Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells

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Botulinum neurotoxins (BoNTs) cause botulism by entering neurons and cleaving proteins that mediate neurotransmitter release; disruption of exocytosis results in paralysis and death. The receptors for BoNTs are thought to be composed of both proteins and gangliosides; however, protein components that mediate toxin entry have not been identified. Using gain-of-function and loss-of-function approaches, we report here that the secretory vesicle proteins, synaptotagmins (syts) I and II, mediate the entry of BoNT/B (but not BoNT/A or E) into PC12 cells. Further, we demonstrate that BoNT/B entry into PC12 cells and rat diaphragm motor nerve terminals was activity dependent and can be blocked using fragments of syt II that contain the BoNT/B-binding domain. Finally, we show that syt II fragments, in conjunction with gangliosides, neutralized BoNT/B in intact mice. These findings establish that syts I and II can function as protein receptors for BoNT/B.

Introduction

Clostridial neurotoxins (CNT) are the most toxic substances known. There are eight related toxins; seven botulinum neurotoxins (BoNT/A–G) and a tetanus neurotoxin (TeNT; Simpson, 1981; Schiavo et al., 2000). Each toxin is composed of a heavy and a light chain; the heavy chain mediates binding to the surface of specific nerve terminals. Once internalized via endocytosis, the light chain is translocated from the lumen of the vesicle into the cytoplasm, where it functions as a zinc-dependent protease (Schiavo et al., 2000). The light chain cleaves one or more components of a conserved membrane fusion complex composed of syntaxin, SNAP-25, and synaptobrevin (syb), thereby blocking exocytosis (Schiavo et al., 1992, 1993; Blasi et al., 1993a,b). Because of their ability to selectively disrupt Ca21-triggered exocytosis, the CNTs have emerged as important tools for the study of membrane fusion and synaptic transmission (Jahn and Niemann, 1994). More recently, BoNTs have become useful for the treatment of muscle dystonias and have emerged as potential biological weapons (Mahant et al., 2000; Arnon et al., 2001).

The first step in the action of CNTs involves binding to receptors on the surface of neurons. Current evidence suggests that the receptors are composed of gangliosides and proteins that cooperate to form high affinity toxin-binding sites. Alternatively, gangliosides may constitute relatively low affinity toxin-binding sites that serve to capture CNTs to facilitate interactions with cell surface receptor proteins (Montecucco, 1986; Nishiki et al., 1996a). Gangliosides are ubiquitous glycosphingolipids in the outer leaflet of plasma membranes. They are classified according to the number and position of sialic acids present in their head groups. Polysialiogangliosides, which are present almost exclusively in neurons and neuroendocrine cells, bind to CNTs with the greatest avidity (Halpern and Neale, 1995). Although a protein component is also clearly involved in toxin–cell recognition, at present, a protein that mediates toxin entry has not been identified (Schiavo et al., 2000).

Biochemical experiments have led to the identification of a handful of CNT-binding proteins. In most cases, these binding proteins do not appear to function as receptors that mediate entry of the toxins. For example, BoNT/A, B, E, and TeNT were reported to bind synapsin I and adducin, respectively (Schengrund et al., 1992, 1993, 1996). Because neither of


Figure 1.   **Screening for interactions between syt isoforms and BoNT/A, B, and E.** (A) Top; schematic diagram of the syt constructs used in the GST pull-down experiments. To facilitate purification, all syt constructs lacked a C2B domain; the arrow indicates the COOH terminus of the truncated syt. The transmembrane domain is indicated by a black rectangle. Middle; GST or the indicated GST fusion proteins were incubated with 30 nM BoNT/B, A, and E, either with (+; 25 μg/ml) or without (−) gangliosides in 100 μl TBS. 17% of the bound materials were analyzed by SDS-PAGE and immunoblotting using anti-CNT pAbs. "Total" corresponds to 80 ng toxin. In the bottom panel, the amount of fusion protein was varied as indicated. (B) Binding assays were performed as in A. NH$_2$-terminal fragments of syts II and IX

