

**MOL #80507**

**G $\beta$ y inhibits exocytosis via interaction with critical residues on SNAP-25**

Christopher A. Wells, Zack Zurawski, Katherine M. Betke, Yun Young Yim, Karren Hyde,  
Shelagh Rodriguez, Simon Alford, and Heidi E. Hamm

Department of Pharmacology (C.A.W., Z.Z., K.M.B., Y.Y.Y., K.H., H.E.H.) Vanderbilt  
University Medical Center, Nashville, TN 37232-6600

Department of Biological Sciences (S.R., S.A.) University of Illinois at Chicago, 840 West  
Taylor St., Chicago, IL 60607, USA.

**MOL #80507**

**Running title: G $\beta$ y inhibits exocytosis via interaction with SNAP-25**

**Corresponding Author:**

Heidi E. Hamm

Department of Pharmacology

Vanderbilt University Medical Center

442 Robinson Research Building

23rd Ave. South @ Pierce

Nashville, TN 37232-6600

Tel.: (615) 343-3533

Fax: (615)-343-1084

E-mail: [heidi.hamm@vanderbilt.edu](mailto:heidi.hamm@vanderbilt.edu)

Number of text pages: 36

Number of tables: 2

Number of figures: 8

Number of references: 56

Number of words in the Abstract: 217

Number of words in the Introduction: 542

Number of words in the Discussion: 1492

**Abbreviations:**

5-HT, 5-Hydroxytryptamine; EDTA, ethylenediaminetetracetic acid; EGTA, ethylene glycol tetraacetic acid; GPCR, G-protein coupled receptors; GST, glutathione-S-transferase; HEPES-KOH, potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; MIANS, 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid; RT, room temperature; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TFA, trifluoroacetic acid

**MOL #80507**

***Abstract***

Spatial and temporal regulation of neurotransmitter release is a complex process accomplished by the exocytotic machinery working in tandem with numerous regulatory proteins. G-protein  $\beta\gamma$  dimers regulate the core process of exocytosis by interacting with the SNARE proteins SNAP-25, syntaxin 1A, and synaptobrevin.  $G\beta\gamma$  binding to ternary SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein REceptors) overlaps with synaptotagmin's calcium-dependent binding, inhibiting synaptotagmin-1 binding and fusion of the synaptic vesicle. To further explore the binding sites of  $G\beta\gamma$  on SNAP-25, peptides based on the sequence of SNAP-25 were screened for  $G\beta\gamma$  binding. Peptides that bound  $G\beta\gamma$  were subjected to alanine scanning mutagenesis to determine their relevance to the  $G\beta\gamma$ -SNAP-25 interaction. Peptides from this screen were tested in protein-protein interaction assays for their ability to modulate the interaction of  $G\beta\gamma$  with SNAP-25. A peptide from the C-terminus, residues 193-206, significantly inhibited the interaction. Additionally, Ala mutants of SNAP-25 residues from the C-terminus of SNAP-25, as well as from the amino terminal region decreased binding to  $G\beta_1\gamma_1$ . When SNAP-25 with 8 residues mutated to alanine was assembled with syntaxin 1A, there was significantly reduced affinity of this mutated t-SNARE for  $G\beta\gamma$ , but it still interacted with synaptotagmin-1 in a  $Ca^{2+}$ -dependent manner and reconstituted evoked exocytosis in BoNT/E-treated neurons. However, the mutant SNAP-25 could no longer support 5-HT-mediated inhibition of exocytosis.

**MOL #80507**

***Introduction***

Release of neurotransmitter into the synapse is a complex, regulated process involving core exocytotic machinery proteins, accessory proteins that play roles in docking and priming the vesicle, ion channels, calcium sensors, and pre-synaptic inhibitory G-protein coupled receptors (GPCRs). Previous studies over the past 20 years have shown that the G $\beta\gamma$  subunit, part of the heterotrimeric G protein complex activated by GPCRs, has a variety of effectors that it can interact with and regulate when dissociated from G $\alpha$  upon GPCR activation (Blackmer et al., 2005; Cabrera-Vera et al., 2003; Clapham and Neer, 1997; Gautam et al., 1998; Gerachshenko et al., 2005; Smrcka, 2008; Vanderbeld and Kelly, 2000). In the presynapse, G $\beta\gamma$  has been shown to be an important regulator of neurotransmission through interactions with calcium channels (Dolphin, 2003; Hille, 1994; Ikeda and Dunlap, 1999) and with the secretory machinery itself ((Blackmer et al., 2005; Blackmer et al., 2001; Gerachshenko et al., 2005), reviewed by Betke et al. 2012). Specifically, G $\beta\gamma$  binds directly to the ternary SNARE complex (a trimer of SNAP-25, syntaxin 1A, and synaptobrevin), as established in biochemical as well as *in vitro* assays (Blackmer et al., 2005; Blackmer et al., 2001; Delaney et al., 2007; Gerachshenko et al., 2005; Photowala et al., 2006; Yoon et al., 2007; Yoon et al., 2008; Zhang et al., 2011; Zhao et al., 2010).

The first (Blackmer et al., 2001) insight into direct interaction between G $\beta\gamma$  and SNARE proteins was derived from studies utilizing botulinum toxin A (BoNT/A) (Blackmer et al., 2001). BoNT/A cleaves the C-terminal 9 amino acids from SNAP-25 (Binz et al., 1994; Schiavo et al., 1993). Treatment of presynaptic reticulospinal neurons with BoNT/A disrupted the ability of serotonin to inhibit excitatory postsynaptic action potentials (Gerachshenko et al., 2005). Furthermore, a 14-amino acid peptide based on the C-terminus of SNAP-25 was able to block G $\beta\gamma$ -mediated inhibition (Blackmer et al., 2005; Gerachshenko et al., 2005). Reductions in binding between G $\beta\gamma$  and BoNT/A cleaved SNAP-25 compared to uncleaved SNAP-25 confirmed the importance of the G $\beta\gamma$ /SNAP-25 interaction (Yoon et al., 2007; Zhao et al., 2010). In addition to binding to SNAP-25, Yoon et al. showed that G $\beta\gamma$  also binds individually to the other SNARE proteins, syntaxin 1A and synaptobrevin, as well as to the t-SNARE

**MOL #80507**

dimer (SNAP-25 with syntaxin 1A) and to the ternary SNARE complex (Yoon et al., 2007). Presynaptic  $G\beta\gamma$  inhibition of secretion has been identified in neurons (Blackmer et al., 2001; Delaney et al., 2007; Zhang et al., 2011), chromaffin cells (Yoon et al., 2008), and pancreatic  $\beta$  cells (Zhao et al., 2010).

The specific binding sites on each of the component proteins of the  $G\beta\gamma$ -SNARE interaction have yet to be determined. Removal of the C-terminal 26 residues of SNAP-25 (Yoon et al., 2007) disrupted its interaction with  $G\beta\gamma$ . In this manuscript, we have mapped the binding sites involved in the interaction of  $G\beta\gamma$  with SNAP-25 using peptides of 15 amino acids that cumulatively span the full length-sequence of SNAP-25 for interaction with  $G\beta_1\gamma_1$ . We found 8 residues in SNAP-25 that were important for binding  $G\beta_1\gamma_1$ . By mutating corresponding residues in full-length SNAP-25, we found a decreased interaction with fluorescently-labeled  $G\beta_1\gamma_1$ . This mutant SNAP25, when introduced into lamprey neurons, was no longer able to mediate the effect of 5-HT on presynaptic inhibition of exocytosis.

**MOL #80507**

***Materials and Methods***

**Plasmids.** The open reading frame for SNAP-25 was subcloned into the glutathione-S-transferase (GST) fusion vector pGEX-6p-1 (GE Healthcare) for expression in bacteria. Mutagenesis of SNAP-25 was accomplished via the overlapping primer method. The SNAP-25(8A) mutant was subcloned from pGEX-6p-1 into the pRSFDuet-1 plasmid, a dual expression vector that contains cDNAs for both full-length syntaxin 1A and SNAP-25 that results in concomitant expression and formation of t-SNARE complexes (kindly provided by E. Chapman). Plasmids were verified to contain desired mutations via Sanger sequencing utilizing BigDye Terminator dyes and resolved on an ABI 3730 DNA Analyzer (Applied Biosystems).

**Antibodies.** The antibody for rabbit G $\beta$  (T-20; sc-378), was obtained from Santa Cruz Biotechnology, Inc. The HRP-conjugated goat anti-rabbit antibody was obtained from Perkin-Elmer (NEF812001EA). The anti-synaptotagmin-1 antibody subclone 41.1 was obtained from Synaptic Systems (105 011). Anti-GST (goat) Antibody DyLight™ 800 Conjugated (#600-145-200) and the anti-mouse IgG (goat, H&L) Antibody IRDye700DX® Conjugated Pre-adsorbed (#610-130-121) were both from Rockland Immunochemicals, Inc.

**SNAP-25 Protein Purification.** Recombinant bacterially-expressed glutathione-S-transferase (GST) fusion proteins were expressed in *Escherichia coli* strain Rosetta 2 (EMD Biosciences). Protein expression was induced with 100  $\mu$ M isopropyl  $\beta$ -D-1-thiogalactopyranoside for 16 h at room temperature. Bacterial cultures were pelleted and washed once with phosphate-buffered saline before undergoing resuspension in lysis buffer (25mM potassium 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate (HEPES-KOH) pH 8.0, 150mM KCl, 5mM 2-mercaptoethanol, 10.66  $\mu$ M leupeptin, 1.536  $\mu$ M aprotinin, 959 nM pepstatin, 200 $\mu$ M phenylmethylsulfonyl fluoride, and 1mM ethylenediaminetetracetic acid (EDTA)). Cells were lysed with a sonic dismembrator at 4°C for 5 min. Lysates were cleared via ultracentrifugation at 26,000 x g for 20 min in a TI-70 rotor (Beckman Coulter). GST-SNAP-25 fusion proteins were purified from cleared lysates by affinity chromatography

## MOL #80507

on GE Sepharose 4 FastFlow (GE Healthcare). Lysates were allowed to bind to resin overnight before being washed once with lysis buffer containing 1% Triton X-100 (Dow Chemical). The resin was then washed once with elution buffer (25mM HEPES-KOH pH 8.0, 150mM KCl, 5mM 2-mercaptoethanol, 0.5% n-octyl glucoside, 1mM EDTA, 10% glycerol). SNAP-25 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 (Pierce) and purity was verified by SDS-PAGE analysis.

**t-SNARE Protein Purification.** For the t-SNARE with full-length syntaxin with SNAP-25, this was expressed using the tandem vector (pRSFDuet-1) previously characterized by Chicka et al. (Chicka et al., 2008). Purification of this SNAP-25/syntaxin 1A dimer was performed as previously described (Tucker et al., 2004) utilizing a 6xHis tag present upon the N-terminus of SNAP-25. After elution, the t-SNARE was dialyzed into a final buffer consisting of: 25mM HEPES-KOH pH 8.0, 50mM NaCl, 5mM 2-mercaptoethanol, 0.5% n-octyl glucoside, 5% glycerol. The n-octyl glucoside was used to prevent aggregation that may occur due to the hydrophobic transmembrane domain of syntaxin 1A that is present in this construct. After dialysis, the purified t-SNARE was divided into aliquots and frozen at -80°C.

**G $\beta$  $\gamma$  Purification.** G $\beta$  $\gamma$ <sub>1</sub> was purified from bovine retina as described previously (Mazzoni et al., 1991). Recombinant G $\beta$  $\gamma$ <sub>2</sub> was expressed in Sf9 cells and purified via a 6xHis tag on G $\gamma$ <sub>2</sub> using Talon™ immobilized metal affinity chromatography (Clontech, Mountain View, CA).

**Peptide array synthesis.** Peptide array synthesis was performed using the Respep SL (Intavis AG) according to standard SPOT synthesis protocols (Eaton et al., 2008; Frank, 2002). Briefly, the robotics-driven computer-directed device (Respep SL) managed complex timing, mixing, additions, and washing of the membrane over the course of the peptide synthesis. Peptides were 15 residues in length. The sequences of the peptides for SNAP-25 were based on the sequence available from the UniprotKB/Swiss Protein Database for human *SNAP-25*, P60880. After the peptides were synthesized coupled to the membranes, membranes were processed with a final side chain de-protection step. Membranes were placed in an acid-safe container in a chemical hood and submerged in a solution of 95%

**MOL #80507**

trifluoroacetic acid, 3% tri-isopropylsilane for 1 hour with intermittent agitation. After the trifluoroacetic acid solution was removed, the membrane was then put through a series of washes: 1) dichloromethane for four 10-minute washes; 2) dimethylformamide for four 10-minute washes, 3) ethanol for two 2-minute washes. The membrane was allowed to dry in the hood. For the alanine-mutagenesis screening of peptides, the peptides were synthesized to be 14 residues in length.

