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Gβγ binds to the extreme C-terminus of SNAP25 to mediate the action of $G_{i/o}$-coupled GPCRs.

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Running title: SNAP25 C-terminal mutants resistant to Gβγ inhibition

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Abbreviations:

GPCR: G protein coupled receptor. Gβγ: G protein betagamma subunit. Gαi: G protein alpha I subunit. SNARE: Soluble N-ethylmaleimide attachment protein receptor. SNAP25: synaptosomal-associated protein of 25 kDa. BoNT/E: botulinum toxin E. Syt1 C2AB: the tandem C2A-C2B domain of synaptotagmin 1. 5-HT: serotonin. GABA: gamma-hydroxybutyric acid.

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Abstract

$G_{i/o}$-coupled G-protein coupled receptors (GPCRs) 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 protein betagamma (Gβγ) subunits. It has previously been shown that proteolytic cleavage of SNAP25 by botulinum toxin A (BoNT/A) reduces the ability of Gβγ 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 SNAP-5 extreme C-terminal mutant that is deficient in its ability to bind Gβγ while retaining normal calcium-dependent Syt1 binding to SNARE and vesicle release. The SNAP25Δ3 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 $5HT_{1b}$-type serotonin receptor to reduce excitatory postsynaptic current (EPSC) amplitudes, an effect previously shown to be mediated through the interaction of Gβγ with SNAP25. Syt1 calcium-dependent binding to SNAP25Δ3 was reduced by a small extent compared to wild-type. We conclude that the extreme C-terminus of SNAP25 is a critical region for the Gβγ-SNARE interaction.

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Introduction

Regulation of neurotransmitter and hormone release is an essential component of homeostasis and plasticity in many systems. Inhibitory G protein-coupled receptors 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 βγ subunits to voltage-gated calcium channels leading to voltage-dependent inhibition of calcium entry(Ikeda, 1996). The ability of G $_{/o}$-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; Yoon *et al*., 2008; Zhao *et al*., 2010; Delaney *et al*., 2007; Iremonger and Bains, 2009; Hamid *et al*., 2014) We have previously demonstrated that inhibition can also occur through the direct interaction of Gβγ with the SNARE protein SNAP25 (Gerachshenko *et al*., 2005; Yoon *et al*., 2007; Blackmer *et al*., 2005). Gβγ competes in a calcium-dependent manner with the fusogenic calcium sensor synaptotagmin 1 for binding sites on SNARE (Yoon *et al*., 2007; Blackmer *et al*., 2005). Upon calcium binding, synaptotagmin 1 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). Synaptotagmin 1 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 Gβγ. Alanine mutagenesis of 8 residues on SNAP25 reduces its ability to bind Gβγ without disrupting its ability to bind Syt1 (Wells *et al*., 2012). Injection of an exogenous mutant SNAP25 containing these 8 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 serotonin's (5-HT) ability to inhibit vesicle release in lamprey reticulospinal axons (Wells *et al*., 2012). Interestingly, data was recently shown supporting the notion that a distinct "microarchitecture" is prevalent at presynaptic 5-HT_{1b} receptors that predisposes them to

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this mode of Gβγ-driven inhibition, while other microarchitectures both within the same synapses and within other types of synapses function through other mechanisms, such as the Gβγ-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 Gβγ to SNAP25.

While the molecular requirements of the Gβγ-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 Gβγ–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 Gβγ-SNARE interaction has been shown to be functionally relevant for a number of presynaptic $G_{i/o}$-coupled GPCRs (Delaney *et al*., 2007; Zhang *et al*., 2011; Glitsch, 2006; Heinke *et al*., 2011; Betke *et al*., 2012). To explore these and other potential areas of therapeutic relevance further, a transgenic model deficient in the Gβγ–SNARE interaction is required. The generation of such a model presents a number of challenges. A knockout-based strategy would be unsuitable. There are 5 Gβ subunits and 12 Gγ subunits (Betke *et al*., 2012) in the human genome, indicating a high degree of redundancy, making knockout or mutagenesis of Gβγ subunits unfeasible(Betke *et al*., 2012). While studies have been conducted pertaining to the distribution of Gβ and Gγ subunits in the brain (Betke *et al*., 2014), it is not currently known whether a specific combination of subunits is responsible for the Gβγ–SNARE interaction. The possibility of multiple effectors for any given Gβγ would also be a confounding factor in such a knockout. A knockout of SNAP25 would also be unsuitable, as SNAP25 knockouts are neonatally lethal (Washburne *et al*., 2002). Finally, the mutations proposed in Wells *et al*, 2012, are also unsuitable for introduction into a transgenic animal, as

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the large number of mutations (eight) spread throughout the eight exons (Oyler *et al*., 1989) makes homologous recombination challenging. Insertion of the 8 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 was required. Here, we have identified an extreme C-terminal mutation suitable for introduction into the native mouse SNAP25 that reduces Gβγ 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 transferase (GST) fusion vector pGEX-6p-1 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for expression in the Rosetta DE3 strain of *Escherchia 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 dyes and resolved on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Antibodies.

The antibody for mouse anti-Syt1 C2AB (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 *Escherichia coli* strain Rosetta DE3 (Merck Millipore, Darmstadt, Germany). SNAP25 protein expression was induced with 100 μM

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isopropyl β-d-1-thiogalactopyranoside (IPTG) for 16 h at 25 C. Syt1 (residues 96–422) protein expression was induced with 400uM IPTG for 8h at 30 C. Bacterial cultures were pelleted and washed once with phosphate-buffered saline before resuspension in 25mM HEPES-KOH, pH 8.0, 150 mM KCl, 5 mM 2-mercaptoethanol, standard concentrations of the protease inhibitors leupeptin, aprotinin, and pepstatin, 200 μM phenylmethylsulfonyl fluoride, and 1 mM EDTA. Resuspended cells were lysed with a sonic dismembranator at 4°C for 5 min. Lysates were cleared via ultracentrifugation at 26,000*g* for 20 min 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 4h before being washed once with resuspension buffer containing 1% Triton X-100 (Dow Chemical, Midland, MI). After centrifugation at 3000*g*, 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*-octyl glucoside, 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.

Gβγ Purification.

