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Gβγ interferes with Ca^{2+}-dependent binding of synaptotagmin to the SNARE complex

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Running title: Gβγ mediated modulation of exocytosis.

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Abbreviations: SNARE; soluble N-ethylmaleimide-sensitive factor attachment protein receptor. [Ca^{2+}]; Ca^{2+} concentration. SNAP25; Synaptosomal-associated protein of 25,000 dalton. MIANS; 2-(4'-maleimidylanilino) naphthalene-6-sulfonic acid. BoNT/A; botulinum toxin A. Synaptotagmin, Syt I (at figure); synaptotagmin I.

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Abstract

Presynaptic inhibitory GPCRs can decrease neurotransmission by inducing interaction of Gβγ with the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Previously, we showed that this action of Gβγ requires the carboxyl terminus of SNAP25 and is downstream of the well-known inhibition of Ca^{2+} entry through voltage-gated calcium channels (VGCC). We proposed a mechanism in which Gβγ and synaptotagmin compete for binding to the SNARE complex. Here, we characterized the Gβγ interaction sites on syntaxin1A and SNAP25 and demonstrated an overlap of the Gβγ- and synaptotagmin I -binding regions on each member of the SNARE complex. Synaptotagmin competes in a Ca^{2+}-sensitive manner with binding of Gβγ to SNAP25, syntaxin1A and the assembled SNARE complex. We predict based on these findings that at high intracellular Ca^{2+} concentrations, Ca^{2+}-synaptotagmin I can displace Gβγ binding, and the Gβγ-dependent inhibiton of exocytosis can be blocked. We tested this hypothesis in giant synapses of the lamprey spinal cord, where 5-HT works via Gβγ to inhibit neurotransmission (Blackmer et al., 2001). We showed that increased presynaptic Ca^{2+} suppresses the 5-HT- and Gβγ-dependent inhibition of exocytosis. We suggest that this effect may be due to Ca^{2+}-dependent competition between Gβγ and synaptotagmin I for SNARE binding. This type of dynamic regulation may represent a novel mechanism for modifying transmitter release in a graded manner based on the history of action potentials that increase intracellular Ca^{2+} concentrations and of inhibitory signals through Gi-coupled GPCRs.

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Introduction

Modification of neurotransmitter release by presynaptic G protein coupled receptors (GPCRs) is ubiquitous and vital to nervous system function. Gβγ inhibits synchronous exocytosis directly at the SNARE complex, the heart of the fusion machinery (Blackmer et al., 2005; Blackmer et al., 2001; Chen et al., 2005; Gerachshenko et al., 2005; Man-Son-Hing et al., 1989; Silinsky, 1984; Takahashi et al., 2001), and consequently modifies the quantal size of release by causing transient fusion of vesicles in both lamprey giant synapses and mammalian chromaffin cells (Chen et al., 2005; Photowala et al., 2006). G proteins may also inhibit exocytosis through inhibition of VGCCs, blunting the influx of Ca^{2+} required for exocytosis (Herlitze et al., 1996; Ikeda, 1996).

Synaptotagmin I is thought to mediate the requirement for Ca^{2+} in the initiation of exocytosis. Prior to the fusion event, vesicles are primed by formation of a stable protein structure, the ternary SNARE complex, comprising the plasma membrane t-SNAREs, SNAP-25 and syntaxin, and the vesicular SNARE (v-SNARE), VAMP2 (Fig1A). Synaptotagmin, as the putative Ca^{2+} sensor, is thought to act as the trigger by which Ca^{2+} influx effects the fusion of primed vesicles (Bai et al., 2004; Maximov and Sudhof, 2005; Wang et al., 2006).

Given its central role in triggering exocytosis, interactions between synaptotagmins and the SNARE complex have been studied extensively. Upon Ca^{2+} binding to its C2 domains (Fig. 1A) during regulated exocytosis, synaptotagmin interacts with phospholipids and the SNARE complex (Perin et al., 1990; Schiavo et al., 1997). This interaction occurs through the H3 SNARE domain of syntaxin1A and the C-terminus of SNAP25 (Bai et al., 2004; Bennett et al., 1992; Chapman et al., 1995; Gerona et al., 2000). While the mechanism by which the SNARE complex initiates fusion is under debate (Rizo et al., 2006), interactions with synaptotagmin may

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cause stabilized ternary SNAREs to zip up, uniting the vesicular and plasma membranes (Margittai et al., 1999; Sorensen et al., 2006). Other evidence suggests that ternary SNARE complex formation is a concerted process with more than one transition state during initial and sustained dilation of a protein fusion pore (Han and Jackson, 2006).

Despite the controversy with regards to the SNARE complex-mediated membrane fusion, widespread consensus holds that synaptotagmin is the Ca^{2+} sensor for stimulated exocytosis and may provide the cue for full fusion versus kiss-and-run fusion. Mutagenesis studies, for example, indicate that fusion is inhibited in the presence of synaptotagmin mutants with diminished SNARE interactions (Bai et al., 2004). Furthermore, over-expression of different isoforms of synaptotagmin in PC12 cells leads to different fusion pore opening durations, suggesting that the interaction of synaptotagmin with the SNARE complex and the membrane impacts the nature of fusion pore formation and dilation (Wang et al., 2001). Gβγ signaling is another mediator of fusion pore dynamics (Blackmer et al., 2005; Chen et al., 2005; Gerachshenko et al., 2005; Photowala et al., 2006), but it is unclear how the Ca^{2+}-dependent synaptotagmin/SNARE complex interaction is actively modulated by Gβγ (Searl and Silinsky, 2005).

We have shown recently that the nine C-terminal residues of SNAP25 that interact with synaptotagmin provide an important binding site for Gβγ. The resulting Gβγ/SNARE interaction inhibits transmitter release in vertebrate central synapses and mammalian neuroendocrine cells (Blackmer et al., 2005; Gerachshenko et al., 2005). We now identify Gβγ binding sites on the SNARE complex. Additionally, we identify a role for Ca^{2+} and demonstrate competition between Gβγ and synaptotagmin for binding to SNARE complexes. We propose that modulation of exocytosis is subject to convergent and complementary pathways directed to alteration of

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synaptotagmin/SNARE complex interactions in which presynaptic Ca^{2+} both stimulates fusion and modifies the impact of Gi/o-coupled inhibitory GPCRs on exocytosis.

Materials and Methods

Plasmids

The open reading frames for the SNARE component proteins were sub-cloned into the pGEX6p1 vector (Amersham) for expression in bacteria. Individual truncation mutants were generated via a PCR-based strategy.

SNARE protein purification

Recombinant bacterially-expressed glutathione S-transferase fusion proteins were expressed in *Escherichia coli* strain BL21(DE3). Protein expression was induced with 0.1 mM isopropyl *β*-D-thiogalactoside for 16 hr at room temperature. Bacterial cultures were pelleted, washed with 1×PBS and then resuspend in lysis buffer {50 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 0.1% TritonX-100, 2 mM PMSF, and 1 mM dithiothreitol (DTT)}. Cells were lysed with a sonic dismembrator at 4 ℃. GST-SNAP25 and GST-VAMP2 were purified from cleared lysates by affinity chromatography on glutathione–agarose (Pharmacia), following the manufacturer's instructions. While the proteins were bound to the column, the buffer was exchanged to 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.05% n-octyl *β*-D-glucopyranoside (OG) and 5 mM dithiothreitol. In some cases, the proteins were eluted by cleaving from GST with PreScission protease (GE Healthcare) for 4 hr at 4 ℃ and then dialysed extensively against 20 mM HEPES, pH 7.0, 80 mM KCl, 20 mM NaCl and 0.05% OG. GST-Syntaxin1A (lacking the transmembrane domain) was purified from the sonicated bacterial supernatant by affinity chromatography on

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glutathione–agarose (Pharmacia) in 10 mM Hepes, pH 7.4, 0.05 % OG and 2 mM DTT. Protein concentrations were determined with a Bradford assay kit (Pierce), and purity was verified by SDS/PAGE analysis.

Binary and ternary SNARE complex reassembly

An equimolar ratio (3 μM) of GST-syntaxin1A on glutathione-agarose and SNAP25 were incubated overnight at 4 ℃ in 20 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% OG and 2.0 mM dithiothreitol. The binary t-SNARE complex was washed three times with PBS and eluted from the column by removing GST with PreScission protease (GE healthcare) for 4 hr at 4 ℃. Equimolar protein-protein interaction was confirmed by SDS-PAGE/Coomassie staining analysis. To reassemble ternary SNARE complex, an equimolar ratio (3 μM) of GST-syntaxin1A on glutathione-agarose, VAMP2 and SNAP25 were incubated overnight at 4 ℃ in 20 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% OG and 2.0 mM DTT. The SNARE proteins were washed and eluted from the column by removing GST. SDS- and heat-stable SNARE complex formation was verified by SDS/PAGE analysis with/without a 15 min incubation at 100 ℃.

Gβγ purification and labeling

$G\beta_1\gamma_1$ was purified from bovine retina as previously described (Mazzoni et al., 1991). Recombinant $G\beta_1\gamma_2$ was expressed in Sf9 cells and purified via a His_6 tag on $G\gamma_2$ using Ni-NTA affinity chromatography (Sigma Aldrich). Fluorescence labeling of $G\beta_1\gamma_1$ and binding assays were conducted as described (Phillips and Cerione, 1991). Briefly, purified $G\beta_1\gamma_1$ was dialyzed into labeling buffer (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 150 mM NaCl, 10% Glycerol), then mixed with 2-(4'-Maleimidylanilino) naphthalene-6-sulphonic acid (MIANS) in a 5-fold molar

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excess. The reaction proceeded for 3 hr at 4 ℃ before quenching with 5 mM 2-mercaptoethanol. The MIANS-$G\beta_1\gamma_1$ complex was separated from unreacted MIANS using a PD-10 desalting column (GE Healthcare). MIANS- $G\beta_1\gamma_1$ was stored in aliquots at -80 ºC.

