygen is generated and can travel a short distance in solution to strike an acceptor bead, which will generate 520- to 620-nm light to be detected by the plate reader. (B) Nonspecific binding controls for the Alphascreen assay ($n = 3$). Data are presented as the mean $\pm$ S.E.M.

Syt1 (Wells et al., 2012). We hypothesized that introduction of SNAP25 2A into lamprey reticulospinal axons along with subsequent removal of endogenous SNAP25 could decrease the inhibition of glutamate release into the synapse of lamprey presynaptic 5-HT receptors. To do this, we mutated residue D179 to Lys to make SNAP25 2A resistant to BoNT/E cleavage (Zhang et al., 2002).

Control experiments were first performed to ensure that terminals were filled following an injection into the presynaptic axon. Alexa 594 (1 mM) was included in the presynaptic

electrode solution along with BoNT/E. These were pressure injected into the axon. The postsynaptic neuron was filled with Alexa 488 (25 μ M) by diffusion from the patch pipette. The synaptic response to presynaptic action potentials was recorded in control prior to injection. Dye and BoNT/E were then pressure injected into the axon. The presynaptic axon was imaged using fluorescence microscopy with an excitation peak of 590 nm and a long pass emission filter (610 nm), and the postsynaptic with a 488-nm excitation and a bandpass emission filter (510–550 nm) (Fig. 2Bi). BoNT/E cannot access the primed ternary SNARE complex to cleave SNAP25. Thus, after approximately 5 minutes of recording, 300 pulses were administered at a rate of 1 Hz to remove all remaining primed vesicles (Gerachshenko et al., 2005; Wells et al., 2012). It is clear that, when labeling is present presynaptically, synaptic responses are abolished by BoNT/E (Fig. 2Bii).

It is possible to recover synaptic transmission in terminals in which a recombinant BoNT/E-resistant SNAP25 is co-injected into the presynaptic axon with BoNT/E. In a previous study, a SNAP25 containing the D179K mutation was injected into the presynaptic neuron along with BoNT/E, restoring excitatory postsynaptic current (EPSC) amplitudes to $95 \pm 11\%$ of control. In those experiments, subsequent application of 1 μ M 5-HT reduced EPSC amplitudes to $24 \pm 13\%$ of control, showing that $G\beta\gamma$ can still interact with recombinant SNAP25 introduced into the presynaptic terminal via pipette (Wells et al., 2012).

Fig. 2. The SNAP25 2A mutant supports the inhibitory effect of 5-HT on glutamate release in lamprey spinal neurons. (A) Diagram of assay principle. BoNT/E-resistant SNAP25 is loaded into electrodes along with BoNT/E to cleave endogenous SNAP25 and is injected into the presynaptic giant reticulospinal axon. (Bi) Paired recordings are taken between lamprey reticulospinal axons and neurons of the spinal ventral horn. To demonstrate that injected toxins and proteins have access to the presynaptic terminal, dye was included in the presynaptic (red, Alexa 594) and postsynaptic (green, Alexa 488). An image is shown of the dendrites of the postsynaptic cell and the axon passing through these dendrites after pressure injection into the axon. (Bii) Evoked EPSCs are shown as recorded from the postsynaptic cell in control (black) and after the clearing of docked vesicles through application of 300 stimuli at 1 Hz (red) to show efficacy of BoNT/E. (C) Paired recordings from another cell in which the presynaptic electrode contained BoNT/E and a BoNT/E-resistant SNAP25 2A. After the same treatment, 5-HT is applied in the bath to inhibit EPSCs. Addition of 5-HT (1 μ M) substantially reduced this remaining response by $69 \pm 4\%$ of control amplitudes ($n = 4$).

Fig. 3. The SNAP25 2E mutant exhibits inhibited $G\beta\gamma$ -SNARE binding and inhibited neurotransmission. (A) Alphascreen concentration-response curves for SNAP25 WT and SNAP25 2E. Data normalized to the maximum luminescence signal obtained in each experiment. The EC_{50} for the binding of SNAP25 WT to $G\beta_1\gamma_2$ is 67 nM (95% confidence interval: 56–81 nM). The EC_{50} for the binding of SNAP25 2E to $G\beta_1\gamma_2$ is 116 nM (95% confidence interval: 90–150 nM). (B) Example trace of paired recording of presynaptic neuron injected with SNAP25 2E as in Fig. 2. The chemical portion of the EPSC is reduced (to $23 \pm 10\%$ of control), indicating its inability to restore vesicle release into the synapse. Data presented as the mean $\pm$ S.E.M. of two independent experiments.