$G\beta_1\gamma_1$ was purified from bovine retina according to previously published methods (Mazzoni *et al*., 1991). $G\beta_1$6xHis-γ_2 dimers were expressed in Sf9 cells and purified as the method of Kozasa(Kozasa and Gilman, 1995) with the following exceptions: frozen Sf9 cell pellets were lysed by gentle sonication pulse, 10 seconds on 20 seconds off for 3 minutes at 30% intensity on ice. $G\beta_1$6xHis-γ_2 dimers were affinity-purified from detergent solubilized crude cell membrane using Talon® cobalt resin (Clontech)

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followed by three rounds of dialysis in the following buffer: 20mM HEPES, 100 mM NaCl, 10mM BME, 0.8% OG, 10% glycerol pH 8.0.

Biotinylation.

Purified recombinant SNAP25 or GST was diluted to 1mg/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 6mg in 1mL of H_2O. Biotinylation reagents were added slowly to SNAP25 proteins to a 20:1 molar excess. Reactions were allowed to proceed for 30m at 25 C before removal of excess reagent via two rounds of dialysis in 2L 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 (Pierce, Rockford, IL).

Alphascreen Binding Assays.

Alphascreen luminescence measurements were performed in an EnSpire multimode plate reader (Perkin-Elmer, Waltham MA) at 27°C. Biotinylated SNAP25 was diluted into a final concentration of 20nM 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-Gb1g2 ranging from 1uM to 1nM was made in assay buffer. After incubation while shaking for 5m, Alphascreen Histidine Detection Kit (Nickel Chelate) acceptor beads were added to a final concentration of 20ug/mL in assay buffer. The assay plate was shaken for 30m. At that point, Alphascreen Streptavidin Donor Beads were added to a final concentration of 20ug/mL in low light conditions. The final volume in the assay plate (384-well white Perkin-Elmer OptiPlate) was 25uL. Plates were incubated for 1H at 27 C before being read in the EnSpire. 20nM biotinylated GST in place of SNAP25 with the four highest concentrations of $G\beta_1\gamma_2$ was

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used with as a negative control for non-specific 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.

5ug of GST-SNAP25 protein bound to glutathione-agarose resin was incubated with a 400 μM concentration of purified recombinant Syt1 C2AB domains for 1 h at 4°C and washed 3x with assay buffer (20 mM HEPES, pH 7.2, 80 mM KCl, 20 mM NaCl, and 0.2% *n*-octyl glucoside) in a 1.5-ml Eppendorf tube. Assay buffers would contain either 2mm EGTA or 1mM $CaCl_2$. 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) 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

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of SNAP25 and BoNT/E (65ug/mL). Electrode had impedances from 20 to 50 MΩ. 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 μg/ml; List Biological Laboratories Inc., Campbell, CA) was stored at −20°C in 20 mM HEPES-NaOH pH 7.4, 50 mM NaCl, and 1mg/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, USA, www.graphpad.com)

Results

To explore the binding of a number of different SNAP25 mutants to Gβγ, we developed an Alphascreen assay (Perkin-Elmer) with higher throughput and greater dynamic range. In this assay, biotinylated recombinant mouse SNAP25 (biotinylated non-specifically upon primary amine residues with EZ-Link NHS-SS-biotin) interacts with His-tagged Gβ1γ2 subunits purified from SF9 cells inoculated with baculovirus. When the Gβγ–SNAP25 complex forms, the complex is anchored to an Alphascreen

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streptavidin-conjugated donor bead via the biotinylation on SNAP25 and an Alphascreen Ni-trinitriloacetic acid (NTA) acceptor bead via the His-tag on Gβγ. When the donor bead is illluminated with 680nm 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 520-620nm light in response to singlet oxygen (Fig.1A). High specificity for the Gβγ–SNARE interaction was observed, with minimal signal being generated in the absence of protein, but a large signal when 20nM SNAP25 and 170nM Gβ1γ2 is present in solution. As a control for non-specific binding, 20nM glutathione-S-transferase (GST), a protein that does not interact with Gβγ (Yoon *et al*., 2007), was added to solution. 20nM SNAP25 did not generate a signal in the presence of His-tagged 170nM $G\alpha_i$-GDP as a second non-specific binding control (Fig. 1B).

The SNAP25 8A mutant (14) has 8 Gβγ-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 Gβγ, while no change was observed in the ability of proteins containing these mutations to bind 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 μM) 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

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long pass emission filter (610 nm), the postsynaptic with a 488 nm excitation and a bandpass emission filter (510-550) (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 (Wells et al 2012: Gerachshenko et at 2005). It is clear that when labeling is present presynaptically, synaptic responses were abolished by the BoNT/E (Fig 2Bii).

It is possible to recover synaptic transmission in terminals in which a recombinant BoNT/E resistant SNAP25 is coinjected into the presynaptic axon with the BoNT/E. In a previous study, a SNAP25 containing the D179K mutation was injected into the presynaptic neuron along with BoNT/E, restoring EPSC amplitudes to 95 ± 11 % of control. In those experiments, subsequent application of 1μM 5-HT reduced EPSC amplitudes to 24+/-13% of control, showing that Gβγ can still interact with recombinant SNAP25 introduced into the presynaptic terminal via pipette. (Wells *et al*., 2012).

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 ± 2 % of control amplitudes (n=5). In four of these recordings, subsequent application of 1 μM 5-HT reduced EPSC amplitudes to 33 ± 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 Gβγ–SNAP25 interaction, as measured through in vitro binding assays and the effects of 5-HT on EPSC amplitudes (Wells *et al*., 2012) .

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With the 2A mutant of SNAP25 still supporting the Gβγ–SNAP25 interaction, we sought to generate a set of mutants with a deleterious effect on the interaction with Gβγ. 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 fourfold drop in efficacy and a 1.7-fold drop in potency, with an EC_{50} of 116 nM compared to an EC_{50} of 67nM for wild-type SNAP25. Given this promising result, we made a BoNT/E resistant SNAP25 2E and injected it into reticulospinal axons in a similar manner to 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 ± 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 utilized a GST-pull down approach similar to previously published studies(Wells *et al*., 2014). We made GST-fusions of SNAP25 WT or 2E and tested them for their ability to bind Syt1 in a calcium-dependent manner. 5ug of GST-SNAP25 was incubated on glutathione-sepharose beads with 400nM SNAP25 in the presence of either 1mM Ca^{2+} or the calcium chelator 2mM EGTA. As a control, GST alone was incubated with 400nM SNAP25 WT or SNAP25 2E. After incubation for 1hr, 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.