served as positive and negative controls, respectively, and two chimeric constructs, in which the luminal domains of syts II and IX were swapped, were tested for toxin-binding activity. (C) Binding assays were performed as in A using immobilized syt II 1–87 and the indicated concentrations of BoNT/B. Bound toxin was visualized by staining with Coomassie blue; binding was stoichiometric at saturation. The heavy chain (H) of BoNT/B runs at 100 kD, the light chain (L) runs at 50 kD. The asterisk denotes a proteolytic fragment of GST-syt II 1–87.
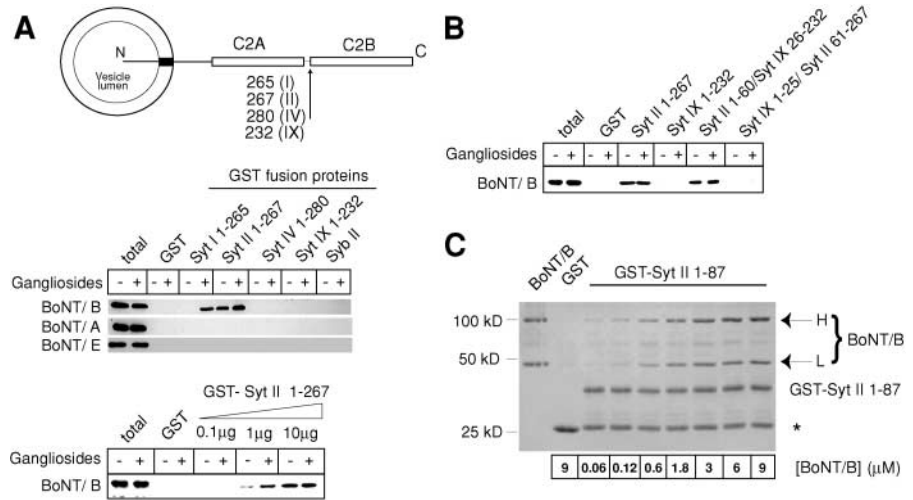
these proteins are exposed to the outside surface of cells, they are unlikely to function as cell surface receptors. TeNT was reported to bind Thy-1, a GPI-anchored plasma membrane protein. However, neurons from mice lacking Thy-1 are still sensitive to TeNT, suggesting that Thy-1 is not essential for TeNT entry into cells (Herreros et al., 2001).

At present, the only remaining candidate receptor proteins for BoNT/B are synaptotagmins (syts) I and II (Nishiki et al., 1994). These are homologous synaptic vesicle membrane proteins thought to function as Ca^{2+} sensors for exocytosis (Schiavo et al., 1998; Chapman, 2002). Syts I and II were reported to bind BoNT/B in the presence of gangliosides; the dissociation constant for the syt I–BoNT/B complex was 2.3 nM and the dissociation constant for syt II–BoNT/B was 0.23 nM (Nishiki et al., 1996a). High affinity binding of BoNT/B to fibroblasts was reconstituted by expression of syt II and incorporation of exogenous gangliosides into surface membranes. However, binding did not result in the cleavage of the BoNT/B target protein, syb II, that had been coexpressed with syt II, indicating that the toxin was not internalized (Nishiki et al., 1996b). Although biochemical experiments clearly established that syt binds to BoNT/B, evidence that binding mediates entry into cells is lacking. Thus, whether this interaction has any functional role remains unknown. More recently, BoNT/A and E have also been reported to bind syt I, albeit in a ganglioside-independent manner (Li and Singh, 1998).

In the current work, we characterized the interactions between BoNT/B, A, and E with syts I and II, and have investigated whether syts I and II function as receptors for BoNT/B. Our data demonstrate that syts I and II mediate BoNT/B entry into PC12 cells and motor nerve terminals. Moreover, the action of BoNT/B on these model systems in vitro and in mice in vivo can be neutralized by syt II fragments that contain the toxin-binding domain.