**Peptide membrane Far-Western.** After membranes had dried from the synthesis de-protection washes, they were first soaked in ethanol for 5 minutes, and then re-hydrated over two washes for 5 minutes in water. The membranes were then blocked with slight agitation for 1 hour in a buffer of tris-buffered saline (TBS) with 5% milk and 0.1% Tween-20 (Sigma-Aldrich). The membranes were then washed 5 times for 5 minutes in TBS with 0.1% Tween-20 on a shaker at RT. The membranes were then incubated overnight at 4°C with G $\beta$ <sub>1</sub> $\gamma$ <sub>1</sub> at a final concentration of 0.44  $\mu$ M in a binding buffer of 20 mM HEPES, pH 7.5, and 5% glycerol. The next morning, membranes were washed at room temperature (RT) on a shaker three times for 5 minutes in TBS with 5% milk and 0.1% Tween-20. Membranes were then exposed to primary antibody against G $\beta$  (T-20, SC-378; Santa Cruz Biotechnology, Inc.) at 1:5000 dilution in TBS with 0.2% Tween-20 with mixing on a shaker table at RT for 1 hour before being washed three times for 5 min each on a shaker table in TBS with 0.1% Tween-20 also at RT. The appropriate secondary antibody was then diluted into TBS to 1:10,000 dilution with 5% milk and 0.2% Tween-20 followed by gentle agitation on a shaker with membranes for 1 hour at RT. Finally, membranes were washed twice for 5-minutes in TBS with 0.1% Tween-20, followed by two 10-minute washes in TBS at RT.

**Chemiluminescence.** The Western Lightning<sup>TM</sup> Chemiluminescence Reagent Plus (NEL104) from Perkin-Elmer and the Immun-Star<sup>TM</sup> WesternC<sup>TM</sup> Chemiluminescence Kit (#170-5070) from Bio-Rad was used to visualize western blots following published protocols. Western blot images were obtained utilizing a Bio-Rad Gel Doc Imager. Images were analyzed for densitometry using ImageJ (available from <http://rsbweb.nih.gov/ij/index.html>). ANOVA calculations comparing alanine mutant



## MOL #80507

peptide spot intensity with wild type peptide reactivity were determined using GraphPad Prism4 software (GraphPad Software, San Diego, CA).

**Peptide synthesis.** Peptides were synthesized using the Respep SL (Intavis AG) using the Tentagel amide resin as the solid support using standard Fmoc/HBTU chemistry. After the last round of synthesis, the peptides on the columns were treated with 5% acetic anhydride in dimethylformamide to acetylate the N-terminus of the peptides. After washing with dimethylformamide followed by chloromethane and overnight drying, the peptides were cleaved from the resin over the course of 3-4 hours using a mixture of 92.5% trifluoroacetic acid (TFA) and 5% tri-isopropylsilane with gentle mixing. The peptides dissolved in the TFA mixture were precipitated using ice-cold tert-butyl methyl ether. After spinning and overnight drying, the peptides were dissolved either in water or a mixture of water with acetonitrile or 0.1% TFA. The dissolved peptides were snap-frozen in an ethanol/dry ice bath followed by overnight evaporation in a vacuum-assisted evaporative centrifuge with cold trap (Centrivap from Labconco). Samples were re-solubilized in a mixture of water/acetonitrile/0.1%TFA, and subjected to preparative HPLC (Gilson) on a reverse phase C18 column (Phenomenex Luna, 30x50mm) at 50ml/min, 10-90% acetonitrile in water gradient with 0.1% TFA over five minutes. Samples were then subjected to LC/MS (Agilent 1200 LCMS) for purity and mass spec identification of the peptide. HPLC fractions were evaporated to retrieve the peptide of interest.

**G $\beta$  $\gamma$  labeling.** Fluorescence labeling of G $\beta$  $\gamma$  $_1$  and binding assays were conducted as described previously (Phillips and Cerione, 1991). In brief, purified G $\beta$  $\gamma$  $_1$  was exchanged via a Centricon 10,000 MW concentrator into labeling buffer (20 mM HEPES, pH 7.4, 5 mM MgCl $_2$ , 150 mM NaCl, and 10% glycerol), then mixed with 2-(4'-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) in a 5-fold molar excess. The reaction proceeded for 3 h at 4°C before quenching with 5 mM 2-mercaptoethanol. The MIANS- $\beta$  $\gamma$  $_1$  complex was separated from unreacted MIANS using a PD-10 desalting column (GE Healthcare). MIANS-G $\beta$  $\gamma$  $_1$  was stored in aliquots at -80°C.

**Fluorescence Binding.** All fluorescence measurements were performed in a fluorescence spectrophotometer (Cary Eclipse; Varian, Inc., Palo Alto, CA) at 17°C. MIANS-G $\beta$  $\gamma$  $_1$  was diluted into

## MOL #80507

0.1 ml of assay buffer (20mM HEPES, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 100 mM NaCl, 1 mM EDTA, and 0.5% n-octyl glucoside) to a final concentration of 20 nM. This assay buffer included 0.5% n-octyl glucoside to limit aggregation of the t-SNARE complexes which contain full-length t-SNARE that includes the hydrophobic transmembrane domain of syntaxin 1A. The MIANS fluorescence was monitored with excitation at 322 nm and emission at 417 nm. Fluorescence changes caused by the addition of SNARE complexes to MIANS-Gβ<sub>1</sub>γ<sub>1</sub> were monitored continuously. The amplitude of the fluorescence reflects the specific site on fluorescently labeled Gβγ and its interaction with each protein. There was no nonspecific binding of the free probe to the SNARE proteins and MIANS-Gβγ was resistant to photobleaching under experimental conditions (data not shown). The EC<sub>50</sub> concentrations were determined by sigmoidal dose-response curve fitting with variable slope.

**GST-pull down Assay.** 5 μg of each GST-SNAP-25 protein immobilized on glutathione-sepharose resin was incubated with 400μM of the C2AB domain of synaptotagmin-1 (residues 96-422) for 1 h at 4°C and washed three times with assay buffer (20 mM HEPES, pH 7.0, 80 mM KCl, 20 mM NaCl, and 0.1% n-octyl glucoside) in a 1.5 mL Eppendorf tube. Immobilized protein complexes were then transferred to a second 1.5mL Eppendorf tube to reduce non-specific binding. The complex was eluted with 20 μl of SDS sample buffer followed by separation via SDS-PAGE. Precipitated Gβ was detected via western blot with a rabbit anti-Gβ antibody (T-20, Santa Cruz Biotechnology, Inc. (sc-378)). Precipitated synaptotagmin-1 C2AB was detected via Western blot using a mouse anti-synaptotagmin-1 antibody (subclone 41.1, Synaptic Systems (105 011)). Western blots were imaged with labeled secondary antibodies: Anti-GST (goat) Antibody DyLight™ 680 Conjugated and the mouse IgG (H&L) Antibody IRDye700DX® Conjugated using the Licor Odyssey imager (Licor Biosciences).

**Electrophysiology and microinjections.** Experiments were performed on the isolated spinal cords or spinal cords and brainstems of lampreys (*Petromyzon marinus*). The animals were anesthetized with tricaine methanesulfonate (MS-222; 100 mg/l; Sigma Chemical, St. Louis, MO), sacrificed by decapitation, and the spinal cord dissected in a cold saline solution (Ringer) of the following composition

## MOL #80507

(in mM): 100 NaCl, 2.1 KCl, 2.6 CaCl<sub>2</sub>, 1.8 MgCl<sub>2</sub>, 4 glucose, 5 HEPES, adjusted to a pH of 7.60.

Procedures conformed to institutional guidelines (University of Illinois at Chicago, Animal Care Committee).

Paired cell recordings were made between reticulospinal axons and neurons of the spinal ventral horn. Axons of reticulospinal neurons were recorded with sharp microelectrodes containing 1M KCl, 5mM HEPES buffered to pH 7.2 with KOH and a SNAP-25 and BoNT/E mixture as defined below. Electrode impedances ranged from 20 to 50 MΩ. Postsynaptic neurons were recorded with patch clamp in voltage clamp conditions. Patch electrodes contained in mM: Cesium methane sulfonate 102.5, NaCl 1, MgCl<sub>2</sub> 1, EGTA 5, HEPES 5, pH adjusted to 7.2 with CsOH.

BoNT/E and proteins were pressure microinjected through presynaptic microelectrodes using a Picospritzer II. Presynaptic recordings were made within 100 μm of the synaptic contact to ensure protein diffusion to the region of the terminal and this was confirmed by injection of fluorescently tagged SNAP-25 protein in separate experiments. Light chain BoNT/E (List. (65 μg/mL)), was stored at -20 °C in 20 mM HEPES, 50 mM NaCl 0.015 mM BSA at pH 7.4. The buffered toxin was diluted as 5μL with 20 μL 2 M KMeSO<sub>4</sub> and 5 mM HEPES and 20μL of solution containing one of three different variants of SNAP-25. SNAP-25 proteins were stored at -20 °C in a buffer containing 25mM HEPES-KOH pH 8.0, 150mM KCl, 5mM 2-mercaptoethanol, 0.5% n-octyl glucoside, 1mM EDTA, and 10% glycerol. The buffered protein mixed with BoNT/E where appropriate was diluted 1:5 with 2 M KMeSO<sub>4</sub> and 5 mM HEPES. Microinjections of buffer solutions do not affect the synaptic response or 5-HT inhibition.

**Protein structure visualization.** All representatives of protein structure were made using the computer program Pymol (Schrodinger, 2010).

**Statistics and curve fitting.** All statistics (Student's t-test) and curve-fitting (sigmoidal dose-response with variable slope) were performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com).

**MOL #80507**

***Results***

**Screening of SNAP-25 Peptides for Interaction with G $\beta$  $\gamma$ .** Removal of varying portions of the C-terminus of SNAP-25 diminished or disrupted binding between G $\beta$  $\gamma$  and SNAP-25 (Yoon et al., 2007). Our ultimate goal was to engineer a SNAP-25 (or t-SNARE) that binds synaptotagmin but no longer binds to G $\beta$  $\gamma$ , as a tool to test the physiologic role of G $\beta$  $\gamma$  interaction with SNARE complexes. To more fully investigate the determinants of interaction between G $\beta$  $\gamma$  and SNAP-25, we searched for small linear sequences from SNAP-25 that would interact with G $\beta$  $\gamma$ .

We used a peptide synthesizer (Respep SL, Intavis) to generate a sequential series of peptides from SNAP-25 on a membrane. This membrane was then exposed to G $\beta_1\gamma_1$ , washed, and then with exposure to an antibody specific for G $\beta$ , and then a secondary antibody that reacts in a chemiluminescent assay, see Figure 1a. Membranes consisted of 15-mer peptides shifting 3 amino acids for each successive peptide spot (1-15, 4-18, 7-21, etc.) cumulatively representing the entire sequence of SNAP-25. Membranes were made and tested in triplicate. For positive controls, peptides that were previously reported to bind G $\beta$  $\gamma$  were spotted on the membrane (SIRK (SIRKALNILGYPDYD) (Scott et al., 2001), QEHA (QEHAQEPERQYMHIGTMVEFAYALVGK) (Weng et al., 1996), and the C-terminus of  $\beta$ ARK (WKELRDAYREAQQLVQRVPMKMKPKRS) (Koch et al., 1993)), as well as a peptide based on the G $\beta$  $\gamma$ -binding sequence from the calcium channel Cav2.2 (GID site: KSPLDAVLKRAATKKSRLDI) (De Waard et al., 2005). The epitope of the primary antibody, the extreme C-terminus of G $\beta_1$ , was used as a positive control. Shown in Figure 1b is a representative image of the spotted membrane for the SNAP-25 protein and the other G $\beta$  $\gamma$  interacting peptides, as well as the antibody binding positive control. As can be seen from the image shown of the SNAP-25 peptides, there are several clusters of consecutive peptides of SNAP-25 that bind G $\beta_1\gamma_1$  (see red circles in Figure 1b). Locations #64-69 on the membrane are intentionally underivatized by peptides during the synthesis to demonstrate non-specific binding of G $\beta$  $\gamma$  or antibodies with the membrane alone. Densitometry was performed on images of the triplicate membranes and normalized to the brightest signal for each membrane, respectively. Figure 1C is a bar

## MOL #80507

graph across all 65 peptides from the sequence of SNAP-25 showing clusters of reactive peptides spanning residues 49-75, 82-108, 121-144, 145-168, and 184-206. The sequences in common for each cluster of peptides are visualized in Figure 1D on the x-ray crystal structure of the ternary complex of SNAP-25, syntaxin 1A, and synaptobrevin (Sutton et al., 1998). The ternary SNARE complex is depicted in a “primed” state with a docked vesicle that has not yet fused, which is the proposed location and state that would bind G $\beta\gamma$  (Blackmer et al., 2005; Yoon et al., 2007). Note that the four helices of the SNARE complex are arranged in parallel to each other with all N-termini of those helices at one end of the complex, and all C-termini at the opposite end. The peptide sequences of SNAP-25 that bind G $\beta\gamma$  in this experiment (shown in red) are clustered on either the N-terminal end of the ternary SNARE complex (labeled ‘N’ in Figure 1D), or the C-terminal end proximal to the transmembrane domains of ternary SNARE (labeled ‘C’ in Figure 1D).