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Finally, we sought to identify Gβγ–binding residues in other positions at the C-terminus of SNAP25. While 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 utilized is unlikely to identify key residues that bear close structural similarity to Ala, such as Gly or Ser. Prior studies with the SNAP25Δ9 construct and BoNT/A show that this truncation has impaired Gβγ binding and reduced ability for 5-HT to inhibit vesicle 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 SNAP25Δ3 mutant, lacking three C-terminal residues, was previously shown to have release properties similar to wild-type SNAP25 (Gil *et al*., 2002; Criado *et al*., 1999), while the SNAP25Δ4 mutant had substantially reduced exocytosis due to the critical residue L203 being truncated in this construct. We tested the ability of recombinant purified SNAP25Δ3 to bind Gβγ. This mutant exhibited a twofold reduction in the efficacy of SNAP25 binding to Gβγ compared to wild-type (Fig.5A). In the same electrophysiological assay utilized for figures 2 and 3, the BoNT/E resistant SNAP25Δ3 was able to restore exocytosis completely, with EPSC amplitudes 99 ± 4 % of control amplitudes prior to BoNT/E treatment. However, the effect of 5-HT was partially abrogated, with 1 μM 5-HT only reducing EPSC amplitudes to 48 ± 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 to prior results obtained with SNAP25 8A(Wells *et al*., 2012) in which inhibition was almost completely lost. Together, these results suggest that SNAP25Δ3 exhibits moderately impaired ability to bind Gβγ. Finally, we tested the ability of GST- SNAP25Δ3 to bind Syt1 in the GST-pull down assay (Fig. 6). A 1.4-fold reduction in calcium-dependent binding was observed for GST- SNAP25Δ3 compared to wild-type ($p<.0001$), despite no reduction in exocytosis relative to wild-type in Fig. 5 Calcium-independent binding was not significantly different from wild-type for GST- SNAP25Δ3 ($p= .065$) Similarly, GST- SNAP25 Δ9 showed significantly impaired Syt1 binding in the presence of 1mM Ca^{2+} compared to wild-type, possibly

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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($p = .152$) binding was observed, comparable to previously published results obtained with BoNT/A(Gerona *et al*., 2000).

Discussion

We have obtained a mutant, SNAP25Δ3, which has impaired binding to Gβγ 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 Gβγ binding to SNAP25 in favor of Syt1 for the SNAP25Δ3 mutant, as maximum Gβγ binding and $G_{i/o}$-coupled GPCR activity is reduced, while 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(Blackmer *et al*., 2005; Wells *et al*., 2012; Gil *et al*., 2002; Criado *et al*., 1999, Shimazaki *et al*., 1996; Fang *et al*., 2015; Chen *et al*., 2001; Sutton *et al*., 1998) (Fig. 7). Many residues at the C-terminus of SNAP25 have been associated with reduced exocytosis in mutation or truncation studies: these include 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 SNAP25Δ3(Criado *et al*., 1999). Our studies echo these results, with SNAP25Δ3 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.

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The goal of these studies is to obtain a mutant with impaired Gβγ-SNARE interaction to evaluate its importance *in vivo*. The SNAP25Δ3 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 Gβγ 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 Gβγ binding residues: while the *in vitro* binding data shows 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 Gβγ 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(Fang *et al*., 2015; Sorensen *et al*., 2006), 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-pull down 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 Gβγ binding in a transgenic animal. The SNAP25Δ3 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).

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Prior studies conducted by our group have shown that removal of the C-terminus of SNAP25 by BoNT/A enable Syt1 to compete more effectively with Gβγ in the presence of Ca^{2+} ions(Blackmer *et al*., 2001; Yoon *et al*., 2007; Blackmer *et al*., 2005). SNAP25Δ9 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. 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 (Tucker *et al*., 2004), even at very low levels of Ca^{2+}. Our results echo those obtained in reconstituted fusion assays, with a reduction in binding at 1mM Ca^{2+}. Furthermore, they support cellular studies in which overexpression of the SNAP25Δ9 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 t-SNAREs made with SNAP25 2E, and to a lesser extent SNAP25Δ3 or SNAP25Δ9. However, in cell-based studies, SNAP25Δ3 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 and C-terminal binding sites were mutagenized. Selective mutagenesis of the N-terminal Gβγ 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 $Gi_{/o}$-coupled GPCR may only occur 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 to SNAP25 Δ3, 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 Gβγ with voltage-

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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 Gβγ to Stx1A for voltage-gated calcium-channel inhibition. Further studies are needed to confirm either or both of these hypotheses, however the effects of Gβγ at Ca^{2+} channels is likely to be synergistic to the inhibition of Ca^{2+} dependent Syt1 binding to the SNARE complex that we observe.

One limitation of our studies is the use of the lamprey, a non-mammalian organism. Several studies in mammalian synapses in this field have been conducted. We have previously shown that the serotonin 1B ($5\text{-}HT_{1b}$) receptor inhibits neurotransmission in rat CA1 hippocampal neurons through the interaction of Gβγ 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 Gβγ 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 Gβγ-SNARE mechanism evolved earlier than the Gβγ-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 a2 adrenergic receptor via the Gβγ-SNARE interaction (Delaney *et al.*, 2007). Other mammalian studies include Zhang *et al*., (2011), where introduction of Gβγ-scavenging peptides into CA3 hippocampal terminals blocked group II mGluR-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 Gβγ-scavening peptides and light-chain

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botulinum toxins to demonstrate the involvement of the Gβγ-SNARE interaction. Early studies with the Gβγ-SNARE interaction in lamprey utilized 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 would 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 Gβγ with SNAP25, a whole-organism model bypasses many of the current limitations of existing models utilized 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.

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

Authorship Contributions:

Participated in research design: ZZ, SR, SA, and HEH

Conducted experiments: ZZ, SR, and SA

Contributed new reagents: ZZ and KH

Performed data analysis: ZZ, SR, and SA

Wrote or contributed to the writing of the manuscript: ZZ, SR, KH, SA, and HEH

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FOOTNOTES:

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FIGURE LEGENDS

Fig. 1. The Alphascreen Gβγ–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 Gβγ –SNAP25 complexes are captured on Alphascreen Ni-NTA acceptor beads via the His-tag on Gβγ , while simultaneously being captured on Alphascreen streptavidin donor beads via the biotinylation on SNAP-25. If 680nm 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 520-620nm light to be detected by the plate reader. B) Non-specific binding controls for the Alphascreen assay. (n=3) Data presented as mean + S.E.M .