Results

A region within the luminal domain of syts I and II mediates direct interactions with BoNT/B

To assay for direct syt–BoNT interactions, fragments of syts I and II were immobilized as GST fusion proteins and used as an affinity matrix to pull down BoNT/A, B, or E in the presence and absence of gangliosides. For these experiments, we included two other syt isoforms, IV and IX, as well as full-length syb II, as negative controls. The structures of the syt fragments are shown in Fig. 1 A (top). Because each fragment contains a transmembrane domain, binding assays included 0.5% Triton X-100; thus, gangliosides were presented as mixed micelles. In contrast to a previous paper (Li and Singh, 1998), we did not observe detectable binding of BoNT/A or E to any of the immobilized proteins (Fig. 1 A, middle), even when relatively high concentrations of BoNT/A and E were used (300 nM; unpublished data), indicating that these toxins do not bind to the syt fragments used in our assays.

Under identical conditions, we observed that BoNT/B binds syts I and II. Although syt I–BoNT/B interactions were strictly dependent on gangliosides, syt II bound BoNT/B in the absence of gangliosides (Fig. 1 A, middle). Reducing the concentration of bead-immobilized GST–syt II fusion protein revealed that gangliosides can enhance syt II–BoNT/B interactions (Fig. 1 A, bottom), but this interaction is clearly less dependent on gangliosides. These findings are consistent with previous data showing that syt II binds BoNT/B more tightly than does syt I (Nishiki et al., 1996a); presumably, the higher affinity syt II–BoNT/B interaction is less reliant on gangliosides. Syt I/II–BoNT/B interactions are specific because binding to an analogous region of syt IV or syt IX, or to full-length syb II, was not detected (Fig. 1 A, middle).

If syts I and II are physiologically relevant receptors for BoNT/B, binding must be mediated by the region of syt
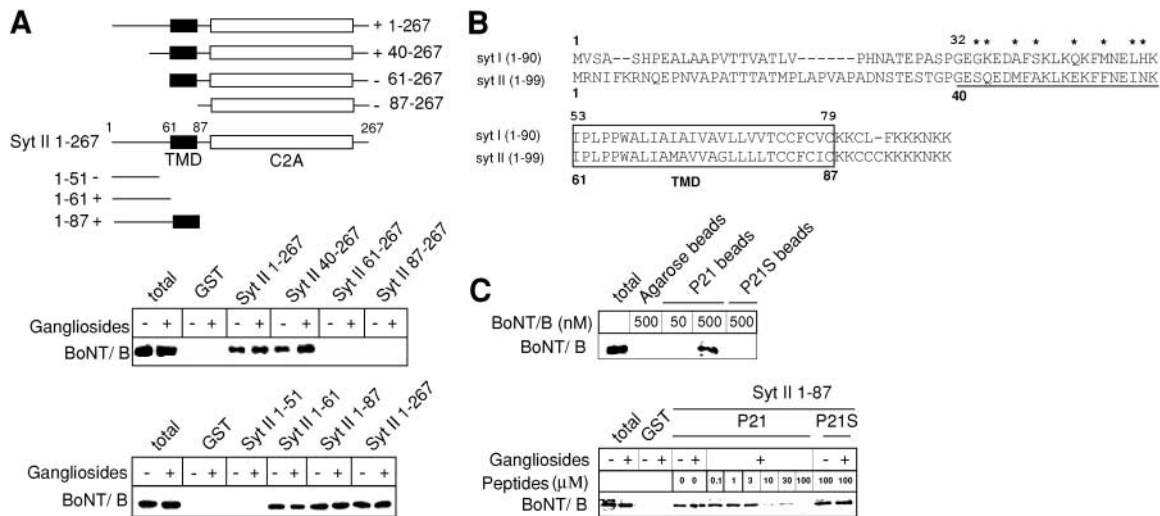
Figure 2.   **Mapping the BoNT/B-binding site within the luminal domain of syt II.** (A) Binding assays were performed as in Fig. 1 A, using the indicated syt II truncation mutants. The top shows a schematic of the truncation mutants where (+) denotes binding and (−) denotes lack of binding. (B) Sequence of the amino terminus of syts I and II. The underlined region (residues 40–60 in syt II; residues 32–52 in syt I) is critical for binding BoNT/B; the asterisks indicate sequence differences. The transmembrane domain (TMD) is boxed. (C) Top; a peptide, P21, corresponding to residues 40–60 of syt II, plus a COOH-terminal cys residue was conjugated to agarose beads and used to pull-down toxin as described in A; a scrambled peptide, P21S, served as a negative control. Bottom; P21 (but not P21S) blocked the interaction between BoNT/B and the 1–87 fragment of syt II. Binding assays were performed as in A, but as a function of the indicated P21 or P21S concentration.