We repeated that experimental format in this study using the BoNT/E-resistant SNAP25 2A. This was injected into axons along with BoNT/E. Paired recordings of EPSCs were then conducted between the injected reticulospinal axons and their synaptic target neurons of the spinal ventral horn (Fig. 2A). As before, 300 action potentials were evoked to deplete the primed vesicle pool. From these data, it is clear that SNAP25 2A can support evoked synaptic transmission, because EPSC amplitudes recovered to $81 \pm 2\%$ of control amplitudes ($n = 5$). In four of these recordings, subsequent application of $1 \mu\text{M}$ 5-HT reduced EPSC amplitudes to $33 \pm 5\%$ of the amplitude after injection and application of higher-frequency stimulation. This was not different from prior studies with SNAP25 containing the D179K mutation alone (Wells et al., 2012) (Fig. 2B). Together, these data indicate that the SNAP25 2A mutant is still capable of forming fusion-competent SNAREs and partaking in exocytosis. Furthermore, the SNAP25 2A mutant still supports the G $\beta\gamma$ -SNAP25 interaction, as measured through in vitro binding assays and the effects of 5-HT on EPSC amplitudes (Wells et al., 2012).

With the 2A mutant of SNAP25 still supporting the G $\beta\gamma$ -SNAP25 interaction, we sought to generate a set of mutants with a deleterious effect on the interaction with G $\beta\gamma$. Since residues R198 and K201 are positively charged and Ala is electrostatically neutral, we hypothesized that mutating these positively charged residues to negatively charged residues may have a larger effect. We generated a R198E K201E double mutant containing two Glu residues, SNAP25 2E. Purified recombinant SNAP25 2E had a substantially reduced ability to interact with G $\beta_1\gamma_2$ as measured in the Alphascreen assay (Fig. 3A), with a 4-fold drop in efficacy and a 1.7-fold drop in potency, with an EC₅₀ of 116 nM compared with an EC₅₀ of 67 nM for wild-type (WT) SNAP25. Given this promising result, we made a BoNT/E-resistant SNAP25 2E and injected it into reticulospinal axons in a manner similar to that shown in Fig. 2. Using the same approach of eliminating primed vesicles inaccessible to BoNT/E after the injection, we

demonstrated that the SNAP25 2E mutant could only support a substantially reduced evoked neurotransmission in this system. The peak amplitude of the response was reduced to $23 \pm 10\%$ of the control amplitude (Fig. 3B). We hypothesized that SNAP25 2E may have had altered Syt1 binding as a result of the dramatic changes to the electrostatic character of the C terminus of SNAP25. To test this, we used a GST-pulldown approach similar to those in previously published studies (Wells et al., 2012). We made GST fusions of SNAP25 WT or 2E and tested them for their ability to bind Syt1 in a calcium-dependent manner. Five micrograms of GST-SNAP25 was incubated on glutathione-sepharose beads with 400 nM Syt1 C2AB in the presence of either Ca²⁺ (1 mM) or the calcium chelator EGTA (2 mM). As a control, GST alone was incubated with 400 nM SNAP25 wild type (WT) or SNAP25 2E. After incubation for 1 hour, complexes were washed to remove unbound SNAP25 and analyzed via SDS-PAGE and Western blot. Antibodies against Syt1 and GST were used for detection (Fig. 4A). Both SNAP25 WT and SNAP25 2E bound Syt1 in a calcium-dependent manner. We observed a 4.6-fold (Student's *t* test, $P < 0.001$) reduction in calcium-dependent binding for SNAP25 2E relative to SNAP25 WT. No reduction in calcium-independent binding for SNAP25 2E was observed relative to SNAP25 WT ($P = 0.076$) (Fig. 4B). These data suggest that the lack of evoked neurotransmission seen in the 2E mutant may be due to impaired Syt1 calcium-dependent binding.