Based on these results, peptides that bound G $\beta\gamma$  in this screen were then subjected to alanine-scanning mutagenesis. Full-length SNAP-25 peptides were compared to the corresponding mutagenized peptides. For each series of horizontal spots, the initial spot was the wild type sequence of a given peptide followed by spots with a single alanine mutation in the first through fourteenth residue, individually. After averaging the results of three independent experiments, residues were identified within the sequence that had reduced ability to bind G $\beta\gamma$  when mutated to alanine in comparison to wild-type peptide. The sequences for these peptides are listed in Table 1. The bar graphs demonstrating the average densitometry of all peptides with loss of binding in a mutant are shown in Figure 2b. This resulted in a list of nine amino acids that had disrupted binding when mutated to alanine in their respective peptides. The resulting nine amino acids, when mapped to the crystal structure of the ternary SNARE, again show two clusters at either end of the coiled-coil helical SNARE (see Figure 2c). There is one cluster near the published interaction site at the C-terminus of SNAP-25 (Yoon et al., 2007) including two residues within the extreme C-terminus of SNAP-25: Arg198 and Lys201. In addition to the those amino acids in SNAP-25 important for peptide binding shown in the x-ray crystal structure near the C-

## MOL #80507

terminus of SNAP-25, there are 2 amino acids (Asp99 and Lys102) in the linker region between the two helices of SNAP-25 in close proximity to the palmitoylation sites of SNAP-25 (Cys 85, 88, 90, and 92) that are not in the x-ray crystal structure, but are shown in Figure 2c in cartoon form. Interestingly, when mapped to the ternary SNARE structure, residues Gly63 and Met64 that had significant reduction in binding when mutated to alanine in the 59-72 SNAP-25 peptide (see Figure 2) are buried in the interface with syntaxin 1A and synaptobrevin. Because these two residues are not expected to be accessible based on the x-ray crystal structure, these residues were omitted from mutagenesis in studies with full-length SNAP-25 protein. Of the remaining residues, 7 of the residues lose a positive charge when mutated to alanine, and the other two lose a negative charge when mutated to alanine.

**Effect of alanine mutations in full-length SNAP-25.** We next examined the effect of mutating the nine residues of SNAP-25 determined from the peptide screening, on the interaction between full length SNAP-25 and  $G\beta\gamma$ . The order of mutations was chosen primarily to start from the two residues implicated in the region previously published to be important to the  $G\beta\gamma$  - t-SNARE interaction: R198 and K201 of SNAP-25 (Yoon et al., 2007) that we termed “2A.” upon mutagenesis. From that, further mutations of residues in close proximity to R198 and K201 found in both helices, and the loop connecting the two helices, were made including: E62A/R198A/K201A (“3A”), E62A/D99A/R198A/K201A (“4A”), and E62A/D99A/K102A/R198A/K201A (“5A”). Continuing, further mutations were designed beginning at the N-terminal end of the two SNAP-25 helices. This set of mutations includes: E62A/D99A/K102A/R135A/R198A/K201A (“6A”), E62A/D99A/K102A/R135A/R136A/R198A/K201A (“7A”), E62A/D99A/K102A/R135A/R136A/R142A/R198A/K201A (“8A”), and E62A/D99A/K102A/R135A/R136A/R142A/R161A/R198A/K201A (“9A”). The full set of SNAP-25 mutants is defined in Table 2.

We then utilized a sensitive and quantitative fluorescence assay to compare the ability of wild-type SNAP-25 and these mutated SNAP-25 proteins to bind  $G\beta_1\gamma_1$ . Purified  $G\beta_1\gamma_1$  was labeled with MIANS, an environmentally sensitive fluorescent probe. MIANS fluorescence will increase in a local

**MOL #80507**

hydrophobic environment, as would be anticipated in protein-protein interaction. In Figure 3a, 20 nM MIANS-G $\beta_1\gamma_1$  fluorescence increased when exposed to increasing amounts of wild type SNAP-25 saturating with an EC<sub>50</sub> of 0.35  $\mu$ M SNAP-25. Mutations 2A-5A display decreased affinity for G $\beta\gamma$  (see Figure 3A and Table 2). The EC<sub>50</sub> for the 5A mutant is 1.2  $\mu$ M. The mutations (3A) – (5A) appeared to behave similarly in this assay. Further cumulative mutations, 6A-9A, result in limited enhancement over baseline fluorescence of MIANS-G $\beta\gamma$  compared to the increase achieved by wild type SNAP-25 (see Figure 3B, Table 2).

To test the importance of the N-terminal region (R135A, R136A, R142A, and R161A) alone, a construct was made that incorporated only these mutations in the SNAP-25 protein. As seen in Figure 3C, as compared to wild type SNAP-25, this mutant (N4A) had a decreased maximal fluorescence, and its EC<sub>50</sub> was 0.20  $\mu$ M (95% C.I.: 0.10-0.40 $\mu$ M) as compared to 0.40  $\mu$ M (95% C.I.: 0.25-0.64  $\mu$ M) for wild type. This suggests that in interaction between G $\beta\gamma$  and SNAP-25 is altered (loss of fluorescence enhancement) but the high affinity C-terminal binding site is maintained.

**SNAP-25 C-terminal peptide inhibits SNAP-25 – G $\beta\gamma$  interaction.** Mutations of full-length SNAP-25 suggested a complex binding mechanism between SNAP-25 and G $\beta\gamma$  that extends beyond the C-terminus of SNAP-25. Peptides identified previously in screens synthesized and screened in the fluorescence intensity assay of 20 nM MIANS-labeled G $\beta_1\gamma_1$  with a fixed amount (0.3  $\mu$ M) of SNAP-25 that gives half maximal response (n=3) were tested for their ability to inhibit interaction between SNAP-25 and MIANS-G $\beta_1\gamma_1$ . Four of the five peptides showed no effect (see Figure 4). Only the C-terminal peptide (SNAP-25<sub>193-206</sub>) was able to inhibit the interaction significantly. This is the same peptide that was able to functionally inhibit serotonin-mediated inhibition (via G $\beta\gamma$ ) in lamprey central synapses (Gerachshenko et al., 2005). A modified SNAP-25<sub>193-206</sub> peptide in which residues R198 and K201 were mutagenized to Ala was unable to inhibit the SNAP-25-mediated fluorescent enhancement of MIANS-G $\beta_1\gamma_1$  in the same fashion as the wild-type SNAP-25<sub>193-206</sub> peptide (see Figure 4). It is also possible that

## MOL #80507

the other peptides adopt a conformation in solution that differs from its structure in the intact protein or tethered to a membrane.

**Effect of SNAP-25 mutations on synaptotagmin binding.** Calcium-dependent synaptotagmin binding to t-SNARE has been shown to mediate exocytotic fusion (Brose et al., 1992; Chapman et al., 1995; Chapman and Jahn, 1994; Geppert et al., 1994; Mehta et al., 1996). To confirm that mutant SNAP-25 can still interact with synaptotagmin, the GST-fusion proteins of wild-type SNAP-25 or the mutants 5A-9A immobilized to glutathione-sepharose beads were exposed to synaptotagmin-1 C2AB, either in the presence of the calcium chelator 2mM EGTA or 1mM CaCl<sub>2</sub>. As can be seen in Figure 5a, wild type SNAP-25 is capable of binding to synaptotagmin-1 in the presence of calcium with an increase in bound synaptotagmin-1, as expected. There is no statistically significant decrease in calcium-dependent binding between the SNAP-25 mutants and synaptotagmin-1 with progressive mutation of SNAP-25 residues when compared with binding to wild type SNAP-25 (Figure 5b, Student's t-test,  $p > 0.05$ ).

Synaptotagmin binding to SNAP-25 and syntaxin 1A can also occur in a calcium-independent manner (Gerona et al., 2000; Mahal et al., 2002; Nishiki and Augustine, 2004; Rickman and Davletov, 2003). In the presence of 2mM EGTA, the interaction between synaptotagmin-1 and mutant SNAP-25(6A) (Student's t-test,  $p < 0.05$ ) and SNAP-25(9A) (Student's t-test,  $p < 0.01$ ) is reduced relative to wild type SNAP-25. Binding was most dramatically reduced for the SNAP-25 9A mutant suggesting that that calcium-independent binding of synaptotagmin-1 to SNAP-25 is at least in part mediated by R161. To test this, the single mutation R161A was made in wild type SNAP-25 and pull down experiments were performed with synaptotagmin-1 C2AB. As can be seen in the western blots in Figure 5c and densitometry measured over three separate experiments in Figure 5d, mutation of this one residue resulted in a significant decrease in calcium-independent binding of synaptotagmin-1 when compared to wild type SNAP-25 (Student's t-test,  $p < 0.01$ ), however there was no difference in calcium-dependent binding to synaptotagmin-1 between SNAP-25 and SNAP-25(R161A). These results for SNAP-25(R161A) are similar to the results for SNAP-25(9A).



**MOL #80507**

**t-SNARE formed with syntaxin 1A and SNAP-25(8A) has decreased affinity for G $\beta$  $\gamma$ .** We investigated whether SNAP-25 mutations would reduce G $\beta$  $\gamma$  binding when assembled with syntaxin 1A into a t-SNARE complex. Increasing concentrations of t-SNARE consisting of full-length syntaxin 1A and SNAP-25 increased fluorescence of MIANS-G $\beta$  $\gamma$ . The EC<sub>50</sub> was 0.13  $\mu$ M (n=4, 95% C.I. 0.067-0.26  $\mu$ M) (see Figure 6), similar to the previous results shown earlier and those reported by Yoon et al. (Yoon et al., 2007). Using a subcloning strategy, 8 of the alanine mutations were introduced with a single reaction into the SNAP-25 sequence in a vector that contains both full-length syntaxin 1A and SNAP-25 that is used to dually express both proteins simultaneously to promote efficient t-SNARE formation. When t-SNARE consisting of syntaxin 1A with this subcloned SNAP-25(8A) mutant, termed t-SNARE(8A), was used in this assay, an identical maximal fluorescence enhancement as wild-type t-SNARE was observed (see Figure 6), but this t-SNARE(8A) had a four-fold lower affinity for MIANS-G $\beta$  $\gamma$  with a measured EC<sub>50</sub> of 0.58  $\mu$ M (n=4, 95% C.I. 0.47-0.70 $\mu$ M).

**SNARE complexes formed with SNAP-25 (8A) in neurons has impaired ability to support G $\beta$  $\gamma$  mediated inhibition.** If G $\beta$  $\gamma$  inhibits exocytosis by interacting with t-SNARE, the mutant SNAP-25 that interacts less well with G $\beta$  $\gamma$  should decrease the effect of G $\beta$  $\gamma$  on exocytosis. To test this hypothesis, mutant SNAP-25s were injected into lamprey neurons to test its ability to impair serotonin-mediated inhibition of vesicle release, which is mediated by G $\beta$  $\gamma$  (Blackmer et al., 2001; Gerachshenko et al., 2005). Synaptic responses were evoked at 30 s intervals by evoking presynaptic action potentials with brief depolarizing current pulses (2ms, 1-3 nA). After obtaining at least 10 responses serotonin (1 $\mu$ M) was bath applied to the preparation and a further series of EPSCs were evoked. Serotonin significantly reduced the EPSC amplitudes to 24  $\pm$  8 % of the control amplitude. SNAP-25 and SNAP-25 (8A) was injected into presynaptic axons using pressure pulses. We confirmed that SNAP-25 protein was injected by imaging injected SNAP-25 that had been fluorescently labeled in four paired cell recordings (data not shown).