that is exposed outside of cells (i.e., the luminal domain) during cycles of exocytosis and endocytosis. To clarify how BoNT/B binds to syt II, we first used syt II and syt IX chimeras. Swapping the luminal domains of these proteins was sufficient to transfer the BoNT/B-binding activity from syt II to syt IX (Fig. 1 B), indicating that BoNT/B binding is mediated by the luminal domain of syt II. Consistent with this finding, a shorter fragment of syt II, composed of only the luminal and transmembrane domain (residues 1–87), mediated stoichiometric binding of the toxin (Fig. 1 C).

Truncation analysis was used to further map the toxin-binding site of syts I and II. Within the luminal domain of syt II, residues 40–60, which are adjacent to the transmembrane domain, are critical for toxin binding (Fig. 2 A). We note that fragment 61–267 of syt II can bind gangliosides via its transmembrane domain (residues 61–87; Kozaki et al., 1998), yet this fragment fails to bind BoNT/B in the presence of gangliosides (Fig. 2 A, middle). These data suggest that gangliosides do not directly mediate toxin binding under our assay conditions, but rather cooperate with the luminal domain to form high affinity BoNT/B-binding sites. The analogous membrane proximal region of syt I (residues 32–52) was also critical for binding of BoNT/B (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200305098/DC1). This segment is highly conserved between syts I and II (Fig. 2 B); minor sequence differences may account for the differences in affinity for BoNT/B (Nishiki et al., 1996a). We note that the isolated luminal domain of syt II (residues 1–61) but not syt I (residues 1–53), bound to BoNT/B (Fig. 2 A and Fig. S1). This result is likely due to the strong ganglioside requirements for syt I–BoNT/B interactions; deletion of the transmembrane domain of syt I abolishes ganglioside binding (Kozaki et al., 1998), and thereby decreases BoNT/B-binding activity.

The mapping experiments we have just described suggest that residues 40–60 of syt II comprise the BoNT/B-binding domain. To test this directly, a synthetic peptide (P21) corresponding to this segment of syt II was immobilized on beads and used as an affinity matrix. This peptide binds directly to BoNT/B, although less avidly than do longer fragments of syt II because detectable binding required higher concentrations of the toxin (Fig. 2 C, top). A scrambled version of this peptide, P21S, served as the negative control. Furthermore, P21, but not P21S, was able to competitively inhibit syt II–BoNT/B interactions (Fig. 2 C, bottom). P21 also inhibited syt I–BoNT/B interactions (unpublished data). Together, these experiments establish that residues 40–60 of syt II largely mediate binding of BoNT/B.

Syt I mediates ganglioside-dependent binding and entry of BoNT/B into PC12 cells

The experiments described above demonstrate that syts I and II bind to BoNT/B through a conserved region in their ectodomains. However, the key question is whether syt I or syt II mediate entry of the toxin; namely, are they functional protein receptors for BoNT/B? To address this question, we first used PC12 cells, a neuroendocrine cell line that serves as a model system to study Ca^{2+}-triggered exocytosis. These cells express the substrates for all of the CNTs, but are resistant to entry of BoNT/B, probably due to lack of functional toxin receptors (Lomneth et al., 1991). PC12 cells express syt I and IX and trace levels of syt IV (Zhang et al., 2002). Because syts IX and IV do not bind BoNT/B and syt I binds only in the presence of gangliosides (Fig. 1 A, middle), toxin resistance could be due to the fact that these cells contain low levels of gangliosides as compared with neurons (Walton et al., 1988). We tested this idea by preloading exogenous gangliosides into the plasma membrane of wild-type PC12