Finally, we sought to identify G $\beta\gamma$ -binding residues in other positions at the C terminus of SNAP25. Although the peptide-mapping approach previously used identified several important residues, the lack of higher-order structure achieved by short peptides may lead to false-negative results. Furthermore, the Ala-scanning approach previously used is unlikely to identify key residues that bear close structural similarity to Ala, such as Gly or Ser. Prior studies with the SNAP25 $\Delta 9$ construct and BoNT/A show that this truncation has impaired G $\beta\gamma$ binding and reduced ability for 5-HT to inhibit vesicle

Fig. 4. The SNAP25 2E mutant exhibits inhibited synaptotagmin 1 calcium-dependent binding. (A) Western blot images of GST-pulldown assay. The LI-COR Odyssey system was used for simultaneous imaging of GST and Syt1. The upper blot shows samples in the presence of 2 mM EGTA, whereas the lower blot is taken in the presence of 1 mM CaCl₂. Red IRDye800-labeled bands (the I800 channel) are representative of GST (26 kDa) or GST-SNAP25 (51 kDa). Green IRDye700-labeled bands (the I700 channel) are representative of Syt1 C2AB (37 kDa). (B) Densitometry of bands in each sample. Densitometry was performed by LI-COR Odyssey software. The amount of Syt1 C2AB present in each sample is normalized to the amount of GST or GST-SNAP25 present to correct for loading discrepancies. The resulting amount of Syt1 C2AB pulled down is then plotted as a percentage of the Syt1 pulled down by WT SNAP25. White bars represent conditions containing 2mM EGTA, while black bars represent conditions containing 1mM Ca²⁺. Error bars represent the mean $\pm$ S.E.M. Values measured by two-tailed Student's *t* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $n = 3$).

release (Yoon et al., 2007). Furthermore, this mutant has impaired SNARE complex zippering (Fang et al., 2008). Our intent was to make a smaller truncation mutant that did not exhibit these deficiencies in SNARE complex formation. The SNAP25 Δ 3 mutant, lacking three C-terminal residues, was previously shown to have release properties similar to wild-type SNAP25 (Criado et al., 1999; Gil et al., 2002), whereas the SNAP25 Δ 4 mutant had substantially reduced exocytosis due to the critical residue L203 being truncated in this construct. We tested the ability of recombinant purified SNAP25 Δ 3 to bind G β γ . This mutant exhibited a 2-fold reduction in the efficacy of SNAP25 binding to G β γ compared with the WT (Fig. 5A). In the same electrophysiological assay used for Figs. 2 and 3, the BoNT/E-resistant SNAP25 Δ 3 was able to restore exocytosis completely, with EPSC amplitudes $99 \pm 4\%$ of control amplitudes prior to BoNT/E treatment. However, the effect of 5-HT was partially abrogated, with $1 \mu\text{M}$ 5-HT only reducing EPSC amplitudes to $48 \pm 11\%$ of control ($n = 3$). 5-HT was significantly less effective than in wild-type conditions (Fig. 5B) while still showing an intermediate effect compared with prior results obtained with SNAP25 8A (Wells et al., 2012), in which inhibition was almost completely lost. Together, these results suggest that SNAP25 Δ 3 exhibits moderately impaired ability to bind G β γ . Finally, we tested the ability of GST-SNAP25 Δ 3 to bind Syt1 in the GST-pulldown

Fig. 5. The SNAP25 Δ 3 mutant shows both impaired G β γ binding and an impaired inhibitory effect of 5-HT on glutamate release. (A) Alphascreen concentration-response curves for SNAP25 WT and SNAP25 Δ 3. The EC₅₀ for the binding of SNAP25 WT to G β ₁γ₂ is 76 nM (95% confidence interval: 64–91 nM), whereas the EC₅₀ for SNAP25 Δ 3 binding to G β ₁γ₂ is 89 nM (95% confidence interval: 75–105 nM). A 2-fold decrease was observed in the maximum luminescence signal generated by SNAP25 Δ 3 compared with SNAP25 WT. Data are normalized to the maximum luminescence signal obtained in each experiment. (B) Example trace of paired recording of presynaptic neuron injected with SNAP25 Δ 3 as in Fig. 2. After 300 stimuli, EPSC amplitudes recovered to 95% of preinjection amplitudes, indicating the ability of the mutant to restore SNAP25-dependent exocytosis. 5-HT ($3 \mu\text{M}$) reduced EPSC amplitudes to only $48 \pm 11\%$ of EPSC amplitudes recorded in the absence of 5-HT ($n = 3$).

