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RAPID REPORT

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# Syntaxin Requirement for $Ca^{2+}$-Triggered Exocytosis in Neurons and Endocrine Cells Demonstrated with an Engineered Neurotoxin

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Supporting Information

**ABSTRACT:** Botulinum neurotoxins cleave synaptic SNAREs and block exocytosis, demonstrating that these proteins function in neurosecretion. However, the function of the SNARE syntaxin remains less clear because no neurotoxin cleaves it selectively. Starting with a botulinum neurotoxin that cleaves both syntaxin and SNAP-25, we engineered a version that retains activity against syntaxin but spares SNAP-25. These mutants block synaptic release in neurons and norepinephrine release in neuroendocrine cells, thus establishing an essential role for syntaxin in $Ca^{2+}$-triggered exocytosis. These mutants can generate syntaxin-free cells as a useful experimental system for research and may lead to pharmaceuticals that target syntaxin selectively.

A large body of experimental work supports the view that soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) function in $Ca^{2+}$-triggered exocytosis of neurotransmitters and hormones.[1,2] However, for one of the three synaptic SNAREs, the plasma membrane protein syntaxin, unequivocal support for such a role is lacking. For the two other synaptic SNAREs, SNAP-25 and synaptobrevin (also known as VAMP), experiments with botulinum neurotoxin (BoNT) and tetanus toxin have provided some of the most convincing evidence that supports a function in exocytosis.[3] Most of these toxins selectively cleave synaptobrevin or SNAP-25, and they all block exocytosis. However, none of them cleave syntaxin selectively. BoNT/C1 is the only one that cleaves syntaxin, but it also cleaves SNAP-25. Genetic studies have confirmed the roles of SNAP-25[4] and synaptobrevin,[5] and by providing cells for testing mutants on null backgrounds, these knockouts have allowed researchers to investigate the roles of these two proteins in remarkable detail.[6,7] While loss of syntaxin from invertebrates impairs synaptic transmission,[8] ablation of different exons of the syntaxin 1A gene either leaves synaptic transmission normal[9] or is embryonic lethal.[10] Thus, for vertebrates, the function of syntaxin has yet to be rigorously tested, and a null system for detailed mutagenesis studies is still unavailable.

All of the BoNT serotypes have a similar overall structure, with an ~100 kDa heavy chain that mediates cell surface binding and entry and an ~50 kDa light chain that acts as a selective protease. The homology between the various BoNT light chains indicated that we can use the crystal structure of the BoNT/A−substrate complex[11] to locate residues important for substrate recognition and specificity within the BoNT/C1 structure[12] (Figure 1A). We created more than 150 mutants in these and other parts of the BoNT/C1 light chain and assayed their proteolytic activity by using a lentiviral vector to introduce the encoding DNA into neurons. Western blot analysis was then used to test for proteolysis of syntaxin and SNAP-25 (Supporting Information).

Extracts from control cells (cultured rat hippocampal neurons not transfected with a BoNT/C1 light chain) contained syntaxin 1A and 1B, which appeared as two closely spaced bands, and SNAP-25, which appeared as a single band (Figure 1B). Transfection with the wild-type BoNT/C1 light chain reduced the amount of full-length protein and produced lower-molecular mass cleavage products. Most of the mutants we created exhibited dual specificity or no activity (Supporting Information), but two were found to cleave only syntaxin and spare SNAP-25 (lanes 3 and 4 of Figure 1B). These two highly selective mutants were designated BoNT/C1α-3W and BoNT/C1α-51. BoNT/C1α-3W was generated by mutating the three residues in the S1′ pocket (L200, M221, and I226) (Figure 1A, red) to tryptophan. BoNT/C1α-51 was another triple mutant, 51T/52N/53P, in a region that has not previously received attention. Wild-type BoNT/C1, BoNT/C1α-51, and BoNT/C1α-3W reduced the syntaxin bands by 52.3, 60.5, and 73.3% of the control, respectively, while only wild-type BoNT/C1 reduced the density of the SNAP-25 band (Figure 1C). No cleavage of synaptobrevin was seen with BoNT/C1α-51 in one experiment (data not shown). None of the many other mutants we tested exhibited such clear selectivity for syntaxin over SNAP-25. These two mutants can thus serve as tools for testing the role of syntaxin. A third mutant, the inactive form denoted BoNT/C1γ, has mutations in two critical catalytic residues (R372A and Y375F). This mutant had no proteolytic activity (lane 5 of Figure 1B) and was used as a control for transfection and actions unrelated to catalysis.