Fluorescence binding and competition assay

All fluorescence measurements were performed in a fluorescence spectrophotometer (Cary Eclipse) at room temperature. Generally, MIANS-$G\beta_1\gamma_1$ was diluted into 0.5 ml of assay buffer (20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 0.1 M NaCl, 1 mM EDTA) to a final concentration of 20 nM. The MIANS fluorescence was monitored at excitation 322 nm and emission 417 nm. All proteins purified as GST fusion proteins were cleaved from GST with PreScission protease (GE healthcare) prior to analysis. The fluorescent changes caused by the addition of SNARE complexes were monitored continuously. Note that the amplitude of the fluorescence increase is not a measure of the affinity of the complex, but rather reflects the specific site on fluorescently-labeled Gβγ of interaction with each protein. There was no non-specific binding of the free probe to the SNARE proteins and MIANS-Gβγ was resistant to photobleaching under experimental conditions (data not shown). For Gβγ/synaptotagmin competition assays, various concentrations of synaptotagmin with SNARE proteins were added to labeled $G\beta_1\gamma_1$ with the noted Ca^{2+} concentrations, and fluorescence changes were monitored. The EC_{50} concentrations were determined by sigmoidal curve fitting using GraphPad Prism.

GST pull down assay

GST-SNAP25, GST-VAMP2 and GST-syntaxin1A (5 μg of each protein) were incubated with various amount of $G\beta_1\gamma_2$ proteins for 1 hr at 4 ºC and washed three times with assay buffer (20

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mM Hepes, pH 7.0, 80 mM KCl, 20 mM NaCl and 0.1% OG). The complex was eluted with 20 µl of SDS sample buffer followed by separation via SDS-PAGE. Precipitated Gβ was detected using western bloting with a rabbit anti-pan Gβ antibody (Santa Cruz, T-20).

Electrophysiology and microinjection

Lamprey spinal cord preparations (*Petromyzon marinus*) were performed as previously described (Wickelgren, 1977). Whole-cell patch-clamp recordings were achieved with a modified blind technique (Blanton et al., 1989) using pipettes (5 to 10 MΩ) containing solution of the following composition: 102.5 mM $MeSO_4$, 1 mM NaCl, 1 mM $MgCl_2$, 5 mM HEPES, 0.05 mM EGTA, brought to a pH of 7.4 with KOH. All paired recordings to measure the effect of protein injection in this study were made at a minimum of 5 min after injection and less than 100 µm from the presynaptic injection site. Recombinant $G\beta_1\gamma_2$ protein was stored at -20 ^oC in a solution containing 100 mM $MeSO_4$, 5 mM HEPES, 0.05 mM EGTA at pH of 7.4. Concentrations ranged from 3 - 5 mg/ml. For microinjection into the presynaptic terminal, 50% by volume of 1 M $KMeSO_4$ was added to the $G\beta_1\gamma_2$ containing solution. Protein was pressure microinjected through presynaptic microelectrodes using Picospritzer II.

Imaging

Fluorescence images were recorded with a modified confocal microscope (Bio-Rad MRC600) run with custom software available at http://alford.bios.uic.edu. Reticulospinal axons were labeled retrogradely with a dextran amine-conjugate form of the Ca^{2+}-sensitive dye Fluo-4 (high affinity; Molecular Probes). The dye was applied using a suction pipette fitted to the cut end, immediately after the end of the spinal cord was cut. The tissue was then incubated overnight for

the dye to be transported throughout the axons. Images were collected at high speed by scanning a laser over a single line at 500 Hz. Imaging data were analysed using ImageJ software on a Macintosh computer. ImageJ was used to calculate the brightness value (range of possible values, 10 bit) for each pixel in a field of view. For each individual axon of interest, the brightness values were measured, and after background subtraction, images were normalized to the baseline level of fluorescence to give $(F+1)/F$ values.

Calibration of the Ca^{2+} sensitive dyes

The dye sensitivity to Ca^{2+} was determined using the same optical path (confocal microscope) that was used to measure Ca^{2+} transients in the tissue. Dyes (5 μM) were prepared in blends of two Ca^{2+} buffer standards (10 mM K_2EGTA, 100 mM KCl and 30 mM MOPS) and (10 mM CaEGTA, 100 mM KCl and 30 mM MOPS) both at pH 7.2 (Molecular Probes) to make an 11 point standard curve. The absolute Ca^{2+} values of the mix were corrected for the low temperatures used in this study (10 °C). The dye buffer mix was placed between cover slips cooled from below with a liquid cooling system to 10 °C and imaged from above with the 40X water immersion lens.

Western blots are representative of at least three independent experiments. Data points are presented as mean ± SEM (* indicates $p < 0.01$ and ** indicates $p < 0.001$, two-tailed Student's t-test).

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Results

Gβγ interacts with individual SNARE proteins as well as ternary SNARE complex.

To understand the relationship between Gβγ and the SNARE complex in the modulation of exocytosis, we characterized sites of interaction by two complementary *in vitro* approaches, GST pulldowns and fluorescent binding assays. In GST pulldowns, both components of the t-SNARE (syntaxin1A and SNAP25) interacted with Gβγ individually. Additionally, the vesicular SNARE component, VAMP2, was found to interact directly with Gβγ (Fig. 1B). To demonstrate that the GST pulldown assay provides specific binding, we performed pulldown assay with Gα subunit and heterotrimeric G protein that contains Gαβγ . The Gβγ subunit, but not the Gα subunit or the heterotrimeric G proteins, interacts with SNARE proteins, suggesting that G protein interaction with SNARE occurs upon inhibitory G protein-coupled receptor activation that induces Gα and Gβγ dissociation (supplementary figure S1).

To quantify the relative contributions to Gβγ binding by the individual SNARE components, we employed a sensitive and quantitative fluorescence assay for measuring protein-protein interactions (Phillips and Cerione, 1991). $G\beta_1\gamma_1$ was labeled with the environmentally-sensitive fluorescent probe MIANS. This probe serves to report interaction with a binding partner because it undergoes enhanced fluorescence when the local environment becomes more hydrophobic. In these assays, labeled Gβγ (final concentration 20 nM) was used, while interactions with different concentrations of individual SNARE proteins or complexes were assessed through fluorescence intensity changes. Therefore, apparent affinities were estimated by determining concentrations of binding proteins at which MIANS fluorescence reached 50% of maximum (EC_{50}).

This assay was used to quantify the interaction of Gβγ with the ternary SNARE complex and its constituent proteins (Fig. 1C, D). The ternary SNARE complex indeed caused a dose-

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dependent increase in the fluorescence of MIANS-Gβγ, consistent with our previous findings that Gβγ and the SNARE complex interact *in vitro* (Blackmer et al., 2005; Blackmer et al., 2001) (Fig. 1C, EC_{50} = 0.27 μM). Similarly, we tested the apparent Gβγ-binding affinities of the individual SNARE components (Fig. 1D). Each of the three components caused a fluorescence increase, confirming direct interactions. The relative affinities followed the order syntaxin1A (EC_{50} = 0.33 μM), VAMP2 (0.94 μM) and SNAP25 (1.07 μM).

Gβγ interacts with the syntaxin1A H3 motif and the C-terminus of SNAP25.

Syntaxin1A has two major regions, the H3 SNARE domain and the modulatory Habc region (Fig. 2A). Truncation mutants representing the two major syntaxin1A regions were generated (Fig. 2A) and EC_{50} values for binding to 20 nM MIANS-labeled Gβγ were determined. The syntaxin1A H3 domain showed a right-shifted binding curve (EC_{50} = 0.89 μM; Fig. 2B) relative to the entire molecule (EC_{50} = 0.33 μM). Syntaxin1A (1-190) did not induce a significant Gβγ fluorescence change. These results, which were confirmed by GST pulldown assays (data not shown), indicate that Gβγ binds to the syntaxin1A H3 SNARE domain. However, since Gβγ bound to the H3 domain with an affinity lower than for intact syntaxin1A, we cannot rule out a masked minor contribution of the Habc domain, or that the isolated domain does not fold optimally (Jarvis et al., 2002).

Recent evidence suggests that nine amino acids from the C-terminus of SNAP25 are crucial for interaction with Gβγ as well (Blackmer et al., 2005; Gerachshenko et al., 2005). BoNT/A treatment, which removes nine C-terminal residues of SNAP25 (SNAP25Δ9) (Fig. 2C), diminishes Gβγ-mediated inhibition of exocytosis (Blackmer et al., 2005; Gerachshenko et al., 2005). Additionally, a ternary SNARE complex comprising VAMP2, syntaxin1A and

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SNAP25Δ9, shows reduced Gβγ binding compared to the wild type SNARE complex (Gerachshenko et al., 2005). Here, we quantified the direct interaction of Gβγ with the C-terminus of SNAP25. A truncation mutant of SNAP25 that mimics the BoNT/A cleavage product (SNAP25Δ9) was analyzed for its binding to Gβγ. Relative to full-length SNAP25, SNAP25Δ9 showed significantly decreased affinity for Gβγ (Fig. 2D).