Fig. 6. Syt1 calcium-independent binding is slightly reduced in the SNAP25 Δ 3 mutant. (A) Western blot images of GST-pulldown assay as in Fig. 4. SNAP25 Δ 9 immunoblot is not shown. (B) Densitometry for Syt1 C2AB pulled down by GST-SNAP25 WT, Δ 3, or Δ 9 in the presence of 2 mM EGTA (white bars) or 1 mM Ca²⁺ (black bars). Values for Syt1 C2AB pulldown measured by two-tailed Student's *t* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $n = 3$).

assay (Fig. 6). A 1.4-fold reduction in calcium-dependent binding was observed for GST-SNAP25 Δ 3 compared with the wild type ($P < 0.0001$), despite no reduction in exocytosis relative to the wild type in Fig. 5. Calcium-independent binding was not significantly different from the WT for GST-SNAP25 Δ 3 ($P = 0.065$). Similarly, GST-SNAP25 Δ 9 showed significantly impaired Syt1 binding in the presence of 1 mM Ca²⁺ compared with the wild type, possibly suggesting that Syt1 utilizes one or more of the C-terminal residues of SNAP25 for calcium-dependent binding (Gerona et al., 2000). A 1.8-fold reduction in Syt1 calcium-dependent binding (Student's *t* test, $P < 0.001$), but not calcium-independent binding ($P = 0.152$), was observed, comparable to previously published results obtained with BoNT/A (Gerona et al., 2000).

Discussion

We have obtained a mutant, SNAP25 Δ 3, that has impaired binding to G β γ and reduced ability to support the actions of an inhibitory G_{i/o}-coupled GPCR upon vesicle fusion. The studies conducted here support a perturbed competition between Syt1 and G β γ binding to SNAP25 in favor of Syt1 for the SNAP25 Δ 3 mutant, as maximum G β γ binding and G_{i/o}-coupled GPCR activity is reduced, whereas exocytosis is unaffected. Given the results in Fig. 4, it would be plausible that R198 and K201 may be important for this interaction, but

SNAP25 8A does not exhibit impaired calcium-dependent binding to Syt1 (Wells et al., 2012). Neutral Ala mutations demonstrably have a smaller effect than charge-reversal mutations in these studies. A structural model of the importance of key residues in the C terminus of SNAP25 illustrates some of the numerous regulatory mechanisms acting upon exocytosis in the C terminus of SNAP25 (Shimazaki et al., 1996; Sutton et al., 1998; Criado et al., 1999; Chen et al., 2001; Gil et al., 2002; Blackmer et al., 2005; Wells et al., 2012; Fang et al., 2015) (Fig. 7). Many residues at the C terminus of SNAP25 have been associated with reduced exocytosis in mutation or truncation studies, including R198 (Fang et al., 2015), K201 (Fang et al., 2015), M202 (Gil et al., 2002), and L203 (Criado et al., 1999). The three C-terminal residues have not, with no significant difference being detected between chromaffin cells expressing wild-type SNAP25 or SNAP25 Δ 3 (Criado et al., 1999). Our studies echo these results, with SNAP25 Δ 3 being able to support exocytosis in neurons to levels similar to pre-BoNT/E-treated controls, much like the BoNT/E-resistant full-length SNAP25 (Wells et al., 2012). There are also two important residues for exocytosis upstream of the BoNT/A cleavage site: the phosphorylation site at S187 and the SNARE-forming residue at N188.

The goal of these studies is to obtain a mutant with impaired $G\beta\gamma$ -SNARE interaction to evaluate its importance in vivo. The SNAP25 Δ 3 mutant is suitable to introduce into the endogenous SNAP25 transcript via current genome-editing technologies, such as the CRISPR/Cas9 system. One issue of concern is that none of the three individual residues in the extreme C terminus of SNAP25 were identified as being important for binding $G\beta\gamma$ in our previous peptide-mapping approach (Wells et al., 2012). The Ala-scanning approach may miss critical residues and is not optimal for identifying the