cells. As shown in Fig. 3 A, detectable toxin binding was observed only when cells were loaded with gangliosides. Then, we determined whether the toxin can enter ganglioside-treated cells. To monitor entry, we assayed for cleavage of the cytoplasmic substrate of BoNT/B, syb II (Schiavo et al., 1992), by immunoblot analysis using anti-syb II antibodies. Cleavage of syb II by BoNT/B occurred only when cells were first preloaded with gangliosides (Fig. 3 B). These data are consistent with a model in which syt I and gangliosides cooperate to mediate the binding and entry of BoNT/B, and are in agreement with biochemical data showing that the toxin binds to syt I only in the presence of gangliosides. To further test this model, we took advantage of a PC12 cell line that lacks syt I (Syt I⁻; Shoji-Kasai et al., 1992). This cell line is still capable of Ca^{2+}-triggered exocytosis, presumably via the redundant action of syt IX (Fukuda et al., 2001; Zhang et al., 2002). As shown in Fig. 3 C, BoNT/B failed to cleave syb II in ganglioside-loaded syt I⁻ PC12 cells. These data indicate that gangliosides plus syt I are both needed for toxin entry.

We also assayed for entry of BoNT/A and E into PC12 cells. Entry was monitored by assaying for cleavage of their substrate SNAP-25 (Blasi et al., 1993a; Schiavo et al., 1993). BoNT/A cleaves SNAP-25 between residues 197–198, thereby removing nine amino acids; BoNT/E cleaves between residues 180–181 and removes 26 residues (Schiavo et al., 1993). Incubation of cells with nM concentrations of BoNT/A and E resulted in similar degrees of cleavage of SNAP-25 in both wild-type (unpublished data) and syt I⁻ cells (Fig. 3 D). Thus, both toxins are able to enter syt I⁻ PC12 cells that have not been preloaded with gangliosides. These experiments demonstrate that syts I and II are not required for entry of BoNT/A and E into PC12 cells, and that syt I⁻ cells are competent to take up at least some CNTs.

Syt II is sufficient to mediate entry of BoNT/B into PC12 cells

To determine directly whether syt II can function as a receptor for BoNT/B, we took advantage of the observation that this syt isoform is able to bind BoNT/B to some extent in the absence of gangliosides (Fig. 1 A). We generated PC12 cell lines that stably express syt II (Syt II⁺; Fig. 4 A), and observed that they bind BoNT/B without preloading cells with exogenous gangliosides (Fig. 4 B). A key finding was that expression of syt II was sufficient to reconstitute toxin entry into the transfected cells, as shown by the cleavage of syb II (Fig. 4 C). The efficiency of cleavage was proportional to the level of syt II expression, and cleavage was not observed in cells lacking syt II (Fig. 4 C, right). These findings demonstrate that syt II can function as a receptor for BoNT/B without preloading cells with exogenous gangliosides.

To further test whether binding and entry is mediated by direct interactions between BoNT/B and the luminal domain of syt II, we determined whether fragments of syt II that contain the BoNT/B-binding site inhibit toxin action. As shown in Fig. 5 A, fragments corresponding to residues 1–267, 1–87, and 40–60 (P21) of syt II, blocked binding of BoNT/B to syt II⁺ PC12 cells. Syt II fragment 61–267, which lacks the luminal domain, and the scrambled peptide P21S, failed to block binding of the toxin. We note that syt II 1–267 and 61–267 contain an oligomerization domain within residues 61–140, and also bind membranes via their C2A domain, thus forming aggregates (Bai et al., 2000). These aggregates are visualized in Fig. 5 A (bottom panels) using an anti-His₆ antibody that recognizes a His₆ tag present in these recombinant syt fragments. The syt 1–267 fragment also contains bound BoNT/B, as shown via the anti-BoNT/B immunoreactivity in the syt II aggregates (Fig. 5 A, top). In contrast, cell-associated syt II 61–267 aggregates did not contain BoNT/B (Fig. 5 A, bottom).
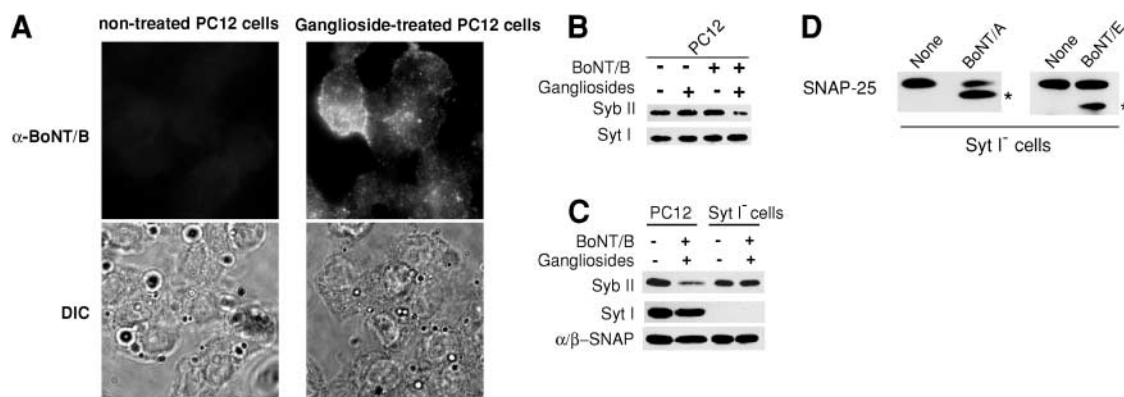