In presynaptic terminals of neurons *in situ* and in cultured PC12 and chromaffin cells, BoNT/E, which cleaves 26 amino acids from the C-terminus of SNAP25 (Fig. 2C), inhibits Ca^{2+}-dependent exocytosis (Chen et al., 2001; Ferrer-Montiel et al., 1998), consistent with an observed loss of interaction between synaptotagmin and SNAP25 (Chen et al., 1999; Gerona et al., 2000). We investigated whether a further truncated SNAP25 (SNAP25Δ26) also weakens binding to Gβγ. Fluorescent binding assays demonstrated that deletion of 26 amino acids caused a great loss of Gβγ binding (Fig. 2D). These data indicate that 26 C-terminal amino acids of SNAP25 are also critical for Gβγ binding.

Gβγ and synaptotagmin compete in binary- and ternary SNARE complexes in a Ca^{2+}-dependent manner.

Interestingly, the Gβγ-binding domains of the SNARE complex, the syntaxin1A H3 domain and the C-terminus of SNAP25, are also known to constitute functionally important Ca^{2+}-dependent synaptotagmin binding sites (Chapman et al., 1995; Gerona et al., 2000). The overlapping of binding sites suggests that there might be competition between Gβγ and synaptotagmin on the complexed SNAREs. To test whether Gβγ competes with synaptotagmin for binding to SNARE proteins in complex, we conducted competition assays *in vitro*. We used a mutant of synaptotagmin I (K326A, K327A) that exhibits wild-type SNARE binding affinity but

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holds a reduced propensity to oligomerize and to form insoluble aggregates in response to high concentrations of Ca^{2+} (Bai et al., 2004). In fluorescent binding assays, synaptotagmin does not interact significantly with MIANS-Gβγ (data not shown). We therefore tested competition between Gβγ and synaptotagmin for binding to the binary t-SNARE complex, comprising syntaxin1A and SNAP25. The interaction between Gβγ and binary t-SNARE complex in the absence of synaptotagmin was normalized to 100%. In 50 μM Ca^{2+}, the interaction between MIANS-Gβγ and 1 μM binary t-SNARE complex was almost completely inhibited by 250 nM synaptotagmin I (Fig. 3A). Increasing synaptotagmin concentrations competed more effectively with Gβγ, resulting in reduced fluorescence changes (Fig. 3B), further indicating that Gβγ and synaptotagmin compete for SNARE binding.

Synchronous vesicle fusion is initiated by Ca^{2+}, but additional Ca^{2+} influx may modify large dense core fusion properties and possibly alter the quantal size of synaptic neurotransmitter release (Elhamdani et al., 2006; Elhamdani et al., 2001; Harata et al., 2006). Since this modulation may reflect the Ca^{2+} sensitivity of synaptotagmin (Morimoto et al., 1995), we tested whether competition with Gβγ is also Ca^{2+} sensitive. We found that the competition between synaptotagmin and Gβγ for binary t-SNARE complex binding is dependent on intracellular Ca^{2+} concentrations. In buffer lacking Ca^{2+}, synaptotagmin caused a slight but statistically significant inhibition of the ability of t-SNAREs to increase the fluorescence of MIANS-Gβγ (Fig. 3C), possibly due to a limited Ca^{2+}-independent interaction between synaptotagmin and t-SNAREs (Bennett et al., 1992; Sollner et al., 1993). However, upon addition of physiologically relevant concentrations of Ca^{2+}, we observed an increased inhibition of Gβγ binding by synaptotagmin, with maximum effectiveness at 50 μM Ca^{2+} (Fig. 3C).

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Interactions of both Gβγ and synaptotagmin with the ternary SNARE complex modify the dilation of the vesicle fusion pore (Bai et al., 2004; Photowala et al., 2006). Thus, we tested the hypothesis that Gβγ competes with synaptotagmin for binding to the ternary SNARE complex. We first measured the Gβγ/SNARE interaction by determining the change in fluorescence of 20 nM MIANS-Gβγ upon addition of the ternary SNARE complex. Addition of 1 μM SNARE complex induced an increase of fluorescence intensity (Fig. 3D). This increase was reduced by inclusion of 250 nM synaptotagmin, indicating that Gβγ and synaptotagmin compete for binding to the ternary SNARE complex. Additionally, competition by synaptotagmin and the resulting reduced fluorescence changes were dependent on the concentration of synaptotagmin (Fig. 3E) and the concentration of Ca^{2+} (Fig. 3F). Together, these data suggest that indeed Gβγ and synaptotagmin compete for binding to the ternary SNARE complex.

Gβγ and synaptotagmin compete for binding to the individual t-SNARES (syntaxin 1A and SNAP25) but not the v-SNARE (VAMP2).

The H3 domain of syntaxin1A and the C-terminus of SNAP25 provide interaction regions for Gβγ and synaptotagmin. Interestingly, we saw significantly more efficient competition between Gβγ and synaptotagmin in the context of binary t-SNAREs than ternary SNARE complexes. Therefore, we measured the individual contributions of SNARE components to competition between Gβγ and synaptotagmin.

First, to determine if Gβγ and synaptotagmin compete for binding to syntaxin1A or SNAP25, the interaction of 20 nM of MIANS-Gβγ with syntaxin1A and SNAP25 was monitored with or without preincubation of the SNARE proteins with 20 nM synaptotagmin. The interactions between Gβγ and SNARE proteins were unaffected by Ca^{2+} in the absence of synaptotagmin

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(data not shown). In the absence of Ca^{2+}, we observed a slight, statistically insignificant decrease in Gβγ binding to both syntaxin1A (Fig. 4A) and SNAP25 (Fig. 4B) upon preincubation of the SNARE proteins with 20 nM synaptotagmin. However, in 100 μM Ca^{2+}, the ability of synaptotagmin to compete for binding to SNAREs was greatly enhanced. Syntaxin1A binding to Gβγ was reduced by 48% (Fig. 4A), and binding of SNAP25 to Gβγ was reduced by 78% (Fig. 4B). Thus, Gβγ competes with Ca^{2+}-synaptotagmin for binding to both syntaxin1A and SNAP25.

We observed a direct interaction between Gβγ and VAMP2 (Fig. 1B and D). An earlier study suggested that synaptotagmin interacts with VAMP2 (Fukuda, 2002), leading to the possibility that this protein forms an additional site of competition between Gβγ and synaptotagmin. However, the existence of a direct synaptotagmin/VAMP2 interaction remains controversial (Chapman et al., 1995). Consequently, we monitored the Gβγ interaction with VAMP2 with or without preincubation of VAMP2 with synaptotagmin in the presence or absence of Ca^{2+}. No alteration in Gβγ binding to VAMP2 by synaptotagmin was observed, suggesting that Gβγ and synaptotagmin do not share a binding region on VAMP2 (Fig. 4C). This result may explain why competition at the ternary SNARE complex appears to favor Gβγ binding as compared to competition at the binary t-SNARE: in the ternary SNARE complex, Gβγ binding is enhanced by an additional binding site within VAMP2 that is unaffected by synaptotagmin I. Thus its ability to compete with Gβγ in the ternary SNARE complex is decreased.

The C-terminus of SNAP25 in the ternary SNARE complex is important for Gβγ - synaptotagmin competition.

Previously, we showed that botulinum toxin A (BoNT/A), which specifically removes the C-terminal nine amino acids from SNAP25, greatly reduced the inhibitory effect of Gβγ on

regulated exocytosis (Blackmer et al., 2001; Gerachshenko et al., 2005). We propose that this reduction can be explained if removal of these residues diminishes Gβγ competition with synaptotagmin for binding to the t-SNAREs and the ternary SNARE complex *in vivo*. Therefore, we examined the contribution of the C-terminus of SNAP25 in the competition between Gβγ and synaptotagmin within the ternary SNARE complex. Importantly, while BoNT/A cleavage of SNAP25 reduced binding of synaptotagmin and Gβγ individually, significant interactions were still observed (Fig. 2D). Thus, it was important to quantify the interactions using the fluorescent binding assay to determine if this cleavage shifted the Gβγ/synaptotagmin competition towards synaptotagmin binding.

To test the relative contributions of the C-terminus of SNAP25 in binding Gβγ and synaptotagmin, we quantified binding to ternary SNARE complexes comprising syntaxin1A, VAMP2 and SNAP25Δ9. Competition by synaptotagmin for Gβγ binding to SNARE complex that contains SNAP25Δ9 was increased compared to the wild type SNARE complex at 50 μM of Ca^{2+} (Fig. 5A). This finding is consistent with a model in which the nine C-terminal amino acids of SNAP25 represent an important determinant mediating exclusion of synaptotagmin binding by Gβγ.

We then determined the concentration dependence of the synaptotagmin competition. The strength of synaptotagmin competition in SNARE complexes formed with SNAP25Δ9 increased dramatically compared to the wild type SNARE complex (Fig. 5B). These results suggest that the C-terminal nine amino acids of SNAP25 are more critical for Gβγ than for synaptotagmin binding. These data support the dramatic loss of inhibitory effect of Gβγ in the BoNT/A treated synapse (Gerachshenko et al., 2005).

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The 5-HT mediated regulation of exocytosis is diminished with high [Ca^{2+}] at the lamprey giant synapse.

What role does Gβγ-synaptotagmin I competition for SNARE complexes play at synapses? At lamprey giant synapses, we have showed that 5-HT inhibits transmitter release through direct Gβγ/SNARE complex interactions. 5-HT mediated Gβγ does not inhibit voltage gated Ca^{2+} channels in this preparation, but directly acts on the ternary SNARE complex to inhibit exocytosis (Blackmer et al., 2001; Gerachshenko et al., 2005; Takahashi et al., 2001). From our *in vitro* study we hypothesize that Gβγ-mediated inhibition requires competition between Gβγ and Ca^{2+}-dependent synaptotagmin binding on the SNARE complex. These findings suggest that the effect of GPCR-mediated activation of Gβγ will be mitigated by raising the synaptic terminal Ca^{2+} concentration.