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 injected into the presynaptic giant RS 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 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 SNAP-25-2A. After the same treatment, 5-HT is applied in the bath to inhibit EPSCs. Addition of 5-HT (1 μM) substantially reduced this remaining response by 69 ± 4% of control amplitudes. (n=4)

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Fig. 3: The SNAP25 2E mutant exhibits inhibited Gβγ-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 67nM (95% C.I.: 56-81nM). The EC_{50} for the binding of SNAP25 2E to $G\beta_1\gamma_2$ is 116nM (95% C.I.: 90-150nM) 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 ± 10% of control), indicating its inability to restore vesicle release into the synapse. Data presented as mean + S.E.M . of two independent experiments.

Fig. 4: The SNAP25 2E mutant exhibits inhibited synaptotagmin 1 calcium-dependent binding**.** A) Western blot images of GST-pull down assay. The LI-COR Odyssey system was used for simultaneous imaging of GST and Syt1. The upper blot shows samples in the presence of 2mM EGTA (black bars), while the lower blot is taken in the presence of 1mM $CaCl_2$ (white bars). Red IRDye800-labeled bands (the I800 channel) are representative of GST (26kDa) or GST-SNAP25 (51 kDa). GreenIRDye700-labeled bands (the I700 channel) are representative of Syt1 C2AB (37 kDa). B) Densitometry of bands in each sample. Densitometry 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 wild-type SNAP25. Error bars represent mean + S.E.M. Values measured by two-tailed Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) (n=3).

Fig. 5: The SNAP25Δ3 mutant shows both impaired Gβγ binding, and an impaired inhibitory effect of 5-HT on glutamate release. A) Alphascreen concentration-response curves for SNAP25 WT and SNAP25Δ3. The EC_{50} for the binding of SNAP25 WT to Gβ1γ2 is 76nM (95% C.I.: 64-91nM), while the EC_{50} for SNAP-25 Δ3 binding to $G\beta_1\gamma_2$ is 89nM (95% C.I.: 75-105nM). A twofold decrease was observed in the maximum luminescence signal generated by SNAP25 Δ3 compared to SNAP25 WT. Data

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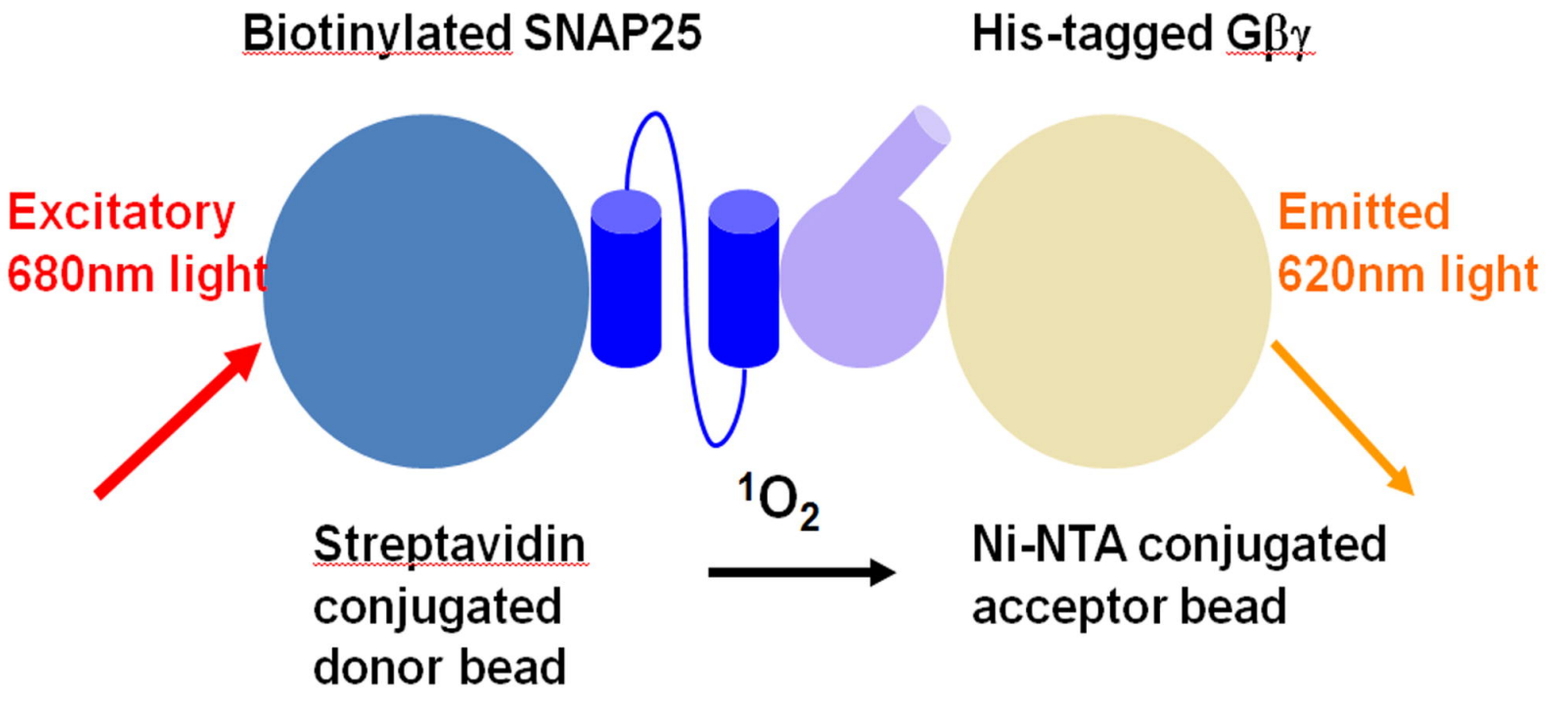
normalized to the maximum luminescence signal obtained in each experiment. B) Example trace of paired recording of presynaptic neuron injected with SNAP25Δ3 as in Fig. 2. After 300 stimuli, EPSC amplitudes recovered to 95% of pre-injection amplitudes, indicating the ability of the mutant to restore SNAP25-dependent exocytosis. 3uM 5-HT reduced EPSC amplitudes to only 48± 11% of EPSC amplitudes recorded in the absence of 5-HT (n=3).

Fig. 6: Syt1 calcium-independent binding is slightly reduced in the SNAP25Δ3 mutant. A) Western blot images of GST-pull down assay as in Fig. 4. SNAP-25Δ9 immunoblot not shown. B) Densitometry for Syt1 C2AB pulled down by GST-SNAP25 WT, Δ3, or Δ9 in the presence of 2mM EGTA (white bars) or 1mM Ca^{2+} (black bars). Values for Syt1 C2AB pulled down measured by two-tailed Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) (n=3).