Figure 3. **Entry of BoNT/B into PC12 cells is dependent on syt I expression and preloading of cells with gangliosides.** (A) PC12 cells were either untreated or preloaded with gangliosides. Cells were then incubated with 50 nM BoNT/B for 48 h, fixed, permeabilized, and stained using a rabbit anti-BoNT/B antibody; the secondary antibody was goat anti–rabbit-FITC. Pre-loading cells with gangliosides resulted in toxin-binding activity. (B) PC12 cells either were (+) or were not (−) preloaded with gangliosides; cells were then incubated with (+) or without (−) 50 nM BoNT/B for 48 h and harvested. 20 μg of each sample was subjected to SDS-PAGE and immunoblot analysis using anti-syb II (Cl 69.1) or anti-syt I (Cl 41.1) antibodies. Pre-loading cells with gangliosides mediated entry of toxin, as evidenced by cleavage of syb II. Syt I was probed to ensure equal loading on the gels. (C) Experiments were performed as in B above, except that wild-type PC12 cells were compared with the syt I⁻ cells. α/β-SNAP was probed to ensure equal loading. In the absence of syt I, BoNT/B cannot enter PC12 cells to cleave syb II, even when cells have been preloaded with gangliosides. (D) Entry of BoNT/A and E into Syt I⁻ PC12 cells. Syt I⁻ PC12 cells were incubated with 30 nM BoNT/A or 50 nM BoNT/E for 48 h; entry was monitored by assaying for cleavage of SNAP-25. Asterisks denote SNAP-25 cleavage products.
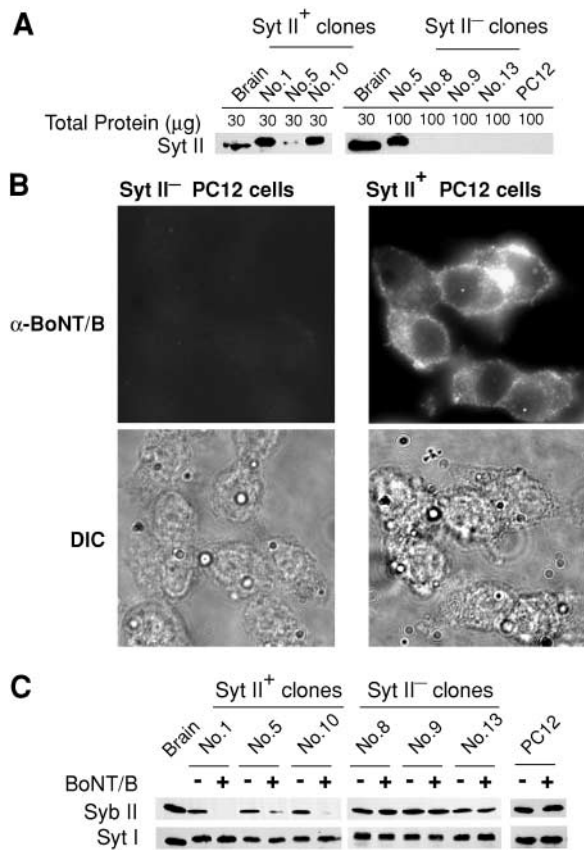
Figure 4.   Syt II mediates entry of BoNT/B into PC12 cells.
(A) Full-length mouse syt II was subcloned into pCDNA3.1(−) and used to transfect PC12 cells. Cells were selected with G418 and several independent monoclonal lines were established and screened for syt II expression by immunoblot analysis using a rabbit anti-syt II antibody; 30 μg of protein from the syt II+ clones and 100 μg from the syt II− clones were loaded onto the gels. Clone no. 1, 5, and 10 expressed syt II (syt II+), clone no. 8, 9, and 13 lacked syt II (syt II−). Because clone no. 5 expressed low levels of syt II (left), a 100-μg sample from this clone was also included in the blots of the syt II− clones to confirm that this clone expresses syt II. (B) Wt or syt II+ (clone no. 1) PC12 cells were decorated with 30 nM BoNT/B as described in Fig. 3 A. (C) Entry of 15 nM BoNT/B into PC12 cells was assayed as described in Fig. 3 B. Entry of the toxin was observed in all syt II+ clones, and was not observed in any of the syt II− clones. As a control, a parental PC12 cell line was analyzed in parallel.