We tested this possibility using paired recordings between giant axons and postsynaptic neurons in lamprey synapse. To increase action potential-evoked intracellular Ca^{2+} concentrations, we raised extracellular Ca^{2+} concentrations (Fig. 6A-C). We first quantified the consequent effect on evoked presynaptic Ca^{2+} entry. Axons were filled with a Ca^{2+} sensitive dye (Fluo-4 dextran high affinity) and imaged with confocal microscopy. Action potential-evoked presynaptic fluorescence transients were imaged under increasing extracellular Ca^{2+} (2.6 mM, 10 mM and 50 mM; Fig. 6B-C and data not shown). The relationship between extracellular Ca^{2+} concentrations and the fluorescence transient was alinear (Fig. 6E). Therefore, to calculate the relationship between extracellular Ca^{2+} and the intracellular Ca^{2+} transient amplitude we determined the peak intracellular Ca^{2+} during the transients. The affinity of the dye (1.2 μM) was obtained from fluorescence curves that showed a 120-fold increase in fluorescence between 0 μM Ca^{2+} and saturation (41 μM) at a physiological temperature. Using these data and the resting

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[Ca^{2+}] in lamprey axons (approximately 100 nM), we estimated the peak transient intracellular Ca^{2+} (Fig. 6E). 50 mM extracellular Ca^{2+} slightly increased basal presynaptic intracellular Ca^{2+} (Fig. 6D). However, the evoked fluorescence transient, and therefore its peak Ca^{2+} concentration, was markedly increased (Fig. 6E). Note that absolute Ca^{2+} concentrations calculated in this way underestimate the concentration at the vesicle fusion site because we cannot image with sufficient resolution to resolve the close spatial relationship between Ca^{2+} channels and the release machinery (Adler et al., 1991). Consequently, we used these data to calculate the relative amplitude of the Ca^{2+} transient at extracellular concentrations of 10 and 50 mM (Fig. 6F) normalized to control (2.6 mM).

We then tested the effect of increased Ca^{2+} on 5-HT-mediated presynaptic inhibition. At a control extracellular Ca^{2+} concentration (2.6 mM), 5-HT (600 nM) reduced the EPSC to 55.9±7.3% of control (n=5; $P<0.05$). The EPSC fully recovered upon 5-HT removal (Fig. 7A, top). 50 mM extracellular Ca^{2+} increased the EPSC amplitude to 129.7±4.7% of control (n=5; $P<0.01$), but 600 nM 5-HT caused no inhibition (Fig. 7A, bottom; EPSC was 97.1±8.1% of the EPSC modified by 50 mM Ca^{2+}; n=5 pairs). At 50 mM extracellular Ca^{2+}, even a saturating dose of 5-HT (10 μM) resulted in only a slight reduction in the EPSC {response was 71.3±0.9% of the control response modified by high Ca^{2+} (n=3, $P>0.05$), compared to a reduction to 20% of control in normal Ca^{2+} (Takahashi et al., 2001)} (Fig. 7B and C). We also confirmed that 5-HT does not change Ca^{2+} influx in lamprey synapses (Blackmer et al., 2001; Takahashi et al., 2001) treated with 50 mM extracellular Ca^{2+} concentration (supplementary figure S2). Thus, increased intracellular Ca^{2+} transients attenuated GPCR mediated presynaptic inhibition.

High extracellular Ca^{2+} attenuates the presynaptic inhibitory effect of Gβγ.

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We have shown that the reduction of the EPSC upon activation of 5-HT receptors is caused by liberated Gβγ (Blackmer et al., 2001; Gerachshenko et al., 2005). Here, we examined the effects of manipulating intracellular Ca^{2+} on EPSC inhibition by free Gβγ introduced through direct injection of purified Gβγ protein into presynaptic axons. We compared Gβγ-mediated inhibition of exocytosis at a control (2.6 mM) and a raised Ca^{2+} concentration (10 mM). 10 mM Ca^{2+} increased the EPSC amplitude (Fig. 8A; to 122±2% of control, n=3). In four more paired recordings we probed the effect of raising Ca^{2+} concentrations on inhibition by presynaptic Gβγ. In control Ca^{2+} (2.6 mM), Gβγ injection significantly reduced the EPSC as previously described (Blackmer et al., 2001) (to 37±12%; Fig. 8B and C). In the same pairs the Ca^{2+} concentration was raised to 10 mM and Gβγ inhibition was reversed (10 mM; Fig. 8B, C; EPSC was 100±9% of control). Thus raised Ca^{2+} transient amplitudes significantly attenuated the inhibitory effect of presynaptic Gβγ. The effects of both 5-HT and presynaptic Gβγ injection are attenuated by enhancing the amplitude of the action potential evoked presynaptic Ca^{2+} transient.

Discussion

In primed vesicles, Ca^{2+} effects exocytosis in part by increasing synaptotagmin's affinity for the primed SNARE complex (Bai et al., 2004; Perin et al., 1990; Schiavo et al., 1997; Wang et al., 2006; Yoshihara and Littleton, 2002). Gβγ liberated by inhibitory Gi/o-coupled GPCRs inhibits neurotransmitter release by at least two mechanisms: inhibition of presynaptic Ca^{2+} entry at many synapses by modulating voltage-gated calcium channels (Herlitze et al., 1996; Ikeda, 1996), as well as interaction with the ternary SNARE complex late in membrane fusion (Blackmer et al., 2005; Gerachshenko et al., 2005). Here we show that Gβγ interacts with SNARE proteins and that this binding is not directly modified by calcium. Gβγ interacts with

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VAMP2 at a site different from synaptotagmin's interaction. Perhaps the Gβγ/VAMP2 interaction strengthens Gβγ binding to the SNARE complex while occlusion of synaptotagmin I binding occurs through the t-SNARE components (syntaxin1A and SNAP25) in the ternary SNARE complex. In addition, Gβγ binds directly to the t-SNAREs SNAP25 and syntaxin1A at similar sites to those synaptotagmin uses to interact with these proteins in a Ca^{2+}-dependent manner. This sets up a potent competition with the Ca^{2+} sensor to inhibit regulated exocytosis. This differential Ca^{2+} requirement can lead to a Ca^{2+}-sensitivity of the Gβγ inhibition of SNARE/synaptotagmin complex formation: at low and moderate Ca^{2+}concentrations, the presence of inhibitory signals generates Gβγ that competes with synaptotagmin for binding to SNARE, leading to inhibition of exocytosis. At high Ca^{2+}concentrations, the affinity of synaptotagmin for SNARE increases while the Gβγ/SNARE affinity remains steady, and thus high synaptic activity overrides the inhibition by Gβγ.

At the synapse, after C-terminal truncation of SNAP-25 with BoNT/A, exocytosis recovers in high Ca^{2+} in many preparations, including the lamprey (Gerachshenko et al., 2005), presumably because high Ca^{2+} enhances the residual synaptotagmin/SNARE interaction that remains after BoNT/A treatment. However, the affinity of Gβγ for the modified SNARE complex is much lower, and Gβγ therefore competes less well with synaptotagmin for the same site, particularly at raised intracellular Ca^{2+}. These findings led to a testable hypothesis: at the synapse, larger evoked Ca^{2+} transients should prevent Gβγ-mediated presynaptic inhibition induced by 5-HT. Accordingly, in the lamprey giant synapse, while 5-HT powerfully inhibits exocytosis through Gβγ interaction with the SNARE complex (Blackmer et al., 2005; Blackmer et al., 2001; Chen et al., 2005; Gerachshenko et al., 2005; Man-Son-Hing et al., 1989; Silinsky, 1984; Takahashi et al., 2001), we observed that enhancing the Ca^{2+} transient reversed this inhibition (Fig. 6-7). These

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data are consistent with the biochemical results showing that inhibition of synaptotagmin/SNARE complex interaction by Gβγ is sensitive to Ca^{2+}. Though Ca^{2+} could have other effects in the synapse, the fact that synaptotagmin is thought to be the major Ca^{2+} receptor for exocytosis (Bai et al., 2004; Maximov and Sudhof, 2005; Wang et al., 2006) suggests that Gβγ may work physiologically by competing with Ca^{2+}-synaptotagmin for SNARE, leading to inhibition of exocytosis. Gβγ also has many other targets, and enhancing intracellular Ca^{2+} could modify the effects of these interactions on exocytosis. The most obvious other Gβγ effector is the voltage-gated Ca^{2+} channel, which has been shown by many investigators to be inhibited by Gβγ. However, Gβγ has no effect on Ca^{2+} entry even at 50 mM extracellular Ca^{2+}, as 5-HT does not modify evoked Ca^{2+} entry (supplementary figure S2).