To evaluate the role of syntaxin in synaptic transmission, we performed patch clamp recordings from cultured hippocampal neurons expressing wild-type BoNT/C1 and BoNT/C1 mutants. Toxins effective in cleaving syntaxin reduced both miniature excitatory postsynaptic currents (mEPSCs) (Figure 2A) and electrically evoked synaptic responses (Figure 2B). The frequency

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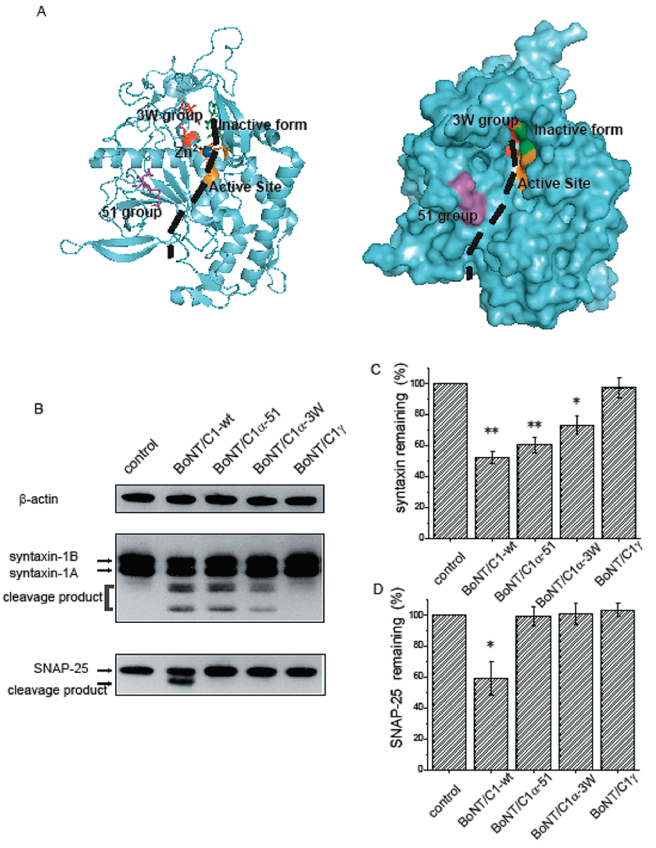


**Figure 1.** (A) Structure of the BoNT/C1 light chain[12] in ribbon (left) and space-filling (right) representations (PDB entry 2QN0). The position of the substrate, indicated by a black dashed curve, was inferred from the BoNT/A−substrate crystal structure.[11] (B) Western blot analysis of extracts from cultured hippocampal neurons blotted for syntaxin and SNAP-25. Untransfected control cells (lane 1) show full-length syntaxin 1A, syntaxin 1B, and SNAP-25. Infection with the wild-type BoNT/C1 light chain cleaves all three proteins (lane 2). BoNT/C1α-51 (lane 3) and BoNT/C1α-3W (lane 4) cleaved syntaxin and spared SNAP-25. BoNT/C1γ exhibited no catalytic activity toward any substrate. (C and D) Gel quantification of BoNT/C1 cleavage. The syntaxin (C) and SNAP-25 (D) band densities were reduced by cleavage in parallel with the appearance of lower-molecular mass cleavage products. Syntaxin and SNAP-25 signals were normalized to the $\beta$-actin control and averaged ($N = 3$). $p < 0.05$ (one asterisk).