More importantly, titration of syt II 1–267 resulted in the dose-dependent protection of syb II cleavage; fragment 61–267 had no protective effect (Fig. 5 B, top). Inclusion of gangliosides increased the efficacy of protection by approximately threefold (Fig. 5 B, bottom), presumably by facilitating the already robust binding of syt II 1–267 to BoNT/B (Fig. 1 A, bottom). This result is consistent with the observation that the binding partner with the highest affinity for BoNT/B is composed of syt II plus gangliosides (Fig. 1 A, bottom; Nishiki et al., 1996a). As a control, mixtures of gangliosides and syt II fragment 61–267 were not able to prevent cleavage of syb II (Fig. 5 B, bottom). P21 also yielded dose-dependent protection, albeit at >10-fold higher concentrations as compared with the 1–267 fragment (Fig. 5 C), presumably because it binds less tightly to BoNT/B than the longer fragments of syt II. There is a concern that low

levels of detergent associated with the transmembrane domains present in some of the syt fragments may affect the uptake and action of the toxin. However, we did not observe any apparent toxicity using these fragments. Also, the ability of fragment 1–267 to block the action of the toxin cannot be due to toxicity from associated detergent, as fragment 61–267 has the same transmembrane domain, yet fails to provide any protection.

Activity-dependent entry of BoNT/B into vesicles that contain syt I

The data above suggest a model in which BoNT/B gains entry into PC12 cells by binding to the luminal domain of syt I or syt II. This model predicts that BoNT/B will follow the internalization of syts I and II from the cell surface and that internalization will be activity dependent. To answer this question, we first demonstrated that an anti-syt I luminal domain antibody (α-syt I$_N$) and BoNT/B can bind to syt I simultaneously (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200305098/DC1). This is the expected result because the antibody recognizes the first 12 amino acids at the NH$_2$ terminus of syt I, whereas the BoNT/B-binding site lies at the COOH-terminal end of the luminal domain.

We took advantage of this finding and determined whether the antibody and toxin are taken up into the same compartment in response to stimulation. PC12 cells were preloaded with gangliosides and depolarized with high [K$^+$] to induce exocytosis of secretory vesicles in the presence of α-syt I$_N$ antibodies and BoNT/B. Exocytosis and endocytosis were allowed to proceed for 10 min, followed by extensive washes to remove surface-bound antibody and toxin. As shown in a confocal section (Fig. 6), both α-syt I$_N$ antibodies and BoNT/B were internalized into the same compartment. Depolarization of cells significantly increased the internalization of both the antibody and BoNT/B; only low levels of internalization, due to spontaneous exocytosis and recycling, were observed in the control (Fig. 6 A).