This Ca^{2+}-dependent regulation may complete a physiological feedback loop, since Gβγ may control both Ca^{2+} influx through voltage-gated Ca^{2+} channels (Herlitze et al., 1996; Ikeda, 1996) and access of the Ca^{2+} effector synaptotagmin to its site on the SNARE. Our data suggest that this competition is elegantly controlled by overlap of the binding sites of Gβγ and synaptotagmin at the SNARE proteins SNAP25 and syntaxin. In a further level of regulation, during repetitive stimulation, buildup of intracellular Ca^{2+} concentration may reverse Gβγ inhibition. Thus, depending on presynaptic Gi/o-coupled GPCR activation and Ca^{2+} influx in response to synaptic activity, there potentially exists a range of efficiencies of exocytosis. Thus we predict that a complex and dynamic regulation of vesicle fusion properties will occur. Finally, at low levels of inhibitory GPCR signaling, Gβγ causes kiss-and-run exocytosis in lamprey synapses (Photowala et al., 2006) and adrenal chromaffin cells (data not shown) (Chen et al., 2005). In hippocampal neurons, sustained stimulus trains forcing high presynaptic Ca^{2+} concentrations change fusion from a state dominated by kiss-and-run to one favoring full fusion (Harata et al., 2006).

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However, the exact Ca^{2+} concentrations required to reverse Gβγ-mediated presynaptic inhibition are likely to exceed those obtained by residual Ca^{2+} during short bursts of stimuli, in which saturating doses of 5-HT remains effective (Takahashi et al., 2001). Thus we speculate that the dynamic relationship between inhibitory GPCRs and presynaptic Ca^{2+} constantly modifies the nature of the vesicle fusion event itself.

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Footnotes

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Figure legends

Fig. 1 Gβγ binds individual SNARE proteins and their ternary complex. *(A) The SNAREs in the primed state and their relationship to synaptotagmin.* We hypothesize that Gβγ counteracts synaptotagmin by interacting with the C-terminal region of the SNARE complex. *(B) Gβγ interacts with SNAP25 and syntaxin1A in a GST pulldown assay (upper panel).* Equimolar amounts of GST (lane 1), GST-SNAP25 (lanes 2-4), or GST-syntaxin1A (lanes 5-7) were incubated with the noted concentrations of $G\beta_1\gamma_2$. Precipitated Gβ was detected with an anti-pan Gβ antibody. Purified Gβγ was included as a loading control (lane 8). *Gβγ interacts directly with VAMP2 (bottom panel),* {GST (lane 1), GST-VAMP2 (lanes 2-5 and lane a Gβγ loading control (lane 6)}. *(C) Quantification of the interaction of Gβγ with the ternary SNARE complex with fluorescent binding.* Purified Gβγ was labeled with MIANS environmental sensitive fluorescence probe. The fluorescence change was monitored by spectrofluorometry using 20 nM labelded Gβγ and various concentrations of SNARE complex. Fluorescence change was normalized by basal level of labeled Gβγ fluorescence and represented as relative fluorescence. Increasing concentrations of the SNARE complex induced enhanced fluorescence of MIANS-$G\beta_1\gamma_1$, indicating formation of a Gβγ/SNARE interaction. The data presented ratio of relative fluorescence (RF) at each different protein concentration (n = 3). *(D) Affinities of individual SNARE components for Gβγ.* Using the fluorescent binding assay, strengths of binding to 20 nM MIANS-Gβγ were found to follow the order syntaxin1A (EC_{50} = 0.33 μM), VAMP2 (0.94 μM), and SNAP25 (1.07 μM).

Fig. 2 Gβγ interacts with the H3 domain of syntaxin1A and C-terminus of SNAP25. *(A) Domain organization of syntaxin1A.* Fluorescent binding assays were performed with three variants of syntaxin1A: 1-266, which contains the Habc and H3 domains but lacks a

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transmembrane region; 1-190, which contains the Habc domain and the Habc-H3 linker region; and 191-266, which contains the H3 domain. *(B) Quantification of the Gβγ/syntaxin1A interaction.* Syntaxin1A variants were cleaved from GST, and interaction with MIANS-Gβγ was detected by increased fluorescence output. The data represented ratio of RF at each different protein concentration. MIANS-Gβγ (20 nM) was found to interact with syntaxin1A (1-266) with an EC_{50} of 0.33 μM and with the H3 domain syntaxin1A (191-266) with an EC_{50} of 0.89 μM, while no significant interaction with syntaxin1A (1-190) was observed. *(C) Domain organization of SNAP25.* SNAP25 contains two SNARE motifs (SN1 and SN2). The C-terminus is cleaved specifically by two botulinum toxins, BoNT/E (between amino acids 180 and 181) and BoNT/A (196 and 197). *(D) Removal of the C-terminus of SNAP25 reduces affinity for Gβγ.* SNAP25 truncation mutants mimicking cleavage by BoNT/A (SNAP25Δ9) or BoNT/E (SNAP25Δ26) were tested for affinity for Gβγ using the fluorescent binding assay. MIANS-Gβγ (20 nM) was found to interact with SNAP25 with an EC_{50} of 1.07 μM and with SNAP25Δ9 (mimics BoNT/A cleavage) with an EC_{50} of 1.83 μM while no significant interaction with SNAP25Δ26 (mimics BoNT/E cleavage).

Fig. 3 Gβγ-mediated inhibition of the synaptotagmin/SNARE interaction is diminished at high Ca^{2+}-concentrations. *(A) Preincubation with synaptotagmin (SytI) abrogates binding of Gβγ to the binary t-SNARE complex, indicating competition.* Binary t-SNARE complexes were assembled with syntaxin1A and SNAP25 in a 1:1 ratio, as shown by SDS-PAGE/Coomassie staining analysis (*inset*). Binding of 1 μM binary t-SNARE to 20 nM MAINS-Gβγ with (+ synaptotagmin) or without (- synaptotagmin) 250 nM synaptotagmin was measured by fluorescence enhancement in 50 μM Ca^{2+}. *(B) Competition for binary t-SNARE binding is dose-*

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dependent. The Gβγ/t-SNARE interaction was monitored as described in *(A)*, and binding in the absence of synaptotagmin was normalized to 100%. % of RF is plotted against synaptotagmin protein concentration in 50 μM Ca^{2+}. *(C) Competition for binary t-SNARE binding is dependent on [Ca^{2+}].* Different concentration of Ca^{2+} (0-100 μM) as noted was applied and % of RF was monitored *(D) Synaptotagmin and Gβγ compete for binding to the ternary SNARE complex.* In vitro reassembled ternary SNARE complex showed an SDS- and heat-stable species (*inset, lane 1)* that dissociated by additional heat treatment (*inset, lane 2)*. Binding of the ternary SNARE complex with MIANS-Gβγ with or without 250 nM synaptotagmin was performed as described for Figure 3A. *(E) Competition for ternary SNARE binding is dependent on the concentration of synaptotagmin. (F) Competition for ternary SNARE binding is dependent on [Ca^{2+}].* Panels A and D depict typical results from at least three experiments. Data are represented as mean ± SEM (n=3).

Fig. 4 Gβγ and synaptotagmin compete for binding to SNAP25 and syntaxin1A but not to VAMP2. *(A) Gβγ competes with synaptotagmin for binding to syntaxin1A.* Competition assays were performed as described for Fig.3. The relative change in fluorescence of 20 nM MIANS-Gβγ in 100 μM Ca^{2+} upon addition of 0.5 μM syntaxin1A was normalized to 100%. Addition of 20 nM synaptotagmin in the absence of Ca^{2+} induced a slight decrease in the Gβγ/syntaxin1A interaction, but inclusion of both 20 nM synaptotagmin and 100 μM Ca^{2+} reduced binding by approximately 50%. *(B) Gβγ competes with synaptotagmin for binding to SNAP25.* 20 nM synaptotagmin caused a slight decrease in the interaction of 20 nM MIANS-Gβγ with 2 μM SNAP25 in the absence of Ca^{2+}, while inclusion of both 20 nM synaptotagmin and 100 μM Ca^{2+} reduced binding by approximately 80%. *(C) Gβγ and synaptotagmin do not compete for binding*

to VAMP2. The change in MIANS-Gβγ fluorescence upon addition of 2 μM VAMP2, normalized to 100%, was not reduced significantly in the presence of 20 nM synaptotagmin, with or without 100 μM Ca^{2+}. Data are presented as mean ± SEM (n = 3).

Fig. 5 The C-terminal nine amino acids of SNAP25 comprise a critical region for competition between Gβγ and synaptotagmin. Gβγ competition with synaptotagmin to bind ternary SNARE complex made up of SNAP25 (black bar) or SNAP25Δ9 (grey bar). Gβγ and SNARE complex binding was normalized to 100% fluorescence in the presence of 1 mM Ca^{2+} and in the absence of synaptotagmin. Competition by synaptotagmin for Gβγ binding to SNARE containing SNAP25Δ9 was increased compared to the wild type SNARE complex *(A) Competition for binding to SNARE (SNAP25Δ9) is more sensitive to [Ca^{2+}].* (B) *Synaptotagmin I competes in a dose dependent manner with Gβγ for binding to SNARE, more potently with SNAP25Δ9*. Data are represented as mean ± SEM (n=3).