importance of residues that bear structural similarities to alanine. It is apparent that mutating R198 and K201 to Ala is inadequate to disrupt the inhibitory effect of the lamprey serotonin receptor. Our results are consistent with previous studies indicating the importance of R198 and K201 as $G\beta\gamma$ -binding residues: although the in vitro binding data show a drop in potency and efficacy, the role of the 2E mutant on $G_{i/o}$ -coupled GPCR-mediated inhibition of exocytosis in cells could not be studied due to the mutant not supporting exocytosis. Other possible mutants that could be considered are the R198E and K201E single mutants, since our data indicate that the 2E double mutant has extremely impaired $G\beta\gamma$ binding (Fig. 3), as well as an impaired secretory phenotype. These single mutants have previously been shown to display an altered secretion phenotype with impaired release frequencies, slower release kinetics, and prolonged duration of the fusion pore (Gil et al., 2002; Fang et al., 2015). The R198Q single mutant also displayed this phenotype (Sorensen et al., 2006; Fang et al., 2015), potentially due to the partial negative charge on this mutant from resonance. Deficiencies identified in Syt1 C2AB calcium-independent or calcium-dependent binding in the GST-pulldown assay for the charge-reversal R198E or K201E mutants may explain the results obtained by these groups. As a result, this makes the positively charged residues R198 and K201 unattractive candidates for our goal of mutagenesis of SNAP25 to decrease $G\beta\gamma$ binding in a transgenic animal. The SNAP25 Δ 3 mutant also leaves the key residues M202 and L203 intact, the former being shown as important for the rapid phase of exocytosis (Sorensen et al., 2006) and the latter being predicted as essential for leucine zipper-mediated protein-protein interactions late in exocytosis (Gil et al., 2002; Sorensen et al., 2006).

Prior studies conducted by our group have shown that removal of the C terminus of SNAP25 by BoNT/A enables Syt1 to compete more effectively with $G\beta\gamma$ in the presence of Ca^{2+} ions (Blackmer et al., 2001, 2005; Yoon et al., 2007). SNAP25 Δ 9 was previously shown to have impaired calcium-dependent binding to Syt1 C2AB domains, which was also observed in our studies (Gerona et al., 2000). Tucker et al. (2004) performed reconstituted membrane fusion assays containing BoNT/A-treated SNAP25 and observed both a rightward shift in the calcium dependence and a reduction in fusion, even at very low levels of Ca^{2+} . Our results echo those obtained in reconstituted fusion assays, with a reduction in binding at 1 mM Ca^{2+} . Furthermore, they support cellular studies in which overexpression of the SNAP25 Δ 9 mutant in chromaffin cells led to slower single-vesicle kinetics and reduced exocytosis (Gil et al., 2002). Other existing data highlight the functional importance of Syt1 calcium-independent binding as a clamp for fusion (Chicka et al., 2008). Our results predict that the stimulatory effect of calcium-bound Syt on fusion would be reduced in a reconstituted fusion assay with vesicles containing target SNAREs (t-SNAREs) made with SNAP25 2E, and to a lesser extent, SNAP25 Δ 3 or SNAP25 Δ 9. However, in cell-based studies, SNAP25 Δ 3 is able to support exocytosis similar to non-BoNT/E-treated controls. The presence of key residues, such as L203, may be required for this effect.

Peptide-mapping approaches have demonstrated the importance of residues on SNAP25 on the SN2 helix located proximally to the N terminus of the SNARE complex (Wells et al., 2012). In that study, both the N-terminal binding sites

Fig. 7. Functional significance of the C terminus of SNAP25. (Left) Three-dimensional crystal structure of ternary SNARE complex obtained through X-ray crystallography (Protein Data Bank ID: 1sfc) (Sutton et al., 1998) with relevant residues highlighted in different colors according to function. (Right) Perspective through SN2 helix of SNAP25. Blue: residues implicated as being important for the $G\beta\gamma$ -SNARE interaction (Wells et al., 2012) (residues S205 and G206 omitted from structure). Yellow residue S187 is phosphorylated by protein kinase C to modulate exocytic events (Shimazaki et al., 1996). Brown residue N188 is important for SNARE complex interactions (Chen et al., 2001). Residues R198 and K201 may also be important for Syt1-SNAP25 calcium-dependent binding. Red: residues implicated as being important for Syt1 calcium-dependent binding (Bai et al., 2004). Magenta residue M202 is important for SNARE formation (Sørensen et al., 2006) and was shown to be important for the rapid phase of exocytosis in chromaffin cells (Gil et al., 2002). Orange residue L203 is predicted to be involved in leucine zipper protein-protein interactions during the last stage of exocytosis (Criado et al., 1999; Gil et al., 2002).