## MOL #80507

We sought to replace endogenous SNAP-25 with a mutant form in the giant synapses. To facilitate this experiment, SNAP-25 was modified to be resistant to BoNT/E by mutating Asp 179 to Lys (Zhang et al., 2002). Either SNAP-25(D179K) or SNAP-25(D179K)(8A) were injected (1mg/ml) along with light chain BoNT/E (25  $\mu$ g/ml) into a presynaptic neuron in the lamprey. After recording the initial responses for 5 minutes, as well as an initial 300 stimuli at 1Hz to remove docked and primed vesicles (Gerachshenko et al., 2005), EPSC were recorded again after 5 minutes of recovery. Injection of BoNT/E alone eliminated synaptic transmission (Figure 7a). By contrast, injection of BoNT/E together with SNAP-25(D179K) partially restored synaptic transmission, showing that the injected SNAP-25 was capable of reconstituting t-SNAREs with endogenous syntaxin1A (Figure 7b). Treatment with 5-HT (1 $\mu$ M) inhibited synaptic transmission to  $24 \pm 13\%$  control (n=3), evidence that injected SNAP-25 could support the normal G $\beta\gamma$ -mediated inhibition of synaptic transmission. To test whether SNAP-25 (8A) can support 5-HT-mediated presynaptic inhibition, SNAP-25(D179K)(8A) was injected presynaptically together with BoNT/E. After a period of 300 Stimuli (1Hz), the EPSC amplitude was recovered to  $73 \pm 9\%$  of control amplitude (n=5) (Figure 7c). Thus, SNAP-25 with the 8 Ala mutations still interacted with the rest of the exocytotic machinery properly. Application of 5-HT had a significantly reduced effect on EPSC amplitude in the presence of SNAP-25(D179K)(8A). This is shown by EPSC amplitudes reduced to  $76 \pm 5\%$  of the amplitude before 5-HT ( $p < 0.01$  compared to inhibition seen using either 5-HT alone or after injection of the protein without including BoNT/E) (Figure 7c). This is evidence that decreasing G $\beta\gamma$ 's affinity for SNAP-25 impairs the normal function of the inhibitory 5-HT receptor to decrease synaptic transmission through the G $\beta\gamma$  subunit.

## MOL #80507

### Discussion

In this study, we have defined the critical residues on SNAP-25 for  $G\beta\gamma$  binding and created a SNAP-25 with minimal affinity for  $G\beta\gamma$  which still interacts in a calcium-dependent manner with synaptotagmin-1. We have confirmed the previously determined C-terminal interaction (Yoon et al., 2007) as well as identified another binding site at the distal end of the coiled-coil of t-SNARE. Additionally, we have shown that one of the  $G\beta\gamma$ -binding residues, R161, is involved in calcium-independent binding to synaptotagmin-1. These two clusters of residues – one at the C-terminus of SNAP-25 (C domain) and a second near the N-terminus of SNAP-25 (N domain) suggest a more complex interaction between  $G\beta\gamma$  and SNAP-25. These data confirm the direct role of  $G\beta\gamma$  regulation of synaptic vesicle release at the exocytotic machinery. SNAP-25 (8A) retains its ability to form SNARE complexes and participate in exocytosis as shown in lamprey neurons, evidence that its interactions with syntaxin, synaptobrevin, and synaptotagmin are not perturbed. The fact that 5-HT-mediated inhibition of EPSCs was dramatically attenuated by SNAP-25 (8A) even while  $G\beta\gamma$ 's *in vitro* interaction with t-SNARE was decreased only 4-fold by this mutant confirms the previous findings of Gerashchenko et al. (2005) that SNAP-25 is critically important for  $G\beta\gamma$ 's inhibition of exocytosis.

Previous work determined the C-terminus of SNAP-25 was important in the binding of  $G\beta\gamma$  both at the molecular level, as well as in synaptic function (Blackmer et al., 2005; Gerachshenko et al., 2005; Yoon et al., 2007; Zhao et al., 2010). Functional studies showed that BoNT/A treatment eliminated the modulatory effect of the  $G_{i/o}$ -coupled 5-HT receptor and  $G\beta\gamma$  on synaptic transmission (Gerachshenko et al., 2005). Biochemical studies showed a decrease in affinity between  $G\beta_1\gamma_1$  and SNAP-25 when its C-terminal 9 amino acids were removed as compared with wild type SNAP-25, but there was complete loss of binding when the C-terminal 26 amino acids were removed, suggesting the major functional binding site of  $G\beta\gamma$  was within those C-terminal 26 amino acids (Yoon et al., 2007). The ability of SNAP-25 C-terminal peptides to inhibit wild-type SNAP-25 binding to  $G\beta_1\gamma_1$  confirmed the importance of the C-

**MOL #80507**

terminus of SNAP-25 (see Figure 4). Peptides derived from the N domain did not compete with the wild-type SNAP-25, reflecting the higher affinity of the C domain.

Blackmer et al. and Yoon et al. showed that Gβγ and synaptotagmin-1 C2AB compete for binding to t-SNARE, and postulated a mechanism by which presynaptic G<sub>i/o</sub>-coupled receptors, via Gβγ, inhibit exocytosis (Blackmer et al., 2005; Yoon et al., 2007). Gβγ binding to ternary SNARE made with SNAP-25 missing its C-terminal 9 amino acids was more sensitive to competition by synaptotagmin-1 than wild type ternary SNARE (Yoon et al., 2007). We have defined key residues in this region as R198 and K201. In addition, we discovered a set of residues which may be nearby this region, D99 and K102, within a loop that is unresolved in the x-ray crystal structures of SNARE complexes, as well as E62, in SNAP-25's first helix (Figure 2). When all of these residues are mutated to Ala (5A), its apparent affinity for Gβγ goes from 0.35 μM to 0.83 μM (Figure 3).

Our work suggests a second region on SNAP-25 important for binding Gβγ, a region on the second helix of SNAP-25 in close proximity to its N-terminus including R135, R136, and R142 (see Figure 8). Alanine substitution of these residues leads to slightly increased affinity (WT: 0.40 μM compared to N4A: 0.20 μM) with a decreased maximal fluorescence (see Figure 3c). Substitution of the C domain as well as the N domain residues (9A) leads to an overall decrease in EC<sub>50</sub> for Gβγ and decreased maximal fluorescence.

We previously showed that the ability of Gβγ to inhibit exocytosis is overcome in high calcium (Yoon et al., 2007); we speculated that the higher affinity of Ca<sup>2+</sup>-synaptotagmin for t-SNARE could overcome Gβγ competition. Remarkably, the residues on SNAP-25 that are important for Gβγ binding do not interfere with calcium-dependent binding of synaptotagmin-1 C2AB (Fig. 5). Previous studies showed three Asp residues (Asp179, Asp186, Asp193) at the C-terminus of SNAP-25 are required for calcium-dependent binding of synaptotagmin-1 to SNAP-25 (Zhang et al., 2002). It is interesting that Gβγ and Ca<sup>2+</sup>-synaptotagmin-1 C2AB bind the same region of SNAP-25, but use different residues for binding this region. There is also a minor calcium-independent binding of synaptotagmin-1 to t-SNARE

**MOL #80507**

(Gerona et al., 2000; Mahal et al., 2002; Nishiki and Augustine, 2004; Rickman and Davletov, 2003). We show that mutating R161 to Ala significantly decreases calcium-independent binding of synaptotagmin-1 to SNAP-25, without impacting calcium-dependent SNAP-25–synaptotagmin-1 interaction (Fig. 5).

Limited docking of G $\beta\gamma$  and t-SNARE structures show that these two clusters of interacting residues on SNAP-25 are too distant for binding between one dimer of G $\beta\gamma$  and one t-SNARE complex. Measuring the x-ray structures of ternary SNARE (PDBID#: 1SFC, (Sutton et al., 1998)) and G $\beta_1\gamma_1$  (PDBID#:1TBG, (Sondek et al., 1996)), respectively, the distance between the most distal mutated SNAP-25 residues, 135 and 201, is  $\sim 90\text{\AA}$ , and the greatest distance across G $\beta_1\gamma_1$  is  $\sim 70\text{\AA}$ . These two binding sites may permit more than one G $\beta\gamma$  dimer to bind a single SNARE complex (greater than 1:1 stoichiometry) whereby two separate dimers occupy the respective binding sites on a single ternary SNARE. Based on other known interactions of SNARE complexes with other proteins, G $\beta\gamma$  may shift from the high- to low-affinity binding sites or vice versa in the context of simultaneous interactions. Alternatively, SNAP-25 or G $\beta\gamma$  may adopt different conformations upon binding than those reported in existing x-ray crystallographic structures of SNARE proteins. To date, however, no structures have been reported for G $\beta\gamma$ -SNARE complexes, t-SNARE alone, or t-SNARE in complex with full-length synaptobrevin during the initial stages of ternary SNARE complex formation. In the other reported structures, SNAP-25 and syntaxin 1A adopted the same conformation regardless of partner (Chen et al., 2002; Kümmel et al., 2011; Pobbati et al., 2004; Sutton et al., 1998).

When a mutated SNAP-25 was expressed and purified in the context of full length syntaxin-1A, the t-SNARE had a reduction in affinity for G $\beta\gamma$  compared to wild type (Figure 6). This result demonstrated that altering the binding affinity of SNAP-25 can change the binding affinity of the t-SNARE complex for G $\beta\gamma$  in the context of t-SNARE with wild-type syntaxin 1A. Furthermore, in the presence of this mutant SNAP-25, G $\beta\gamma$  was dramatically less effective at mediating presynaptic inhibition of vesicle release by 5-HT in lamprey neurons (Figure 7).

## MOL #80507

The importance of the newly identified residues may be in the context of other interactions with the ternary SNARE complex. Numerous other proteins bind to SNARE proteins including calcium channels (Davies et al., 2011), Munc18 (Smyth et al., 2010), tomosyn (Hatsuzawa et al., 2003), and complexin (Tang, 2009). There is evidence of complex interactions, such as between syntaxin 1A, G $\beta$  $\gamma$ , and the calcium channel (Jarvis et al., 2000). In general, there are rearrangements thought to occur within the SNARE proteins throughout the process of regulated exocytosis. One such rearrangement is the transition of syntaxin 1A from a closed to an open position with the relative movement of the H<sub>abc</sub> region away from the remainder of the complex containing the SNARE motifs (Dulubova et al., 1999; Gerber et al., 2008; Hammarlund et al., 2007). Also, there is evidence of two states of SNARE complex: an “unzipped” state that occurs in the docking phase where there is recognition between vesicle and plasma membrane, but the vesicle is not primed and cannot fuse, and the fully “zipped” state that has full association of the SNARE motifs and is fully primed before calcium-triggered synaptotagmin-1 binding and fusion of the two membranes (Di et al., 2012; Nishiki and Augustine, 2004; Pobbati et al., 2006; Rickman and Davletov, 2003). Our results suggest two binding sites for G $\beta$  $\gamma$  and suggest that G $\beta$  $\gamma$  interactions with SNARE complexes could have a role in these and other interactions besides competition for binding with synaptotagmin-1 for SNARE proteins (Yoon et al., 2007). Future studies will determine if residues identified in our studies may have implications in those systems.

The success of the peptide screening technique will allow us to similarly characterize the interaction of G $\beta$  $\gamma$  with syntaxin 1A and synaptobrevin, as well as characterize the binding sites on G $\beta$  $\gamma$  that interact with the SNARE complex. Further work will also be carried out to explore other protein-protein interactions of the exocytotic machinery that might be affected by mutation of these 8 residues on SNAP-25.

In summary, we have established novel residues on SNAP-25 for G $\beta$  $\gamma$  that implies complex binding between the two partners. Mutation of these residues to Ala led to a decreased affinity for G $\beta$  $\gamma$ . This allowed us to critically test the hypothesis that G<sub>i/o</sub>-coupled 5-HT receptors cause inhibition of

**MOL #80507**

vesicle fusion through  $G\beta\gamma$  interaction with SNARE. Mutant SNAP-25 (8A) has loss of binding to  $G\beta\gamma$ , but retains its ability to form SNARE complexes and participate in exocytosis, thereby confirming the direct role of  $G\beta\gamma$  regulation of synaptic vesicle release at the exocytotic machinery.

**MOL #80507**

**Acknowledgements:** We would like to thank Nathan Swentko (Intavis) for his assistance in using the RespepSL. We thank Summer Young (Vanderbilt) for assistance in peptide synthesis. We thank Nathan Kett (Vanderbilt) for assistance in HPLC purification of the peptides. We thank Matthew Duvernay for critical review of the manuscript. Lastly, we thank Ed Chapman for providing the t-SNARE dual-expression vector.