Fig. 7: Functional significance of the C-terminus of SNAP25**.** Left panel: 3-D crystal structure of ternary SNARE complex obtained through X-ray crystallography(PDB: 1sfc) (Sutton *et al.*, 1998) with relevant residues highlighted in different colors according to function. Right panel: Perspective through SN2 helix of SNAP25. Blue: residues implicated as being important for the Gβγ-SNARE interaction[14] (residues S205 and G206 omitted from structure). Yellow residue S187 is phosphorylated by PKC to modulate exocytic events(Shimazaki *et a.l*, 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 (Gil *et al.*, 2002; Criado *et al*., 1999).

Figure 1

A


Biotinylated SNAP25
His-tagged Gβγ
Excitatory
680nm light
Emitted
620nm light
$^1O_2$
Streptavidin
conjugated
donor bead
Ni-NTA conjugated
acceptor bead

B

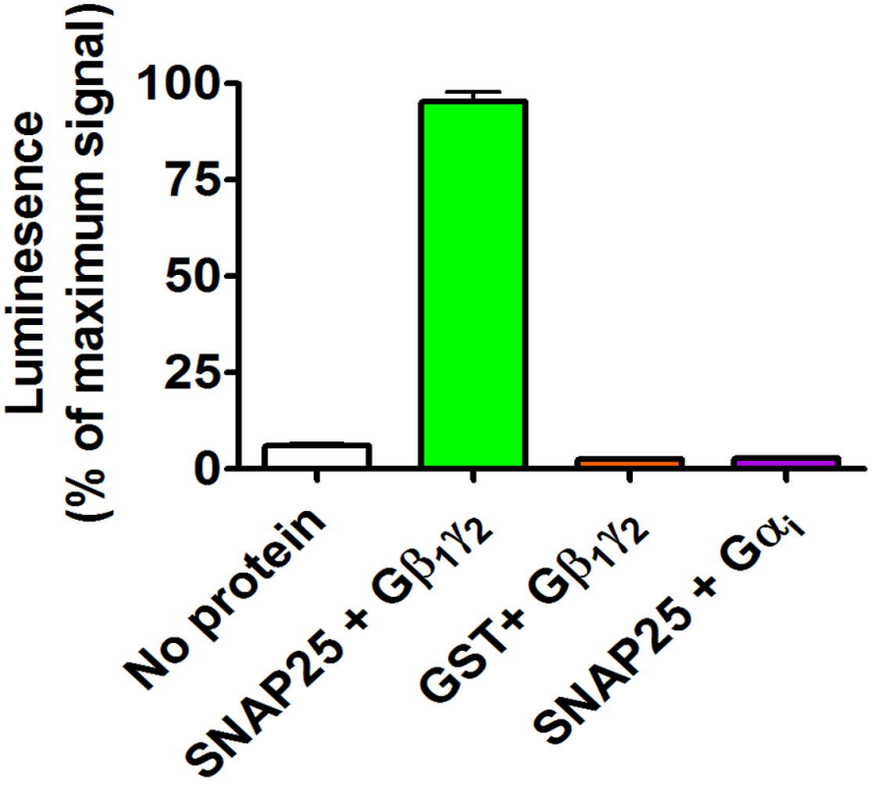

Luminesence
(% of maximum signal)
100
75
50
25
0
No protein
SNAP25 + Gβ1γ2
GST+ Gβ1γ2
SNAP25 + Gαi