and C-terminal binding sites were mutagenized. Selective mutagenesis of the N-terminal $G\beta\gamma$ binding site on SNAP25 has yet to be explored in an electrophysiological model. Two hypotheses can be envisioned as potential outcomes of this experiment. It may be possible that complete removal of the action of an inhibitory $G_{i/o}$ -coupled GPCR only occurs with disruption of both the N-terminal and C-terminal binding sites. The extent of inhibition of 5-HT receptor-mediated inhibition is greater with SNAP25 8A compared with SNAP25 $\Delta 3$, consistent with this hypothesis. Another hypothesis is that N-terminal residues may be important for interaction with other proteins—for example, voltage-gated calcium channels. It has been shown that the interaction of $G\beta\gamma$ with voltage-gated calcium channels is mediated by residues located near the N terminus of the SNARE domain of syntaxin 1A (Jarvis et al., 2002). Existing knowledge of the structure of formed ternary SNARE complexes suggests that these N-terminal residues on SNAP25 would be in close proximity to this region on Stx1A and may facilitate the binding of $G\beta\gamma$ to Stx1A for voltage-gated calcium-channel inhibition. Further studies are needed to confirm either or both of these hypotheses; however, the effects of $G\beta\gamma$ at Ca^{2+} channels is likely to be synergistic to the inhibition of Ca^{2+} -dependent Syt1 binding to the SNARE complex that we observed.

One limitation of our studies is the use of the lamprey, a nonmammalian organism. Several studies in mammalian synapses in this field have been conducted. We have previously shown that the serotonin 1B (5-HT_{1b}) receptor inhibits neurotransmission in rat CA1 hippocampal neurons through the interaction of $G\beta\gamma$ with the C terminus of SNAP25 (Hamid et al., 2014). This inhibition could be overcome via presynaptic injection of the neuron with BoNT/A, much like early studies in lamprey (Gerachshenko et al., 2005). This mechanism of inhibition was found to not be universal across synapses, with other $G_{i/o}$ -coupled GPCRs, such as the GABA_b receptor, acting to inhibit exocytosis via the action of $G\beta\gamma$ on voltage-gated calcium channels. In lamprey, no inhibitory $G_{i/o}$ -coupled GPCRs are known to inhibit release in this manner, potentially implying that the $G\beta\gamma$ -SNARE mechanism evolved earlier than the $G\beta\gamma$ -calcium channel mechanism by its presence in this primitive organism. In Delaney et al. (2007), single-fiber inputs from the nociceptive pontine parabrachial nucleus form glutamatergic synapses with central amygdala neurons. Inhibition of exocytosis at this synapse was shown to be mediated by the α_2 adrenergic receptor via the $G\beta\gamma$ -SNARE interaction (Delaney et al., 2007). Other mammalian studies include that by Zhang et al. (2011), where introduction of $G\beta\gamma$ -scavenging peptides into CA3 hippocampal terminals blocked group II metabotropic glutamate receptor-mediated presynaptic depression of release, and introduction of BoNT/A into Schaffer collateral CA1 synapses reduced induction of long-term depression. These studies are both heavily reliant upon the introduction of $G\beta\gamma$ -scavenging peptides and light-chain botulinum toxins to demonstrate the involvement of the $G\beta\gamma$ -SNARE interaction. Early studies with the $G\beta\gamma$ -SNARE interaction in lamprey used similar approaches (Blackmer et al., 2001; Gerachshenko et al., 2005), and later featured the introduction of recombinant mutants of SNAP25 (Wells et al., 2012). Given the predictive value of the peptide experiments for mammalian studies, we predict that the recombinant SNAP25 experiments would similarly extend to future mammalian studies, indicating predictive power for this approach.

Beyond the pathophysiological consequences of partial disruption of the interaction of $G\beta\gamma$ with SNAP25, a whole-organism model bypasses many of the current limitations of existing models used to study this interaction. One such limitation is the dependence upon BoNT/E to remove endogenous SNAP25. The confounding effects of BoNT/E on the microarchitecture of the synapse will not be present in such a system, enabling study in a more physiologically relevant state.

Acknowledgments

The authors thank Joshua Bauer, of the Vanderbilt University High-throughput Screening Core, for assistance in setting up the Alphascreen assay. The authors thank Ed Chapman, of the University of Wisconsin-Madison, for providing expression vectors for SNAP25 and Syt1 C2AB.

Authorship Contributions

Participated in research design: Zurawski, Rodriguez, Alford, Hamm.
Conducted experiments: Zurawski, Rodriguez, Alford.
Contributed new reagents or analytic tools: Zurawski, Hyde.
Performed data analysis: Zurawski, Rodriguez, Alford.
Wrote or contributed to the writing of the manuscript: Zurawski, Rodriguez, Hyde, Alford, Hamm.

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