**MOL #80507**

**Authorship Contributions:**

Participated in research design: Wells, Zurawski, Betke, Alford, and Hamm

Conducted experiments: Wells, Zurawski, Betke, Yim, Rodriguez, and Alford

Contributed new reagents: Wells, Zurawski, Hyde

Performed data analysis: Wells, Zurawski, Alford

Wrote or contributed to writing of the manuscript: Wells, Zurawski, Alford, and Hamm

**MOL #80507**

## References

- Betke KM, Wells CA and Hamm HE (2012) GPCR mediated regulation of synaptic transmission. *Prog Neurobiol* **96**(3): 304-321.
- Binz T, Blasi J, Yamasaki S, Baumeister A, Link E, Sudhof TC, Jahn R and Niemann H (1994) Proteolysis of SNAP-25 by types E and A botulinum neurotoxins. *J Biol Chem* **269**(3): 1617-1620.
- Blackmer T, Larsen EC, Bartleson C, Kowalchuk JA, Yoon EJ, Preininger AM, Alford S, Hamm HE and Martin TF (2005) G protein betagamma directly regulates SNARE protein fusion machinery for secretory granule exocytosis. *Nat Neurosci* **8**(4): 421-425.
- Blackmer T, Larsen EC, Takahashi M, Martin TF, Alford S and Hamm HE (2001) G protein betagamma subunit-mediated presynaptic inhibition: regulation of exocytotic fusion downstream of Ca<sup>2+</sup> entry. *Science* **292**(5515): 293-297.
- Brose N, Petrenko AG, Sudhof TC and Jahn R (1992) Synaptotagmin - A Calcium Sensor on the Synaptic Vesicle Surface. *Science* **256**(5059): 1021-1025.
- Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR and Hamm HE (2003) Insights into G Protein Structure, Function, and Regulation. *Endocr Rev* **24**(6): 765-781.
- Chapman ER, Hanson PI, An S and Jahn R (1995) Ca<sup>2+</sup> regulates the interaction between synaptotagmin and syntaxin 1. *J Biol Chem* **270**(40): 23667-23671.
- Chapman ER and Jahn R (1994) Calcium-Dependent Interaction of the Cytoplasmic Region of Synaptotagmin with Membranes - Autonomous Function of a Single C-2-Homologous Domain. *J Biol Chem* **269**(8): 5735-5741.
- Chen X, Tomchick DR, Kovrigin E, Araç D, Machius M, Südhof TC and Rizo J (2002) Three-Dimensional Structure of the Complexin/SNARE Complex. *Neuron* **33**(3): 397-409.
- Chicka MC, Hui E, Liu H and Chapman ER (2008) Synaptotagmin arrests the SNARE complex before triggering fast, efficient membrane fusion in response to Ca<sup>2+</sup>. *Nat Struct Mol Biol* **15**(8): 827-835.
- Clapham DE and Neer EJ (1997) G protein beta gamma subunits. *Annu Rev Pharmacol Toxicol* **37**: 167-203.
- Davies JN, Jarvis SE and Zamponi GW (2011) Bipartite syntaxin 1A interactions mediate CaV2.2 calcium channel regulation. *Biochem Biophys Res Commun* **411**(3): 562-568.
- De Waard M, Hering J, Weiss N and Feltz A (2005) How do G proteins directly control neuronal Ca<sup>2+</sup> channel function? *Trends Pharmacol Sci* **26**(8): 427-436.
- Delaney AJ, Crane JW and Sah P (2007) Noradrenaline Modulates Transmission at a Central Synapse by a Presynaptic Mechanism. *Neuron* **56**(5): 880-892.
- Di W, Hu Q, Yan Z, Chen W, Yan C, Huang X, Zhang J, Yang P, Deng H, Wang J, Deng X and Shi Y (2012) Structural basis of ultraviolet-B perception by UVR8. *Nature* **484**(7393): 214-219.

**MOL #80507**

- Dolphin AC (2003) G protein modulation of voltage-gated calcium channels. *Pharmacol Rev* **55**(4): 607-627.
- Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Sudhof TC and Rizo J (1999) A conformational switch in syntaxin during exocytosis: role of munc18. *EMBO J* **18**(16): 4372-4382.
- Eaton WW, Martins SS, Nestadt G, Bienvenu OJ, Clarke D and Alexandre P (2008) The burden of mental disorders. *Epidemiol Rev* **30**: 1-14.
- Frank R (2002) The SPOT-synthesis technique: Synthetic peptide arrays on membrane supports--principles and applications. *Journal of Immunological Methods* **267**(1): 13-26.
- Gautam N, Downes GB, Yan K and Kisselev O (1998) The G-protein betagamma complex. *Cell Signal* **10**(7): 447-455.
- Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF and Sudhof TC (1994) Synaptotagmin I: a major Ca<sup>2+</sup> sensor for transmitter release at a central synapse. *Cell* **79**(4): 717-727.
- Gerachshenko T, Blackmer T, Yoon EJ, Bartleson C, Hamm HE and Alford S (2005) Gbetagamma acts at the C terminus of SNAP-25 to mediate presynaptic inhibition. *Nat Neurosci* **8**(5): 597-605.
- Gerber SH, Rah J-C, Min S-W, Liu X, de Wit H, Dulubova I, Meyer AC, Rizo J, Arancillo M, Hammer RE, Verhage M, Rosenmund C and Südhof TC (2008) Conformational Switch of Syntaxin-1 Controls Synaptic Vesicle Fusion. *Science* **321**(5895): 1507-1510.
- Gerona RRL, Larsen EC, Kowalchuk JA and Martin TFJ (2000) The C Terminus of SNAP25 Is Essential for Ca<sup>2+</sup>-dependent Binding of Synaptotagmin to SNARE Complexes. *J Biol Chem* **275**(9): 6328-6336.
- Hammarlund M, Palfreyman MT, Watanabe S, Olsen S and Jorgensen EM (2007) Open syntaxin docks synaptic vesicles. *PLoS Biol* **5**(8): e198.
- Hatsuzawa K, Lang T, Fasshauer D, Bruns D and Jahn R (2003) The R-SNARE motif of tomosyn forms SNARE core complexes with syntaxin 1 and SNAP-25 and down-regulates exocytosis. *J Biol Chem* **278**(33): 31159-31166.
- Hille B (1994) Modulation of ion-channel function by G-protein-coupled receptors. *Trends Neurosci* **17**(12): 531-536.
- Ikeda SR and Dunlap K (1999) Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. *Adv Second Messenger Phosphoprotein Res* **33**: 131-151.
- Jarvis SE, Magga JM, Beedle AM, Braun JE and Zamponi GW (2000) G protein modulation of N-type calcium channels is facilitated by physical interactions between syntaxin 1A and G betagamma. *J Biol Chem* **275**: 6388-6394.
- Koch WJ, Inglese J, Stone WC and Lefkowitz RJ (1993) The binding site for the beta gamma subunits of heterotrimeric G proteins on the beta-adrenergic receptor kinase. *J Biol Chem* **268**(11): 8256-8260.

**MOL #80507**

- Kümmel D, Krishnakumar SS, Radoff DT, Li F, Giraudo CG, Pincet F, Rothman JE and Reinisch KM (2011) Complexin cross-links prefusion SNAREs into a zigzag array. *Nat Struct Mol Biol* **18**(8): 927-933.
- Mahal LK, Sequeira SM, Gureasko JM and Söllner TH (2002) Calcium-independent stimulation of membrane fusion and SNAREpin formation by synaptotagmin I. *J Cell Biol* **158**(2): 273-282.
- Mazzoni MR, Malinski JA and Hamm HE (1991) Structural analysis of rod GTP-binding protein, Gt. Limited proteolytic digestion pattern of Gt with four proteases defines monoclonal antibody epitope. *J Biol Chem* **266**(21): 14072-14081.
- Mehta PP, Battenberg E and Wilson MC (1996) SNAP-25 and synaptotagmin involvement in the final Ca(2+)-dependent triggering of neurotransmitter exocytosis. *Proc Natl Acad Sci USA* **93**(19): 10471-10476.
- Nishiki T-i and Augustine GJ (2004) Dual Roles of the C2B Domain of Synaptotagmin I in Synchronizing Ca<sup>2+</sup>-Dependent Neurotransmitter Release. *J Neurosci* **24**(39): 8542-8550.
- Phillips WJ and Cerione RA (1991) Labeling of the beta gamma subunit complex of transducin with an environmentally sensitive cysteine reagent. Use of fluorescence spectroscopy to monitor transducin subunit interactions. *J Biol Chem* **266**(17): 11017-11024.
- Photowala H, Blackmer T, Schwartz E, Hamm HE and Alford S (2006) G protein betagamma-subunits activated by serotonin mediate presynaptic inhibition by regulating vesicle fusion properties. *Proc Natl Acad Sci U S A* **103**(11): 4281-4286.
- Pobbati AV, Razeto A, Böddener M, Becker S and Fasshauer D (2004) Structural Basis for the Inhibitory Role of Tomosyn in Exocytosis. *J Biol Chem* **279**(45): 47192-47200.
- Pobbati AV, Stein A and Fasshauer D (2006) N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. *Science* **313**(5787): 673-676.
- Rickman C and Davletov B (2003) Mechanism of Calcium-independent Synaptotagmin Binding to Target SNAREs. *J Biol Chem* **278**(8): 5501-5504.
- Schiavo G, Santucci A, Dasgupta BR, Mehta PP, Jontes J, Benfenati F, Wilson MC and Montecucco C (1993) Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Lett* **335**(1): 99-103.
- Schrodinger, LLC (2010) The PyMOL Molecular Graphics System, Version 0.99rc6.
- Scott JK, Huang SF, Gangadhar BP, Samoriski GM, Clapp P, Gross RA, Taussig R and Smrcka AV (2001) Evidence that a protein-protein interaction 'hot spot' on heterotrimeric G protein betagamma subunits is used for recognition of a subclass of effectors. *EMBO J* **20**(4): 767-776.
- Smrcka AV (2008) G protein betagamma subunits: central mediators of G protein-coupled receptor signaling. *Cell Mol Life Sci* **65**(14): 2191-2214.

**MOL #80507**

- Smyth A, Duncan R and Rickman C (2010) Munc18-1 and Syntaxin1: Unraveling the Interactions Between the Dynamic Duo. *Cell Mol Neurobiol* **30**(8): 1309-1313.
- Sondek J, Bohm A, Lambright DG, Hamm HE and Sigler PB (1996) Crystal structure of a G-protein beta gamma dimer at 2.1A resolution. *Nature* **379**(6563): 369-374.
- Sutton RB, Fasshauer D, Jahn R and Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4A resolution. *Nature* **395**(6700): 347-353.
- Tang J (2009) Complexins, in *Encyclopedia of Neuroscience* (Larry RS ed) pp 1-7, Academic Press, Oxford.
- Tucker WC, Weber T and Chapman ER (2004) Reconstitution of Ca<sup>2+</sup>-Regulated Membrane Fusion by Synaptotagmin and SNAREs. *Science* **304**(5669): 435-438.
- Vanderbeld B and Kelly GM (2000) New thoughts on the role of the beta-gamma subunit in G-protein signal transduction. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **78**(5): 537-550.
- Weng G, Li J, Dingus J, Hildebrandt JD, Weinstein H and Iyengar R (1996) G $\beta$  Subunit Interacts with a Peptide Encoding Region 956-982 of Adenylyl Cyclase 2. *J Biol Chem* **271**(43): 26445-26448.
- Yoon EJ, Gerachshenko T, Spiegelberg BD, Alford S and Hamm HE (2007) Gbetagamma interferes with Ca<sup>2+</sup>-dependent binding of synaptotagmin to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. *Mol Pharmacol* **72**(5): 1210-1219.
- Yoon EJ, Hamm HE and Currie KP (2008) G protein betagamma subunits modulate the number and nature of exocytotic fusion events in adrenal chromaffin cells independent of calcium entry. *J Neurophysiol* **100**(5): 2929-2939.
- Zhang X-l, Upreti C and Stanton PK (2011) G $\beta\gamma$  and the C Terminus of SNAP-25 Are Necessary for Long-Term Depression of Transmitter Release. *PLoS One* **6**(5): e20500.
- Zhang X, Kim-Miller MJ, Fukuda M, Kowalchuk JA and Martin TFJ (2002) Ca<sup>2+</sup>-Dependent Synaptotagmin Binding to SNAP-25 Is Essential for Ca<sup>2+</sup>-Triggered Exocytosis. *Neuron* **34**(4): 599-611.
- Zhao Y, Fang Q, Straub SG, Lindau M and Sharp GW (2010) Noradrenaline inhibits exocytosis via the G protein betagamma subunit and refilling of the readily releasable granule pool via the alpha(11/2) subunit. *J Physiol* **588**(Pt 18): 3485-3498.

**MOL #80507**

**Footnotes:**

This work was supported by the National Institutes of Health National Eye Institute [RO1 EY010291]; the National Institutes of Health National Center for Research Resources [UL1 RR024975-01]; and the National Institutes of Health National Heart, Lung, and Blood Institute [T32 HL007411].