Figure 2

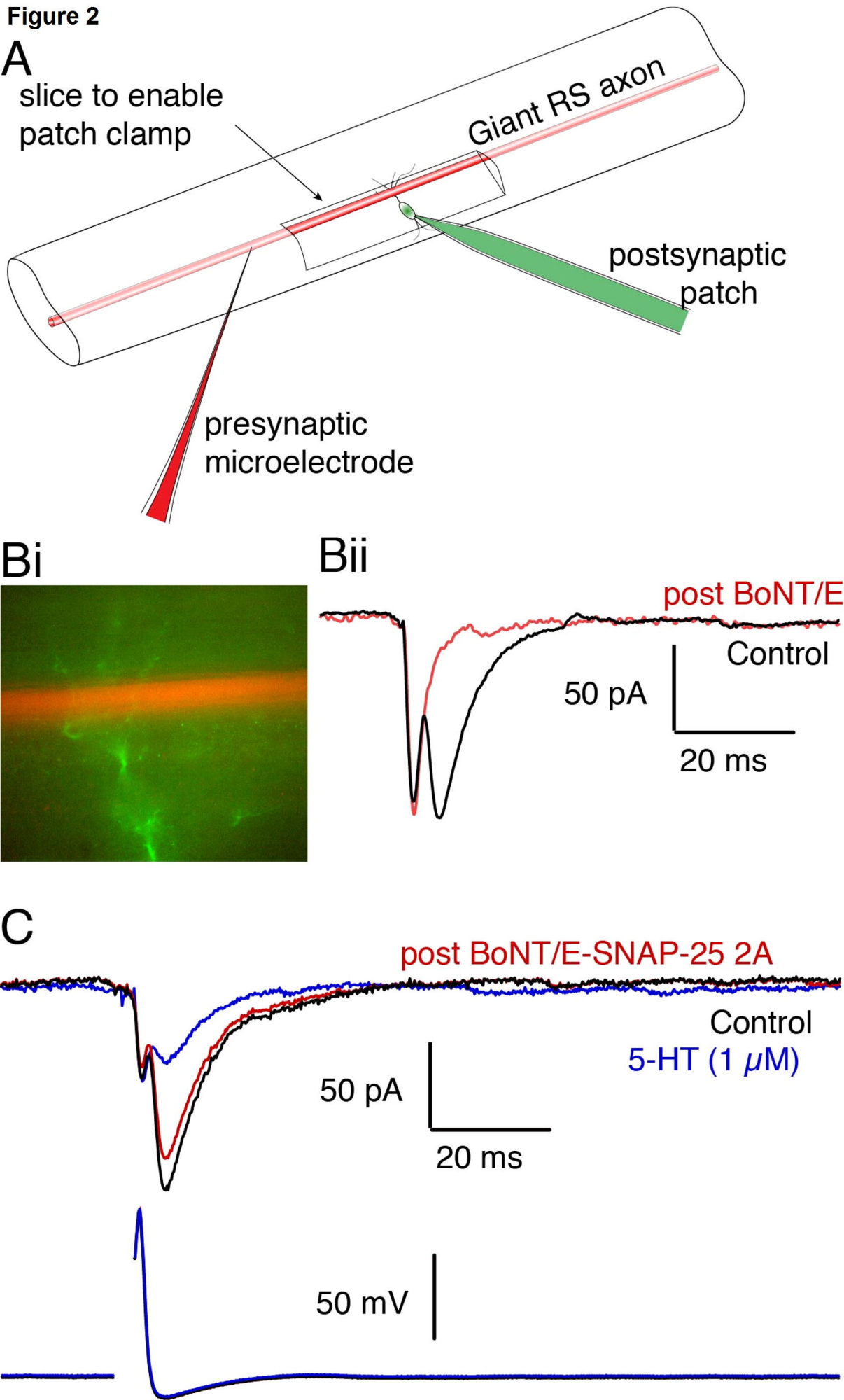

A
slice to enable
patch clamp
Giant RS axon
postsynaptic
patch
presynaptic
microelectrode
Bi
Bii
post BoNT/E
Control
50 pA
20 ms
C
post BoNT/E-SNAP-25 2A
Control
5-HT (1 μM)
50 pA
20 ms
50 mV

Figure 3

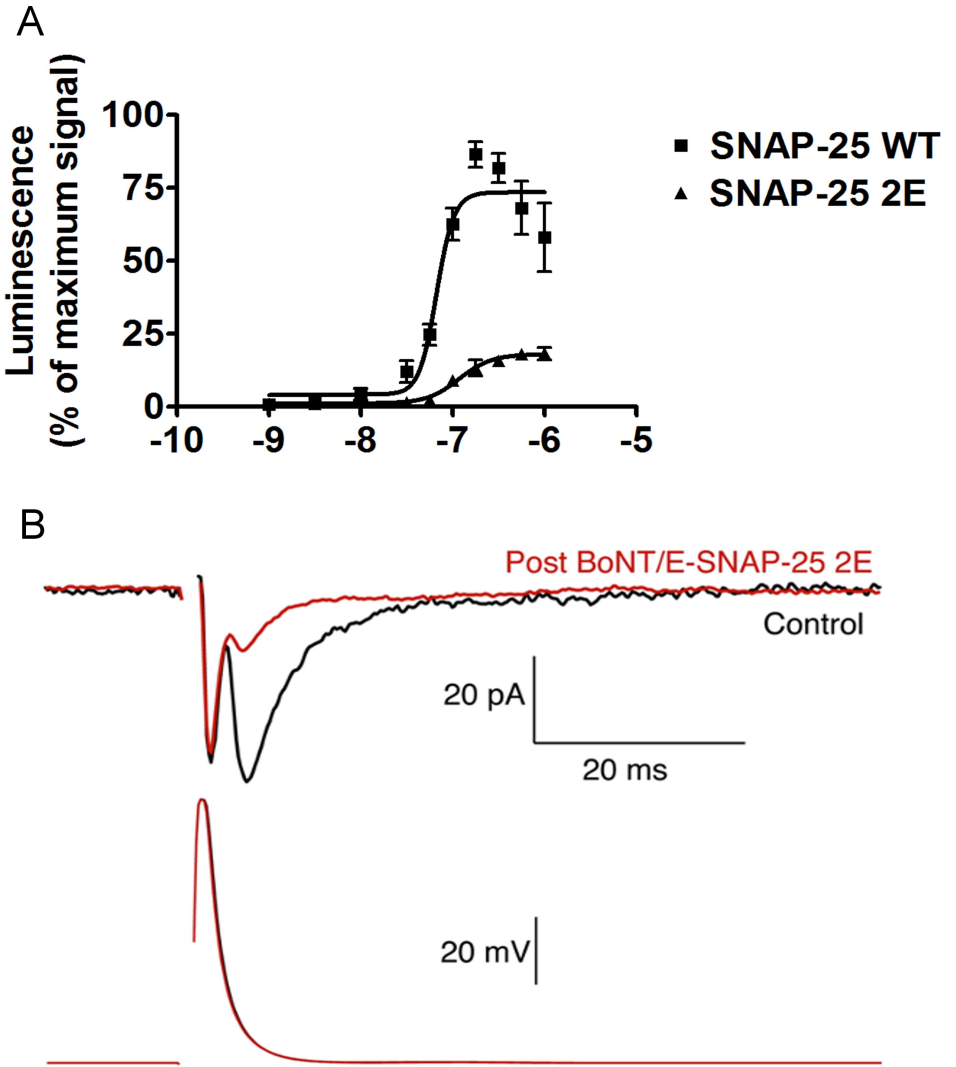

A
Luminescence
(% of maximum signal)
100
75
50
25
0
-10
-9
-8
-7
-6
-5
SNAP-25 WT
SNAP-25 2E
B
Post BoNT/E-SNAP-25 2E
Control
20 pA
20 ms
20 mV