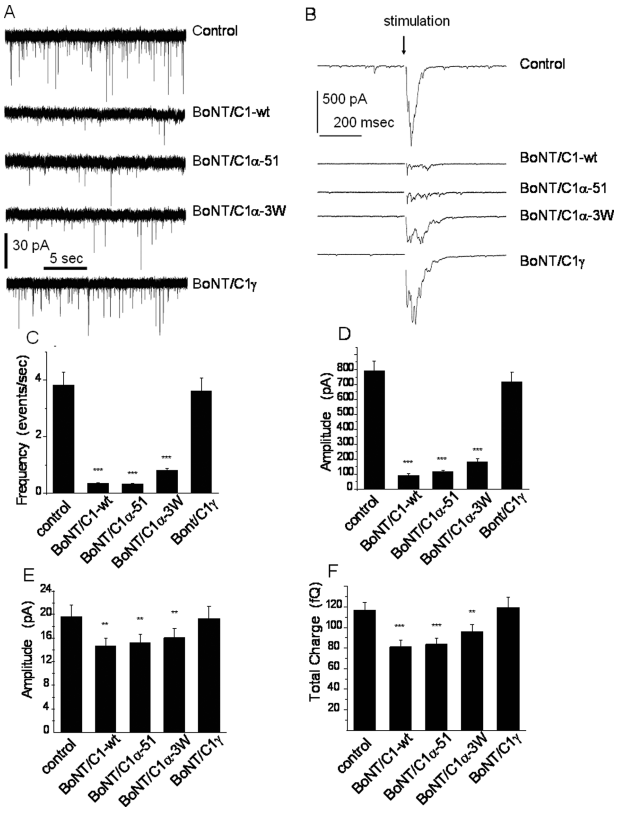


**Figure 2.** Synaptic release. Patch clamp recordings from cultured hippocampal neurons 72−96 h after infection. (A) Miniature excitatory synaptic currents (mEPSCs). (B) Synaptic currents evoked by extracellular stimulation (200 $\mu$A, 0.4 ms). (C) Mean mEPSC frequency ($N =$ 8−10 cells; ~20−300 events per cell). (D) Mean mEPSC amplitude. (E) Mean evoked synaptic current. (F) Mean mEPSC charge. $p < 0.01$ (two asterisks); $p < 0.001$ (three asterisks).

of mEPSCs (Figure 2C) and the amplitudes of evoked synaptic currents (Figure 2D) were reduced approximately 10-fold below controls. BoNT/C1α-51 blocked both forms of release with a similar effectiveness, while BoNT/C1α-3W was somewhat less effective, reducing the levels of spontaneous (Figure 2C) and evoked (Figure 2D) release by ~5-fold. BoNT/C1$\gamma$, the inactive control, produced no significant reduction in the level of spontaneous or evoked release or quantal size (Figure 2A−F). The reduction in the level of synaptic transmission was greater than the reduction in syntaxin levels (compare Figure 2A−D with Figure 1C). This disparity has been noted previously for wild-type neurotoxins and interpreted to mean that the exocytosis of one vesicle requires the cooperative action of multiple SNAREs.[13]

Wild-type BoNT/C1 and the two active mutants both produced a small (~20%) but significant reduction in the amplitude of miniature synaptic currents (Figure 2E) and charge per mEPSC (Figure 2F), indicating that with lower levels of either syntaxin or both syntaxin and SNAP-25, the few vesicles still able to fuse are smaller and contain less glutamate.[14] This could reflect the lower energy barrier for exocytosis of smaller vesicles,[15] which can be overcome by the assembly of fewer SNARE complexes.[16]

To evaluate syntaxin in the exocytosis of dense-core vesicles, we transfected PC12 cells with lentiviral vector DNA. Release was monitored using amperometry, which reveals the release of individual vesicles as spikes[17] (Figure 3A). BoNT/C1 variants that cleave syntaxin blocked depolarization-induced release of norepinephrine from PC12 cells. Depolarized PC12 cells exhibited far fewer spikes following transfection with wild type-BoNT/C1, BoNT/C1α-51, or BoNT/C1α-3W (Figure 3A). The cumulative spike plots (Figure 3B) and overall secretion rates (Figure 3C) indicated a roughly 50-fold reduction; however, the action of BoNT/C1α-3W was somewhat weaker, and the control toxin BoNT/C1$\gamma$ had no effect.