To whom correspondence should be addressed: Heidi E. Hamm, Department of Pharmacology, Vanderbilt University Medical Center, 442 Robinson Research Building, 23rd Ave. South @ Pierce, Nashville, TN, USA, Tel.: (615) 343-3533; Fax: (615)-343-1084; E-mail: [heidi.hamm@vanderbilt.edu](mailto:heidi.hamm@vanderbilt.edu).

**MOL #80507**

**Figure Legends**

**Figure 1. Screening of SNAP-25 peptides for interaction with  $G\beta_1\gamma_1$ .** A) The basic premise of the screening is a “far-western”. The peptide synthesizer creates peptides on a derivatized membrane. With appropriate washes in between steps, the membrane and peptides are sequentially exposed to  $G\beta_1\gamma_1$ , primary antibody for  $G\beta$ , and HRP-conjugated secondary antibody. B) Representative image of a membrane exposed to  $G\beta_1\gamma_1$ . Numbering reflects spots with successive peptides 1-65. Spots 66-69 were left without peptide synthesized on them as negative controls. Shown separately are the peptides spots derived from the sequences for the SIRK peptide, QEHA peptide,  $\beta$ ARK peptide, the  $G\beta\gamma$  binding domain of the calcium channel CaV2.2, and the C-terminus of  $G\beta_1$ . C) Densitometry was performed on the three membranes using ImageJ analysis of the image. Each membrane was normalized to the most intense spot on the membrane. The average of the three membranes was plotted for each set of 65 peptides that span the full length of SNAP-25. The x-axis reflects both the peptide number according to (B) as well as the residue number of the first residue in each respective peptide. Circles reflect clusters of peptides with the highest densitometric signal. D) Representative sequences of the clusters of SNAP-25 peptides that were found are shown in red mapped onto the representation of the x-ray crystal structure of the core SNARE motifs (PDB ID: 2SFC); synaptobrevin, light gray; syntaxin 1A, dark gray; first SNAP-25 helix, green; second SNAP-25 helix, yellow. Syntaxin 1A and synaptobrevin each have a transmembrane domain shown as  $\alpha$ -helices. The black bar inserted into the membrane represents the palmitoylation sites on SNAP-25. The green arc represents the non-structured sequence between the two  $\alpha$ -helices of SNAP-25. This arc includes one of the peptide clusters (red) near the palmitoylation site. “N” signifies the N-terminal end of the helices within the SNARE complex; “C” signifies the C-terminal end of the helices within the SNARE complex.

**Figure 2. Alanine mutagenesis screening of SNAP-25 peptides that bind  $G\beta\gamma$ .** A) Representative image of the alanine screening for SNAP-25 peptides synthesized on a membrane. Five peptides are identified by their sequence number shown on the left. The first spot of each row contains

## MOL #80507

wild type peptide. The next 14 spots to the right are mutant peptides with a single alanine replacement of the residue at position 1, 2, 3...14 for each wild type peptide. B) Densitometry was performed across three separate membranes for each respective peptide and its series of mutants. Mean +/- SD from the three membranes is shown for the five peptides shown in A (Student's t-test; \* -  $p < 0.05$ , \*\* -  $p < 0.01$ , \*\*\* -  $p < 0.001$ ). C) The residues (spheres, red) that had significantly reduced G $\beta\gamma$  binding when mutated to alanine are mapped onto the x-ray crystal structure of ternary snare (PDB ID: 1sfc). The colors signify syntaxin 1A, dark gray; synaptobrevin, light gray; first SNAP-25 helix, green; and second SNAP-25 helix, yellow; unstructured domain between the two SNAP-25  $\alpha$ -helices, green cartoon arc.

**Figure 3. Binding of SNAP-25 and its alanine mutants to MIANS-labeled G $\beta\gamma$ .** A) A fixed concentration of MIANS-G $\beta_1\gamma_1$  (20 nM) was exposed to increasing concentrations of SNAP-25 with resulting increase in fluorescence,  $n=4$ .  $F1/F0$  is the ratio of fluorescence of G $\beta_1\gamma_1$  measured in the presence of SNAP-25 over the fluorescence of G $\beta_1\gamma_1$  in the absence of SNAP-25. The fluorescence was corrected for any intrinsic fluorescence of SNAP-25 at the various concentrations. Finally, all of the curves were normalized to the highest fluorescence achieved by binding of wild type SNAP-25 to G $\beta_1\gamma_1$ . (A) dose-response curves for wild-type SNAP-25, SNAP-25(2A), SNAP-25(3A), SNAP-25(4A), and SNAP-25(5A). To the right of the dose-response curves is a cartoon modified from Figure 2. The red circle denotes the area on the SNARE complex where these mutated residues are located together in the C-terminus. (B) The remaining alanine mutants of SNAP-25 were tested for binding to MIANS-G $\beta_1\gamma_1$ . As compared to wild type SNAP-25, increasing numbers of mutations resulted in decreased  $EC_{50}$  and then decreased maximum fluorescence enhancement of MIANS-G $\beta_1\gamma_1$  (A and B). C) A SNAP-25 mutant with residues in the amino terminal region (R135A, R136A, R142A, and R161A) of the SNAP-25 protein termed N4A. When exposed to fluorescently labeled G $\beta_1\gamma_1$ , this mutant (N4A) had a decreased maximal fluorescence, and its  $EC_{50}$  was 0.20  $\mu\text{M}$  as compared to 0.35  $\mu\text{M}$ . The cartoon in the right portion of (C) shows the region with N-terminal mutated residues.



**MOL #80507**

**Figure 4. Inhibition of Gβγ-SNAP-25 binding by SNAP-25 peptides.** Each peptide (1.5 mM) was added to the fluorescence assay detecting the interaction of 20 nM MIANS-Gβ<sub>1</sub>γ<sub>1</sub> with 0.3 μM SNAP-25. The C-terminal peptide (193-206) was the only peptide to significantly decrease the fluorescence enhancement (Student's t-test, p<0.01; n=3). When the residues R198 and K201 were changed to alanine in that peptide, the peptide was no longer effective at reducing fluorescence enhancement by SNAP-25 (Student's t-test, p>0.05; n=3).

**Figure 5. Binding of SNAP-25 mutants to synaptotagmin-1 by GST pulldowns.** A) GST, GST-SNAP-25 wildtype, and GST-mutant SNAP-25(6A) through SNAP-25(9A) on glutathione beads were exposed to synaptotagmin-1 for 1 hour in either 1mM calcium or 2mM EGTA, washed, and then eluted with sample buffer. Representative blots imaged with Odyssey for simultaneous quantitation of synaptotagmin-1 (green) and GST (red) signal intensity. B) The ratio of normalized synaptotagmin-1:GST signals were averaged over three samples in the two conditions. The results are shown in the bar graph (\*\*, p<0.01 when compared to WT in 2mM EGTA; one-way ANOVA, Tukey's Multiple Comparison post-test). C) GST, GST-SNAP-25 wildtype, and GST-fused mutant SNAP-25(R161A) on glutathione beads were exposed to synaptotagmin-1 for 1 hour in either 1mM calcium or 2mM EGTA, washed, and then eluted with sample buffer. Representative blots imaged with Odyssey for simultaneous quantitation of synaptotagmin-1 (green) and GST (red) signal intensity. D) The ratio of normalized synaptotagmin-1:GST signals were averaged over three samples in the two conditions. The results are shown in the bar graph (\*\*, p<0.01 when compared to WT in 2mM EGTA; Student's t-test).

**Figure 6. Binding of Wild type t-SNARE and SNAP-25(8A) t-SNARE to MIANS-labeled Gβγ.** A fixed concentration of MIANS-Gβ<sub>1</sub>γ<sub>1</sub> (20 nM) was exposed to increasing concentrations of wild-type t-SNARE with resulting increase in fluorescence, closed triangles, n=4. The EC<sub>50</sub> for t-SNARE binding to MIANS-Gβ<sub>1</sub>γ<sub>1</sub> was 0.13 μM (95% C.I., 0.07-0.26 μM). Similarly, the t-SNARE complex of syntaxin 1A with SNAP-25(8A) was exposed to MIANS-Gβ<sub>1</sub>γ<sub>1</sub> with the resulting increase in fluorescence

**MOL #80507**

shown in the figure, open triangles,  $n=4$ . The EC<sub>50</sub> for this complex binding to G $\beta_1\gamma_1$  is 0.58  $\mu\text{M}$  (95% C.I., 0.47-0.70  $\mu\text{M}$ ).

**Figure 7. Effect of SNAP-25 (8A) on presynaptic inhibition in lamprey with 5-HT.** Paired cell recordings were made between lamprey giant reticulospinal axons and postsynaptic ventral horn target neurons. Each recording shown is the mean of at least 10 sequential responses. Overlaid presynaptic action potentials are shown below. A) In a recording in which BoNt/E was included in the presynaptic microelectrode, pressure injection of BoNt/E toxin left synaptic transmission intact (black). A period of 300 stimuli (1Hz) left no remaining chemical EPSC (early component is electrical) following loss of primed toxin resistant vesicles. B) A similar recording in which BoNt/E and SNAP-25 (D179) were included in the presynaptic electrode. A period of 300 stimuli (1Hz) reduced but did not eliminate the EPSC (gray). Addition of 5-HT (1 $\mu\text{M}$ ) substantially reduced this remaining response (blue). C) With BoNt/E and SNAP-25 (D179) (8A) included in the presynaptic pipette. The graph shows peak chemical EPSC amplitudes recorded against time before (closed circles) during (open circles) and after (closed circles) 300 stimuli at 1Hz. Addition of 5-HT (1 $\mu\text{M}$ , blue) failed to inhibit the synaptic response. EPSC examples are means of 10 from before (black), after 300 stimuli (1Hz) gray, and after addition of 5-HT (1 $\mu\text{M}$ , blue).

**Figure 8. G $\beta\gamma$  - SNARE binding model.** Based on the results of this study, G $\beta\gamma$  appears to not only bind at or near the C-terminus of SNAP-25, but there are additional residues distal to the membrane-approximated portion of SNAP-25. Taken in the context of ternary SNARE and its proposed position at a docked synaptic vesicle, a single G $\beta\gamma$  dimer activated by a G $_{i/o}$ -coupled GPCR that is bound to the C-terminus of SNAP-25 would not be able to bind the distal portion of the SNARE complex at the same time. The additional residues appear to have implications in calcium-independent binding of synaptotagmin, but they may also have importance for G $\beta\gamma$  modulation of other interactions with SNARE proteins. These could include calcium channels, tomosyn, complexin, and Munc18.

**MOL #80507**

*Tables*

**Table 1. SNAP-25 peptides found in screening**

SNAP-25 peptides that bound to G $\beta_1\gamma_1$  in the initial screening and had loss of binding when a residue was mutated to alanine are listed below. The residue(s) important for loss of binding is shown in boldface.

Peptide	Peptide Sequence
59-72	RIEEGMDQINKDMK
89-102	VCPCNK <b>LKSSDAYK</b>
130-143	SGGF <b>IRRV</b> TNDARE
151-164	EQVSGIIGN <b>L</b> RHMA
193-206	DEAN <b>QRATK</b> MLGSG

**Table 2. SNAP-25 alanine mutants**

Residues determined to be important for Gβγ binding to SNAP-25 peptides were successively introduced into the native SNAP-25 sequence. Listed are the names given to each SNAP-25 mutant with the corresponding list of residues mutated to alanine. Residues in boldface are the new mutated residue added to the previously made mutant SNAP-25. Max is the maximum fluorescence enhancement (F1/F0) of the non-linear regression for the each mutant, normalized to the maximum enhancement for wild type SNAP-25. (SE – standard error). The N4A mutant contains only the four alanine mutations near the N-terminus of the SNARE complex.