Fig. 6 Raising extracellular [Ca^{2+}] increases intracellular [Ca^{2+}] transients in a presynaptic terminal. *(A) Lamprey giant* axons labeled retrogradely with a Ca^{2+}-sensitive dye (Fluo-4 dextran high affinity) and imaged with confocal microscopy. To quantify rapid action potential-induced Ca^{2+} transients, high-speed line scanning of Ca^{2+} entry (500 Hz) was repeated at the edge of the axon marked with the arrowheads. *(B - F) Raising extracellular [Ca^{2+}] enhances action-potential-induced Ca^{2+} influx.* (B) A single action potential in the axon evoked at the time point marked by the arrowhead caused a localized Ca^{2+} transient in the axon. The line scan is pseudocolored with the lookup table shown below panel (A). The color values were measured and after background substration, images were normalized to the baseline level of fluorescence

{DF/(F+1)}. (C) Increasing the extracellular Ca^{2+} concentration from 2.6 to 50 mM markedly enhanced the amplitude of the Ca^{2+} transient. (D) During wash-in of 50 mM Ca^{2+} the resting fluorescence was monitored at 30 s intervals. A small increase in the resting fluorescence was observed. Fluorescence is recorded to the same scale as (E). (E) The Ca^{2+} transients were measured between the white horizontal lines in (B) and (C) to quantify Ca^{2+} entry. Estimates of recorded Ca^{2+} transient amplitudes were made as described in methods. The calculated Ca^{2+} concentration scale for this estimate is shown to the right. (F) The relative effect of raising extracellular Ca^{2+} concentration on the amplitude of the axonal Ca^{2+} transient is recorded. Note that high extracellular Ca^{2+} concentrations have a proportionately smaller effect on the amplitudes of the recorded intracellular transient.

Fig. 7 Raising extracelluar [Ca^{2+}] decreases 5-HT-mediated inhibition of exocytosis in a presynaptic terminal. *(A) Increasing extracellular [Ca^{2+}] and, therefore the intracelluar [Ca^{2+}] transients abrogates 5-HT-mediated inhibition of exocytosis.* In a paired recording of presynaptic action potentials (upward peaks) and EPSCs (*downward current peaks*), a moderate dose of 5-HT (0.6 μM) applied in normal extracellular [Ca^{2+}] (2.6 mM) caused inhibition of exocytosis (blue). The EPSC recovered following 5-HT washout (top). The effect of 0.6 μM 5-HT is completely abrogated in 50 mM extracellular [Ca^{2+}] (red, bottom). (*B*) Pooled data shows the 5-HT-mediated inhibition in 2.6 mM and 50 mM Ca^{2+} (n=4-5, **, $P < 0.01$; *, $P < 0.05$). In control extracellular Ca^{2+} concentration, 0.6 μM and 10 μM of 5-HT significantly reduced the amplitude (blue). In 50 mM extracellular Ca^{2+}, 0.6 μM 5-HT no longer inhibited synaptic release and a saturating dose of 10 μM of 5-HT slightly decreased the amplitude but was not statistically

significant (red). (C) A comparison of the ability of moderate (0.6 μM) or high (10 μM) doses of 5-HT to inhibit exocytosis reveals that the level of exocytotic inhibition is highly dependent on the levels of both G protein activation and intracellular Ca^{2+}. The relative level of intracellular Ca^{2+} is indicated on the x-axis, while the degree of inhibition of exocytosis was measured as the EPSC response relative to control (no 5-HT) (y-axis).

Fig. 8 High extracellular Ca^{2+} blocks the presynaptic inhibitory effect of Gβγ. *(A) Increased extracellular [Ca^{2+}] causes a slight increase in exocytosis.* A paired cell recording (top, presynaptic action potentials; bottom, resultant EPSCs) demonstrates the effect of raising extracellular Ca^{2+} from 2.6 mM (black trace) to 10 mM (grey). *(B) The effect of presynaptic Gβγ injection is reversed by raising the extracellular Ca^{2+} concentration.* The responses observed in *(A)* were inhibited by injection of Gβγ (blue)**.** Subsequently raising the extracellular Ca^{2+} concentration reversed this inhibition (red). *(C) Pooled data showing the effect of raising extracellular [Ca^{2+}] on the control synaptic responses and on the response in 10 mM Ca^{2+}*. 10 mM extracellular Ca^{2+} enhanced the control response to 122 ± 2% of control (n=3) (grey bar). In a further 4 paired cell recordings presynaptic injection of Gβγ inhibited control responses to 37 ± 12% of control (blue). Raising the extracellular Ca^{2+} concentration to 10 mM caused a recovery to 100 ± 9% of control (red).

Fig. 1

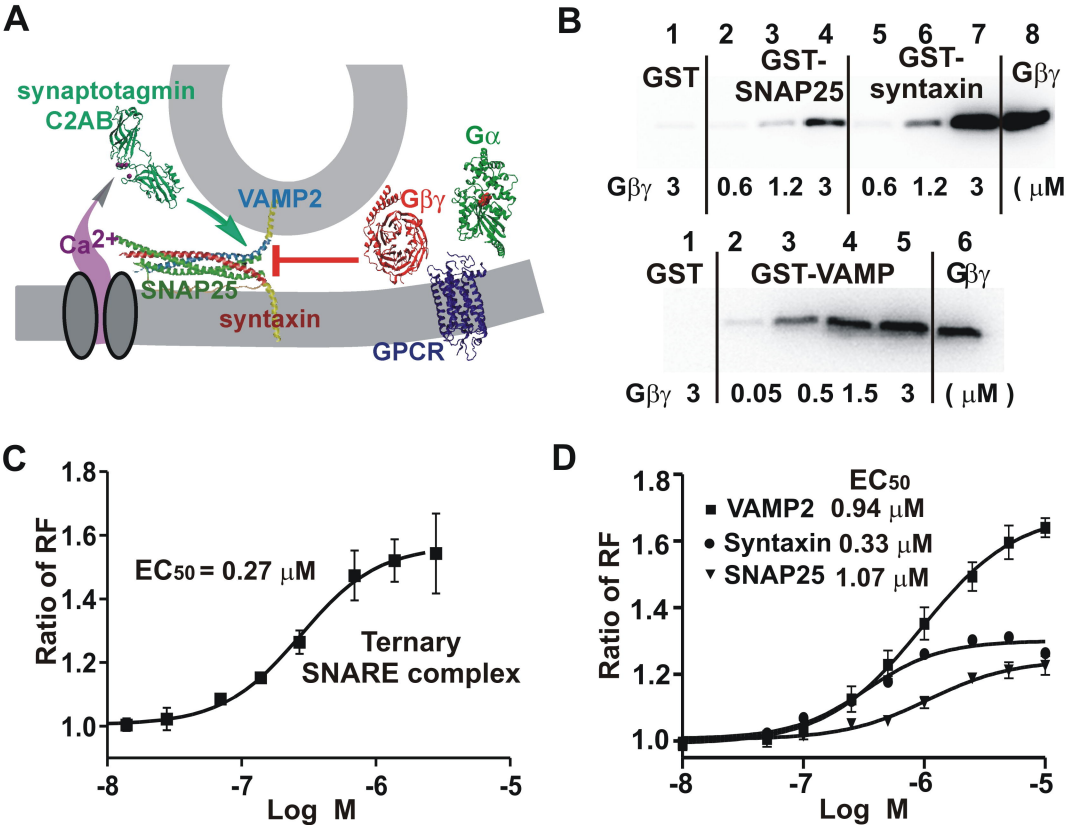

A
synaptotagmin
C2AB
VAMP2
Gβγ
Gα
Ca2+
SNAP25
syntaxin
GPCR
B
1 2 3 4 5 6 7 8
GST
GST-
SNAP25
GST-
syntaxin
Gβγ
Gβγ 3 0.6 1.2 3 0.6 1.2 3 (μM)
1 2 3 4 5 6
GST
GST-VAMP
Gβγ
Gβγ 3 0.05 0.5 1.5 3 (μM)
C
Ratio of RF
EC50 = 0.27 μM
Ternary
SNARE complex
Log M
D
Ratio of RF
EC50
VAMP2 0.94 μM
Syntaxin 0.33 μM
SNAP25 1.07 μM
Log M

Fig. 2

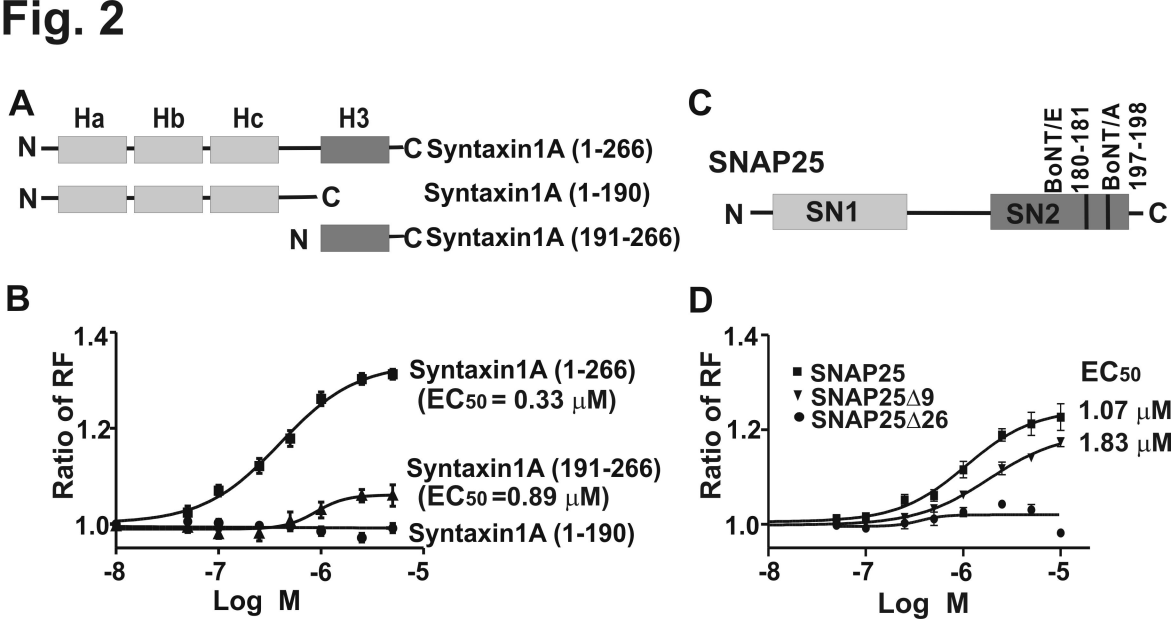

A
Ha
Hb
Hc
H3
N
C
Syntaxin1A (1-266)
Syntaxin1A (1-190)
Syntaxin1A (191-266)
C
SNAP25
BoNT/E 180-181
BoNT/A 197-198
SN1
SN2
B
Ratio of RF
1.4
1.2
1.0
Syntaxin1A (1-266)
(EC50 = 0.33 μM)
Syntaxin1A (191-266)
(EC50 =0.89 μM)
Syntaxin1A (1-190)
-8
-7
-6
-5
Log M
D
SNAP25
SNAP25Δ9
SNAP25Δ26
EC50
1.07 μM
1.83 μM