Figure 4

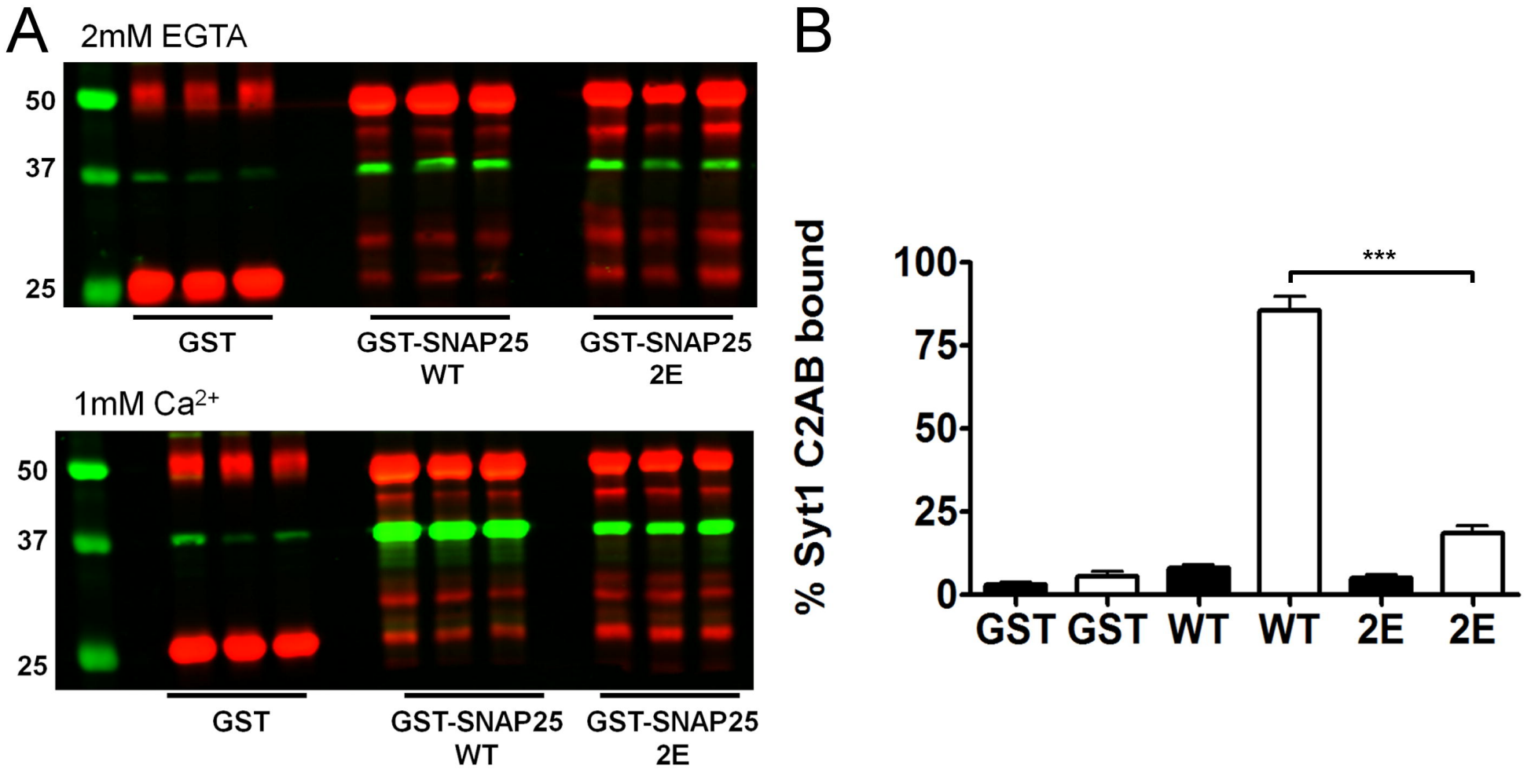
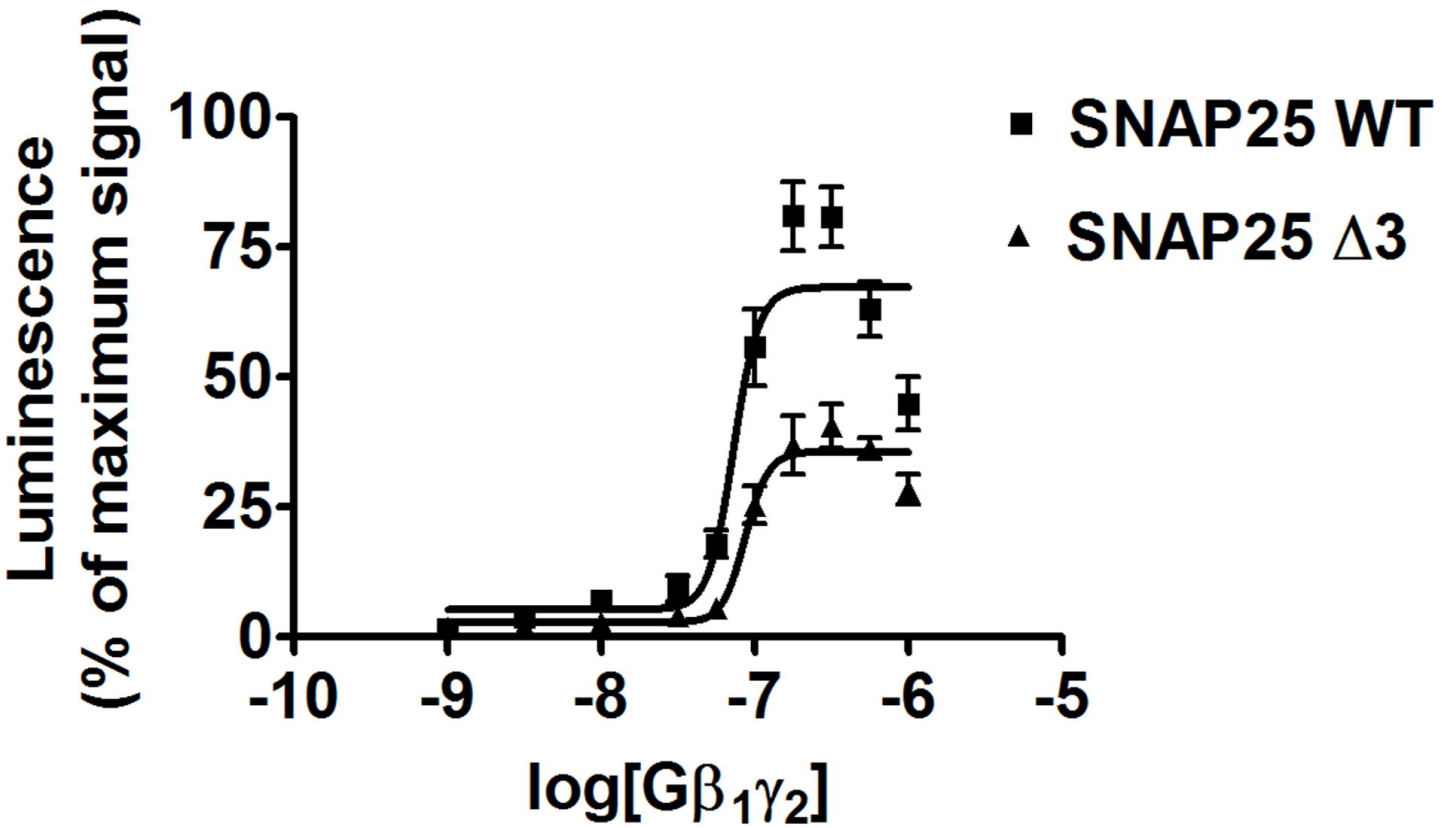