Mutant Name	Residues of SNAP-25 mutated	Log EC <sub>50</sub> (M), (±SE)	EC <sub>50</sub> (M), (±SE)	Max (±SE)
WT	N/A	-6.45(± 0.20)	3.5e-7, (2.2e-7 to 5.6e-7)	100 (±15)
2A	R198A, K201A	-6.18 (± 0.13)	6.6e-7, (4.9e-7 to 8.9e-7)	96 (± 10)
3A	<b>E62A</b> , R198A, K201A	-5.93 (± 0.12)	1.2e-6, (8.9e-7 to 1.5e-6)	122 (± 16)
4A	E62A, <b>D99A</b> , R198A, K201A	-6.12 (± 0.13)	7.6e-7, (5.6e-7 to 1.0e-6)	71 (± 8)
5A	E62A, D99A, <b>K102A</b> , R198A, K201A	-6.08 (± 0.15)	8.3e-7, (5.9e-7 to 1.7e-6)	70 (± 9)
6A	E62A, D99A, K102A, <b>R135A</b> , R198A, K201A	-5.97 (± 0.08)	1.1e-6, (7.4e-7 to 1.2e-6)	88 (± 8)
7A	E62A, D99A, K102A, R135A, <b>R136A</b> , R198A, K201A	-6.12 (± 0.09)	7.6e-7, (6.2e-7 to 8.9e-7)	33 (± 3)
8A	E62A, D99A, K102A, R135A, R136A, <b>R142A</b> , R198A, K201A	-5.87 (± 0.12)	1.3e-6, (1.02e-6 to 1.8e-6)	20 (± 3)
9A	E62A, D99A, K102A, R135A, R136A, R142A, <b>R161A</b> , R198A, K201A	-6.36 (± 0.23)	4.4e-7, (2.6e-7 to 7.4e-7)	25 (± 4)
N4A	R135A, R136A, R142A, R161A	-6.69 (± 0.14)	2.0e-7, (1.5e-7 to 2.8e-7)	44 (± 3)

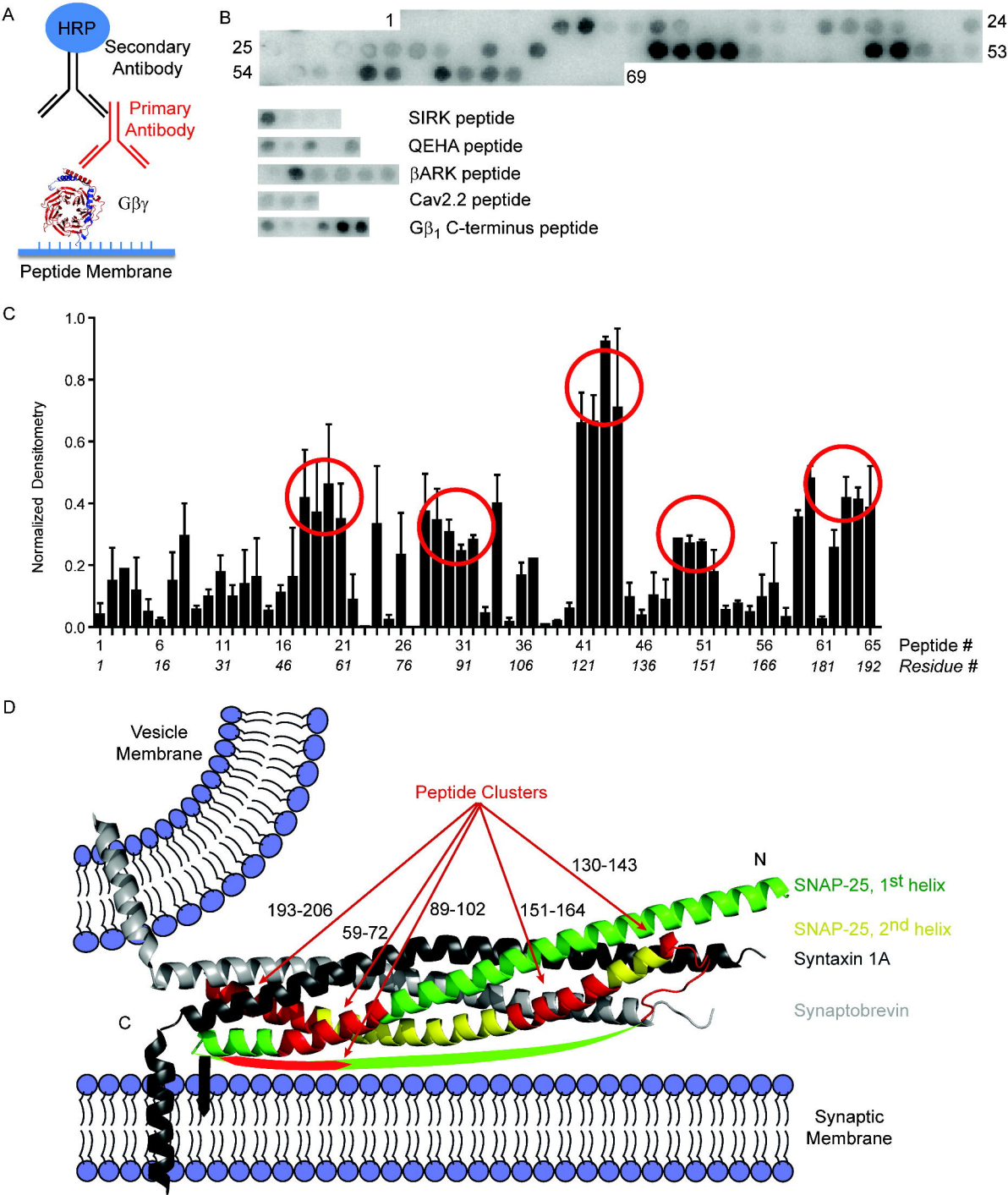


Figure 1

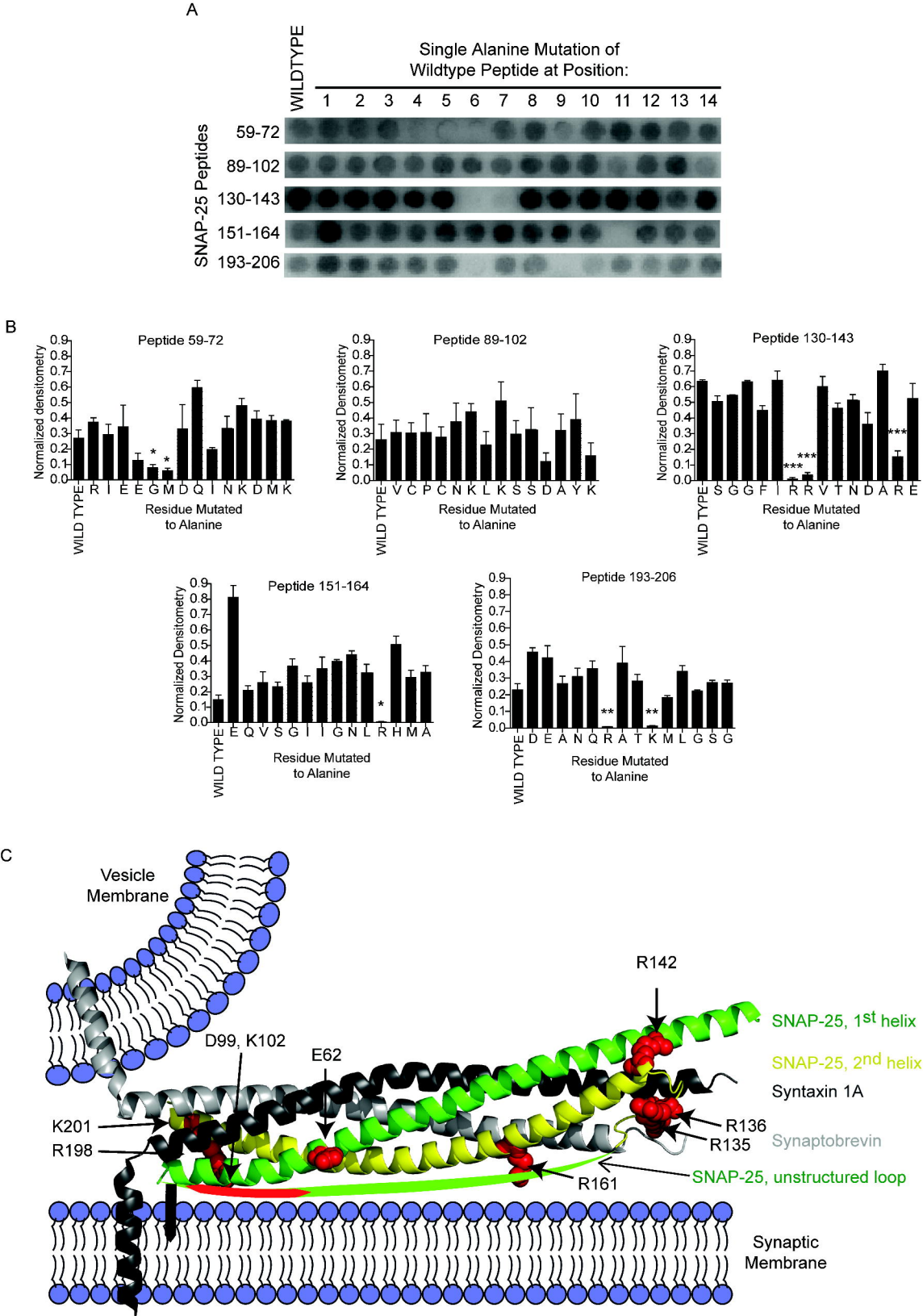
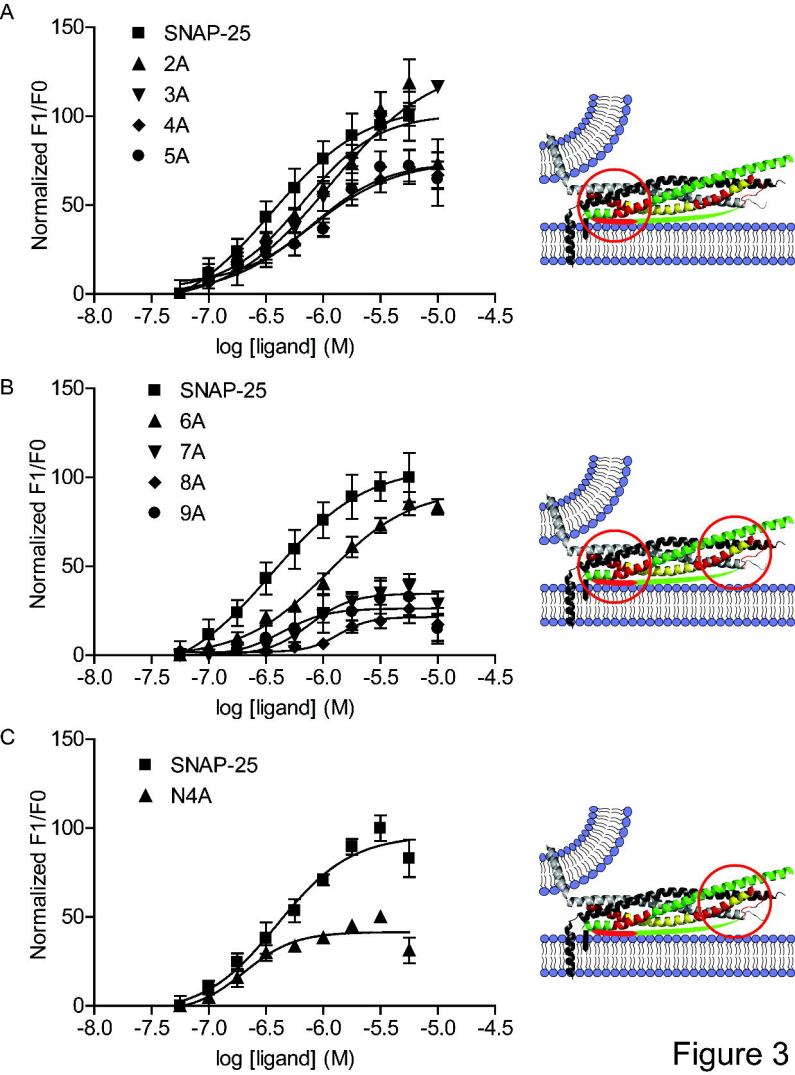