Fig. 3

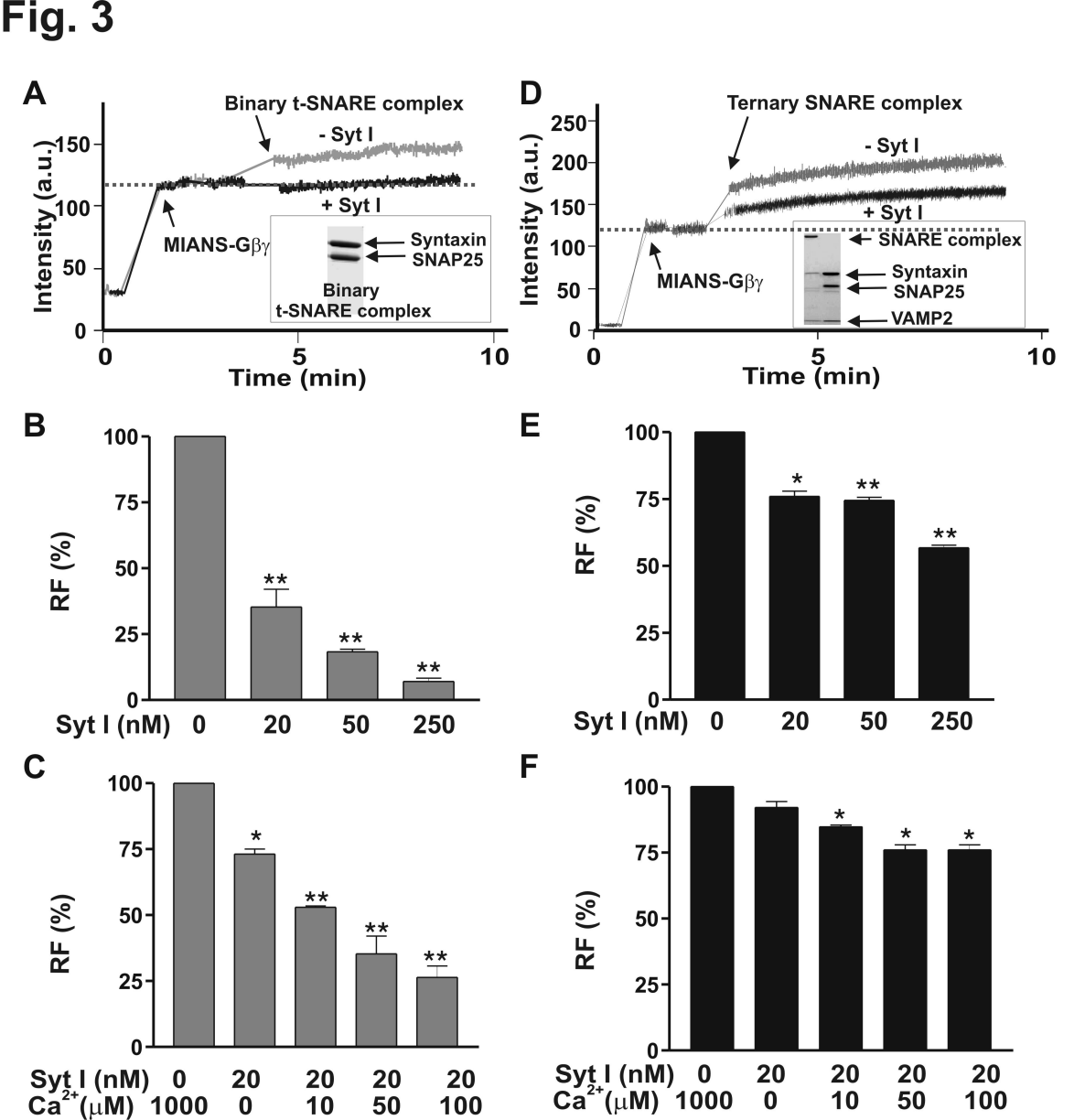

A
Binary t-SNARE complex
- Syt I
+ Syt I
MIANS-Gβγ
Syntaxin
SNAP25
Binary
t-SNARE complex
Intensity (a.u.)
Time (min)
D
Ternary SNARE complex
- Syt I
+ Syt I
MIANS-Gβγ
SNARE complex
Syntaxin
SNAP25
VAMP2
Intensity (a.u.)
Time (min)
B
RF (%)
Syt I (nM) 0 20 50 250
E
RF (%)
Syt I (nM) 0 20 50 250
C
RF (%)
Syt I (nM) 0 20 20 20 20
Ca2+ (μM) 1000 0 10 50 100
F
RF (%)
Syt I (nM) 0 20 20 20 20
Ca2+ (μM) 1000 0 10 50 100

Fig. 4

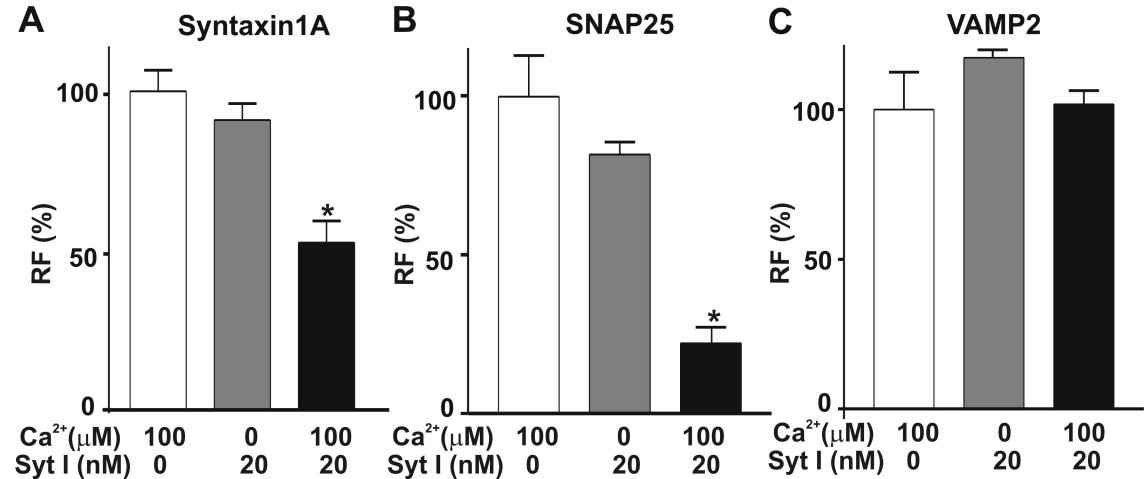

A
Syntaxin1A
B
SNAP25
C
VAMP2
RF (%)
100
50
0
*
Ca2+(μM) 100 0 100
Syt I (nM) 0 20 20