A
2mM EGTA
50
37
25
GST
GST-SNAP25 WT
GST-SNAP25 2E
1mM Ca2+
50
37
25
GST
GST-SNAP25 WT
GST-SNAP25 2E
B
% Syt1 C2AB bound
100
75
50
25
0
***
GST GST WT WT 2E 2E

Figure 5

A


Luminescence
(% of maximum signal)
100
75
50
25
0
-10
-9
-8
-7
-6
-5
log[Gβ1γ2]
SNAP25 WT
SNAP25 Δ3

B

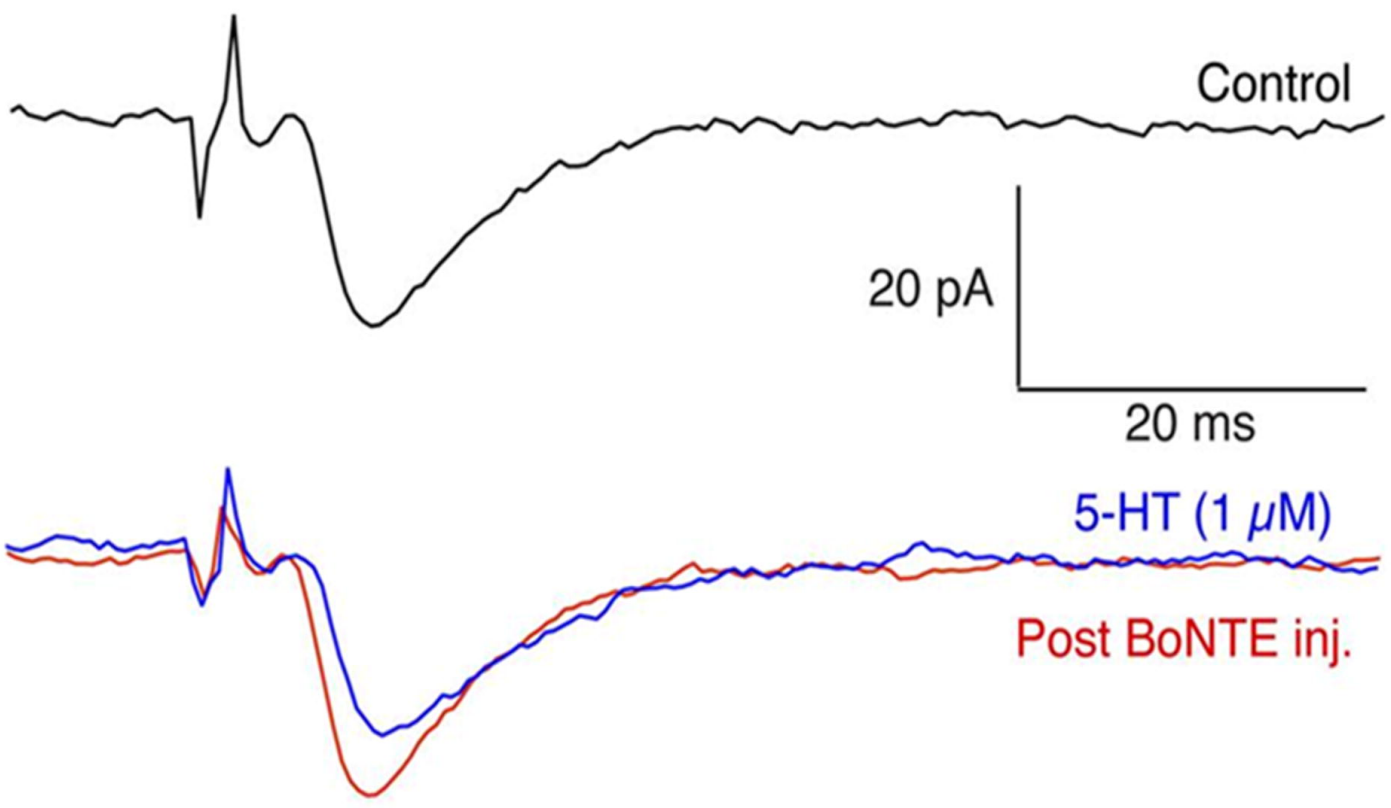
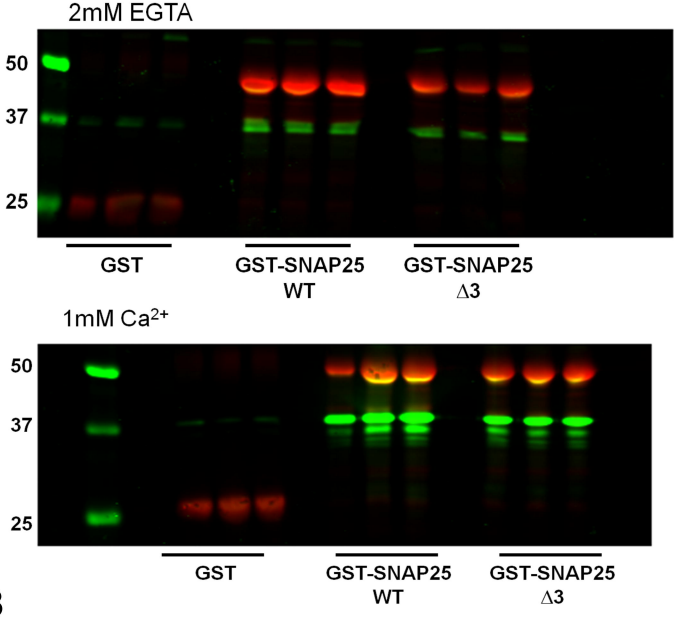

Control
20 pA
20 ms
5-HT (1 μM)
Post BoNTE inj.

Figure 6

A


2mM EGTA
50
37
25
GST
GST-SNAP25 WT
GST-SNAP25 Δ3
1mM Ca2+
50
37
25
GST
GST-SNAP25 WT
GST-SNAP25 Δ3

B

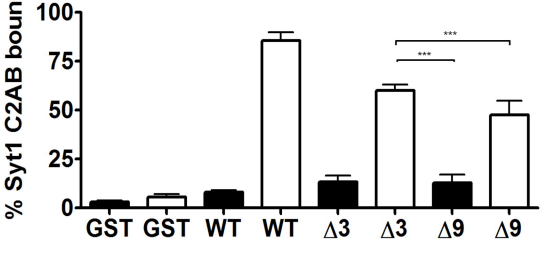

% Syt1 C2AB bound
100
75
50
25
0
***
***
GST GST WT WT Δ3 Δ3 Δ9 Δ9

Figure 7