Figure 2



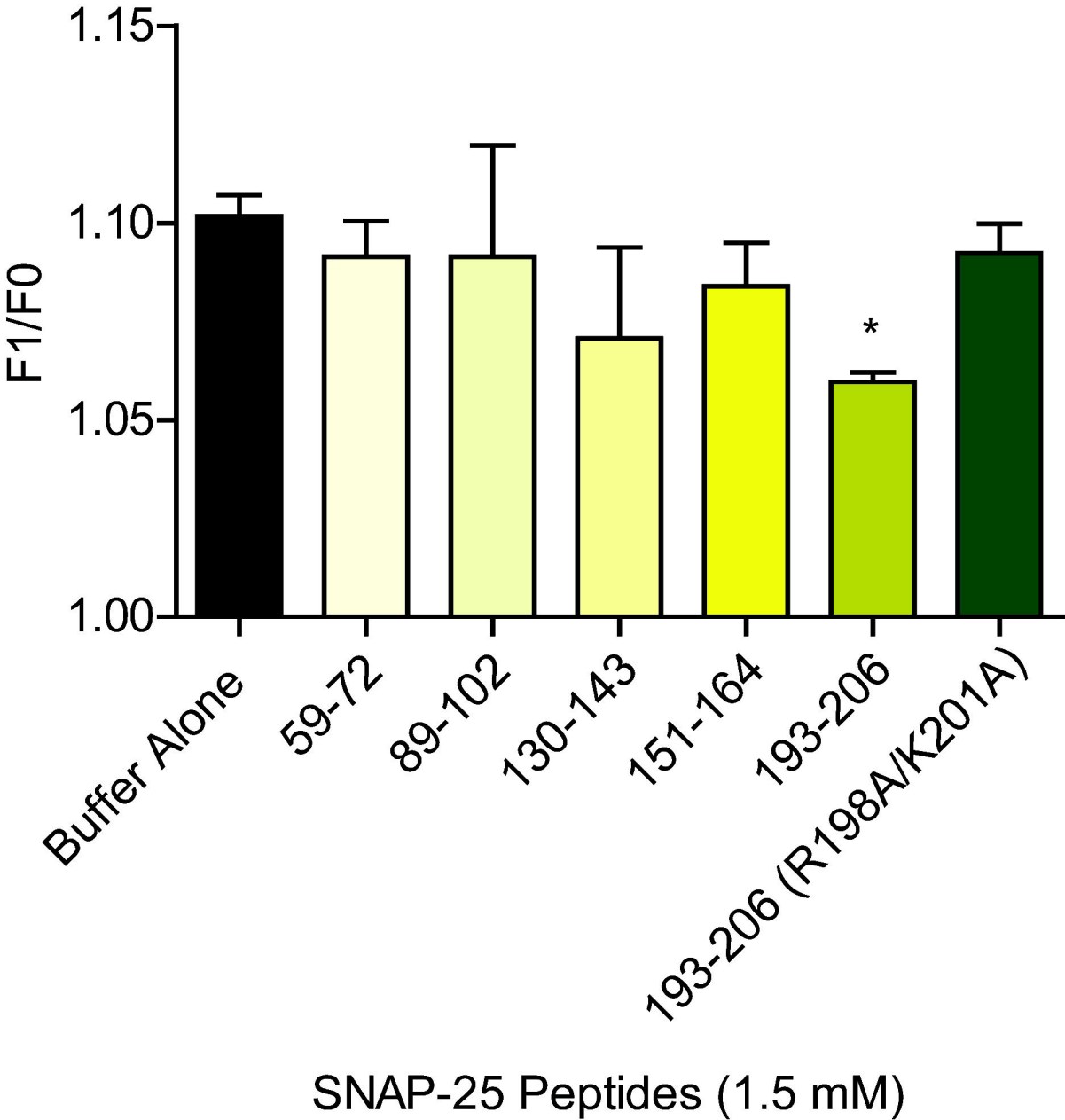


Figure 4



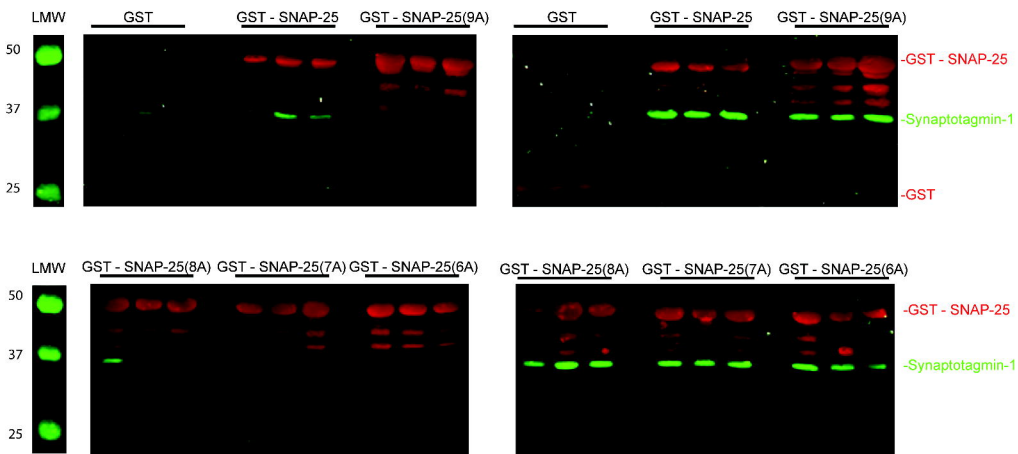
BAIT: GST-SNAP-25

IB: Green - synaptotagmin 1

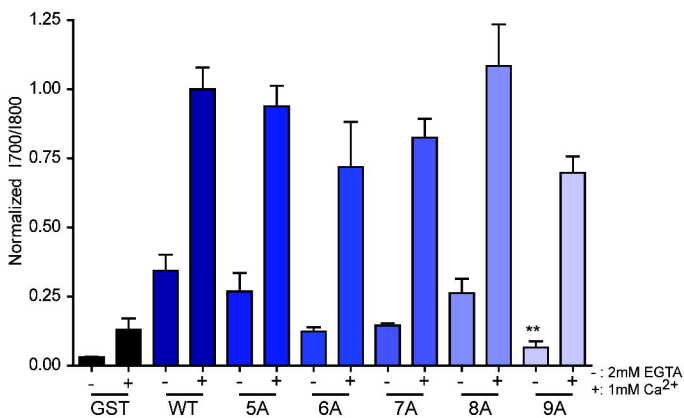
Red - GST

2mM EGTA

1mM Ca<sup>2+</sup>



B



C

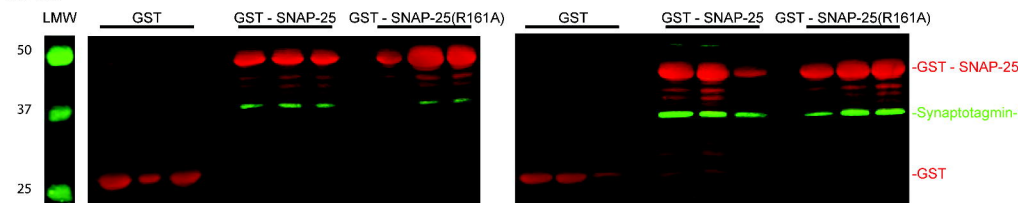
BAIT: GST-SNAP-25

IB: Green - synaptotagmin 1

Red - GST

2mM EGTA

1mM Ca<sup>2+</sup>



D

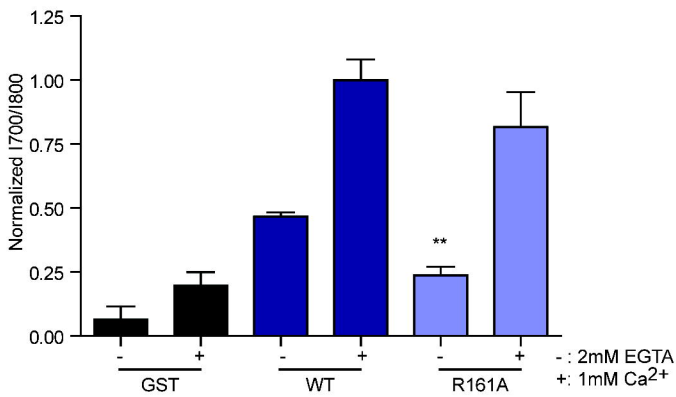


Figure 5

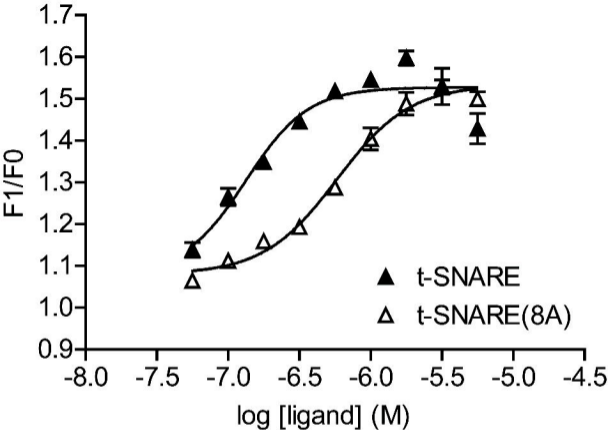
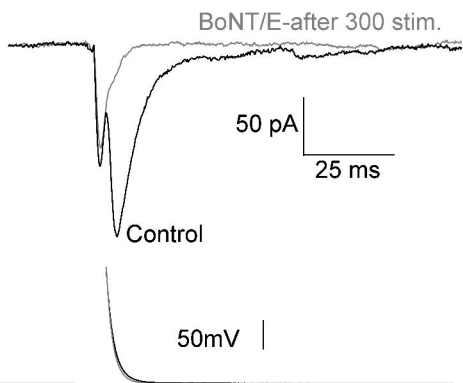
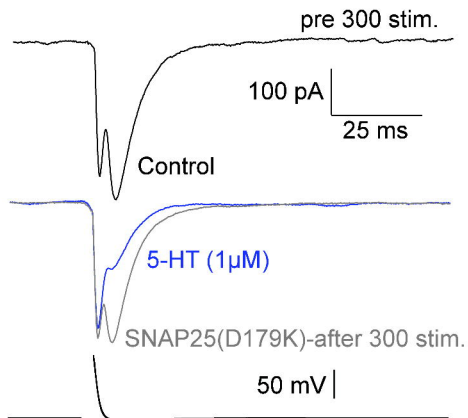


Figure 6

A. following BoNT/E alone



B. following BoNT/E & BoNT/E SNAP-25(D179K)



C. following BoNT/E & BoNT/E SNAP-25(D179K)(8A)

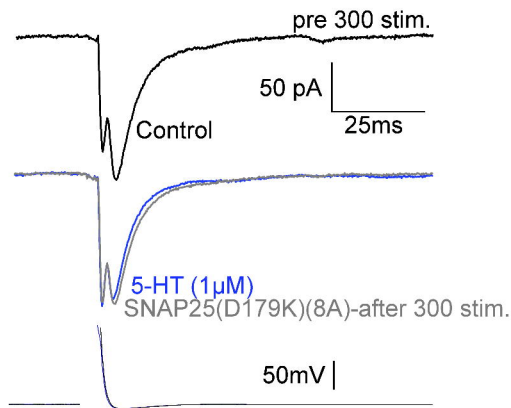
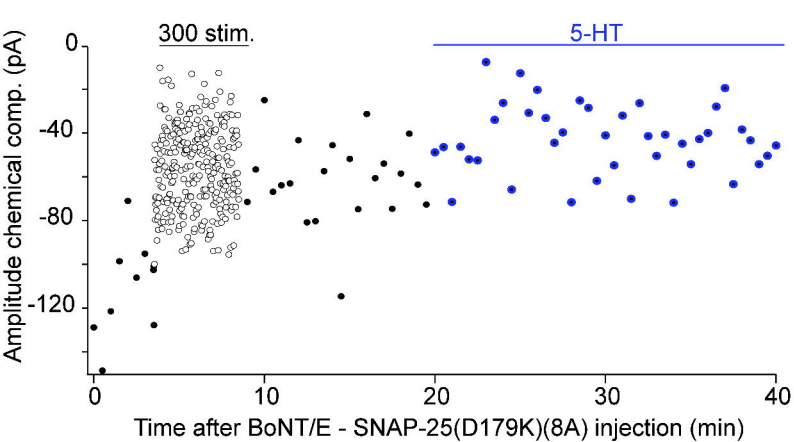


Figure 7

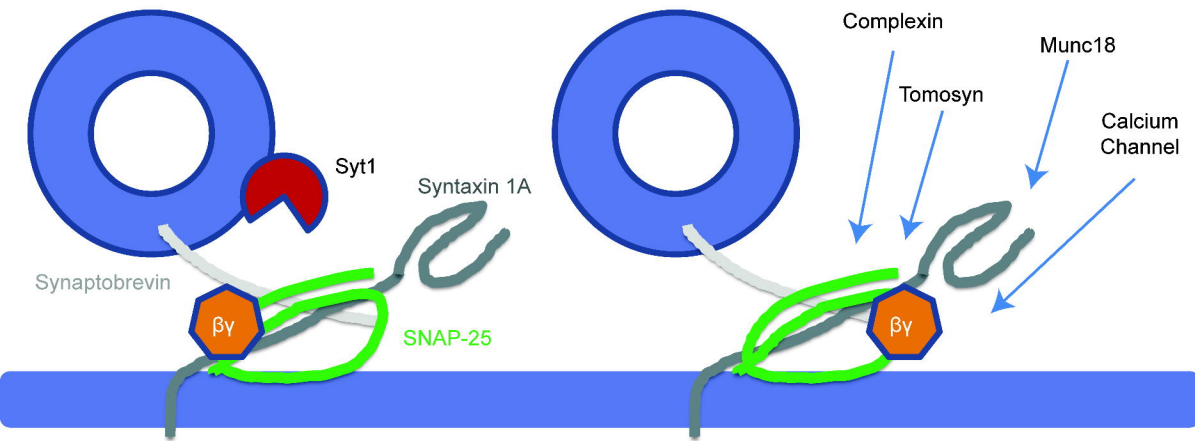


Figure 8