Fig. 5

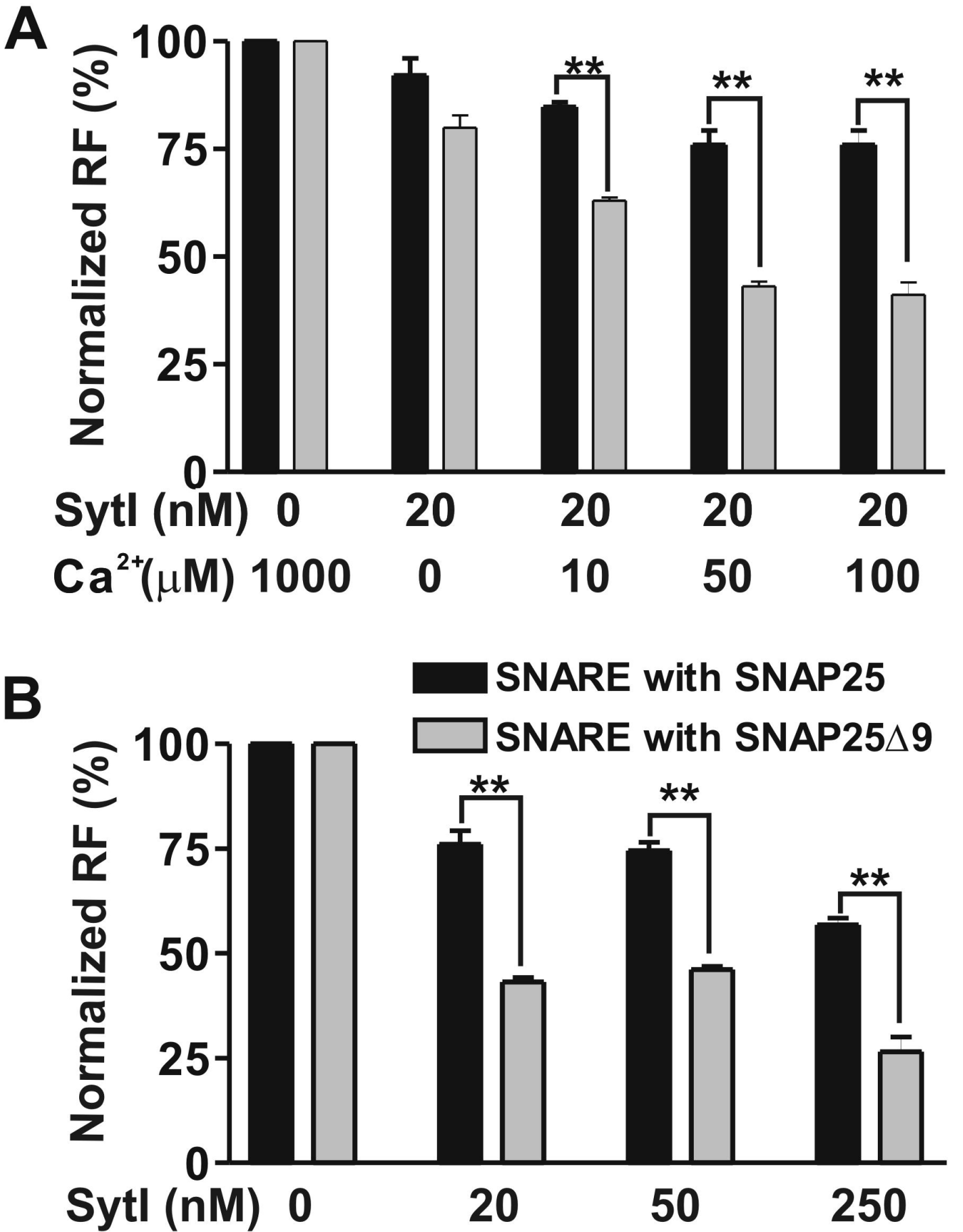

A
Normalized RF (%)
100
75
50
25
0
**
**
**
Sytl (nM) 0 20 20 20 20
Ca2+(μM) 1000 0 10 50 100
B
SNARE with SNAP25
SNARE with SNAP25Δ9
Normalized RF (%)
100
75
50
25
0
**
**
**
Sytl (nM) 0 20 50 250

Fig. 6

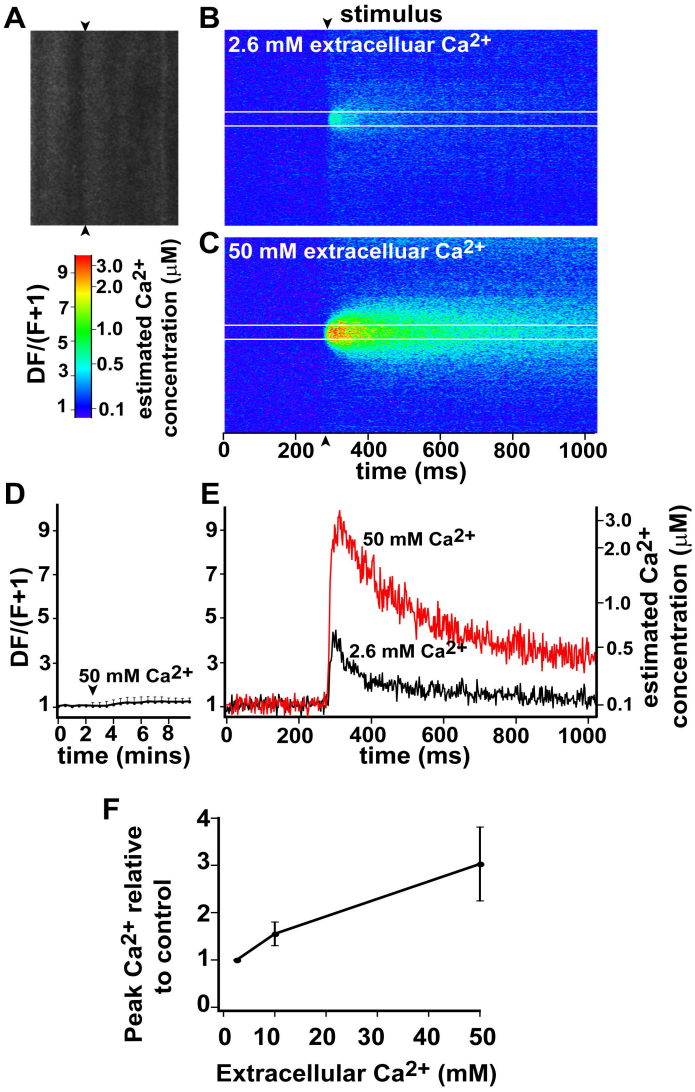

A
B
stimulus
2.6 mM extracelluar Ca2+
C
50 mM extracelluar Ca2+
DF/(F+1)
estimated Ca2+ concentration (μM)
time (ms)
D
50 mM Ca2+
time (mins)
E
50 mM Ca2+
2.6 mM Ca2+
F
Peak Ca2+ relative to control
Extracellular Ca2+ (mM)

Fig. 7

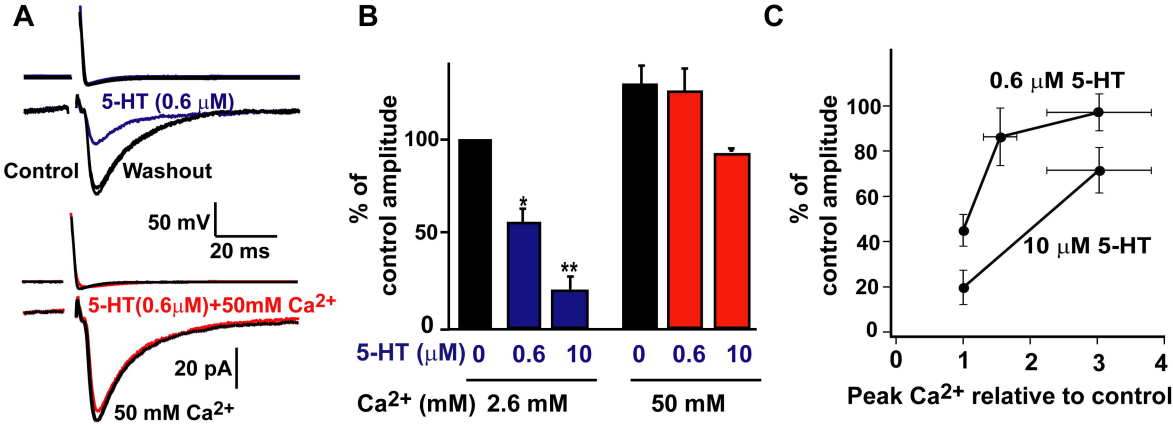
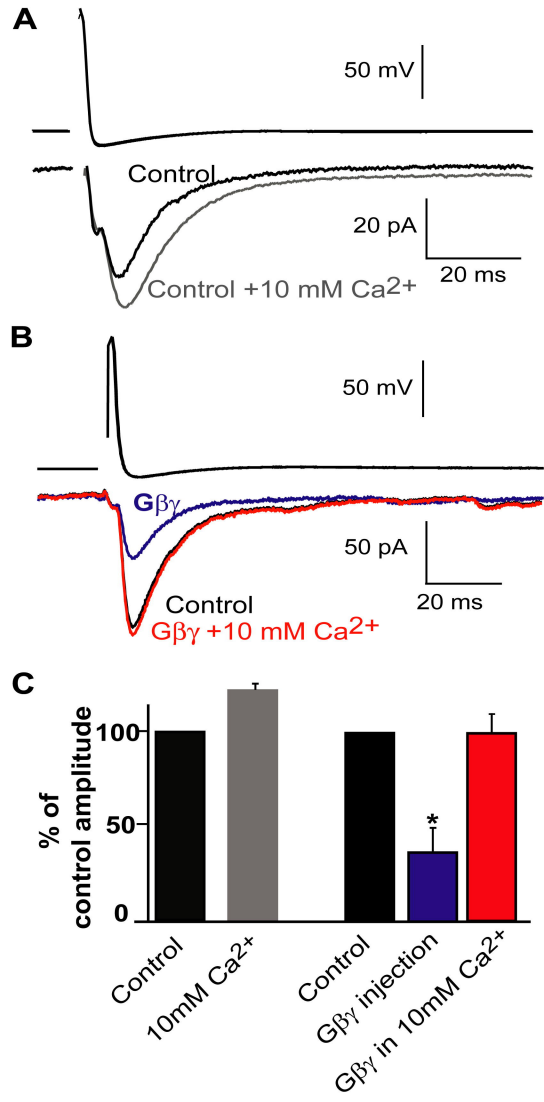

A
5-HT (0.6 μM)
Control
Washout
50 mV
20 ms
5-HT(0.6μM)+50mM Ca2+
20 pA
50 mM Ca2+
B
% of control amplitude
100
50
0
*
**
5-HT (μM) 0 0.6 10 0 0.6 10
Ca2+ (mM) 2.6 mM
50 mM
C
0.6 μM 5-HT
10 μM 5-HT
% of control amplitude
100
80
60
40
20
0
0 1 2 3 4
Peak Ca2+ relative to control

Fig. 8


A
50 mV
Control
20 pA
20 ms
Control +10 mM Ca2+
B
50 mV
Gβγ
50 pA
20 ms
Control
Gβγ +10 mM Ca2+
C
% of control amplitude
100
50
0
*
Control
10mM Ca2+
Control
Gβγ injection
Gβγ in 10mM Ca2+