These experiments establish engineered versions of BoNT/C1 as tools for the acute and selective elimination of syntaxin from cells. Using these reagents, we were able to demonstrate that syntaxin is essential for $Ca^{2+}$-triggered exocytosis of synaptic vesicles in neurons and dense-core vesicles in endocrine cells. Like cleavage of SNAP-25 and synaptobrevin, cleavage of syntaxin 1A and 1B dramatically reduced the level of exocytosis. Our experiments thus place syntaxin on an equal footing with the other synaptic SNAREs as essential components of the exocytotic

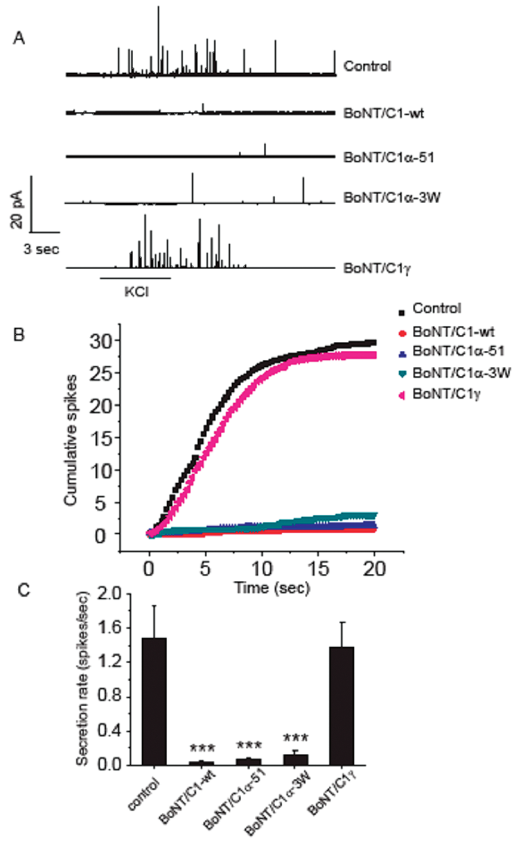


**Figure 3.** Release of norepinephrine from PC12 cells 2−3 days after transfection. (A) Amperometric traces, with exocytosis induced by application of 105 mM KCl (bar below). (B) Cumulative spike counts from traces such as in panel A (control; $N$ = 9−16 cells). (C) Mean frequency of spikes per cell. $p < 0.001$ (asterisks).

apparatus. Furthermore, by providing a syntaxin-free background, the engineered BoNT/C1 light chains developed for this study will serve as valuable tools for detailed studies of the mechanism by which syntaxin functions in exocytosis.

We generated two series of BoNT/C1 light chain variants that are specific syntaxin proteases. These newly engineered BoNT/C1 light chain proteins may also aid in extending the therapeutic potential of the clostridial neurotoxins.[18] BoNT/C1 offers a potential alternative for BoNT/A in BoNT/A nonresponsive patients.[19,20] Furthermore, BoNT/C1α could be useful in the treatment of diseases involving syntaxin, which has a number of functions in addition to exocytosis, including regulation of $K^+$ channels,[21] $Ca^{2+}$ channels,[22] and K-ATP channels.[23] Regulation of K-ATP channels is very important in type II diabetes treatment, many cases of which are caused by hyperactive $\beta$-cells and are treated by K-ATP channel openers to limit electrical activity. Syntaxin 1A downregulates K-ATP channels to make $\beta$-cells more excitable.[24] Cleavage of syntaxin would thus increase K-ATP channel activity to reduce the level of $\beta$-cell firing and restore normal insulin secretion.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Detailed methodology and Western blot analysis of ~150 BoNT/C1 light chain mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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