In contrast to the α-syt I$_N$ antibody, an antibody directed against the cytoplasmic domain of syt I (α-syt I$_C$) was not taken-up (Fig. S2 B, available at http://www.jcb.org/cgi/content/full/jcb.200305098/DC1), demonstrating that staining with the luminal domain antibody is not due to loss of integrity of the cell membranes. Also, α-syt I$_N$ antibodies and BoNT/B were not taken up into syt I− PC12 cells (Fig. S2 B), further establishing that uptake requires the exposure of the syt I luminal domain and is not due to bulk endocytosis. These findings demonstrate that the luminal domain of syt I is exposed on the surface of PC12 cells during exocytosis, and that BoNT/B enters PC12 cells via organelles that contain syt I. This latter observation was further confirmed by the colocalization of BoNT/B with an antibody directed against the cytoplasmic domain (α-syt I$_C$) of syt I (Fig. S2 C).

Similar results were obtained using syt II+ PC12 cells; BoNT/B entered syt I containing vesicles in an activity-dependent manner (unpublished data). We have been unable to localize syt II in the syt II+ cell lines using antibodies currently available. However, syt II is colocalized with syt I on secretory vesicles in brain, and is likely to be targeted to syt I-containing organelles in PC12 cells (Osborne et al., 1999).
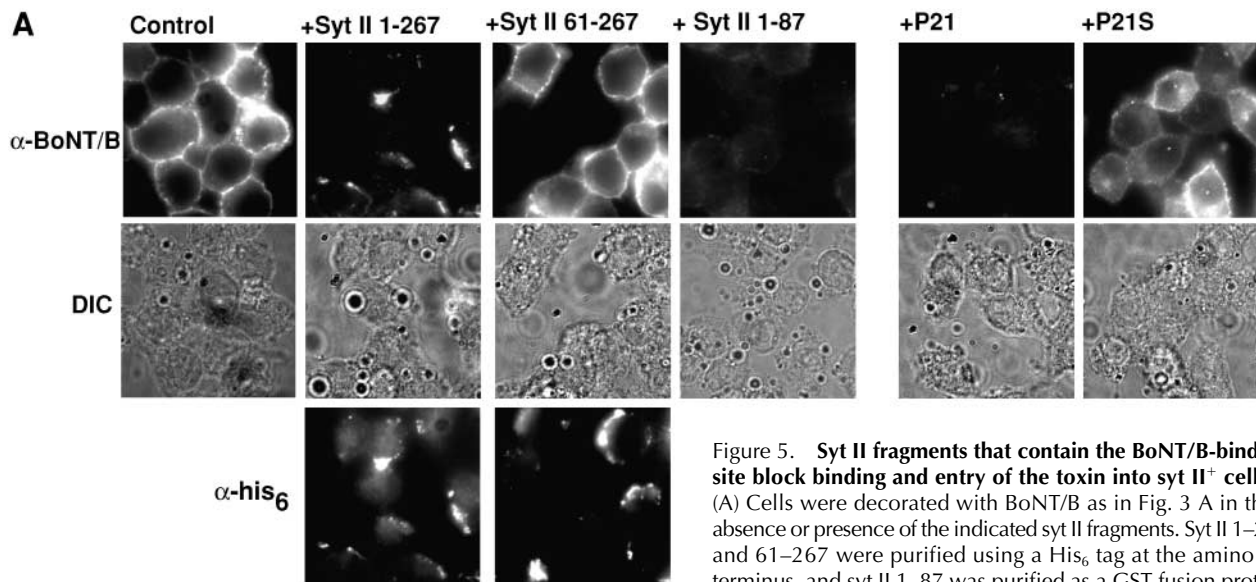
Figure 5. **Syt II fragments that contain the BoNT/B-binding site block binding and entry of the toxin into syt II⁺ cells.** (A) Cells were decorated with BoNT/B as in Fig. 3 A in the absence or presence of the indicated syt II fragments. Syt II 1–267 and 61–267 were purified using a His$_6$ tag at the amino terminus, and syt II 1–87 was purified as a GST fusion protein and eluted from beads using glutathione. Syt fragments 1–267 and 1–87, as well as the P21 peptide (residues 40–60), blocked binding; 61–267 and P21S had no effect. The syt II 1–267 and 61–267 fragments form aggregates bound to cell membranes as visualized with an anti-His$_6$ antibody in the bottom; for syt II 1–267, these aggregates also contained BoNT/B. The final concentrations of recombinant protein and peptides in the media were 960 nM and 10 μM, respectively; the final concentration of BoNT/B was 30 nM. (B) Syt II⁺ PC12 cells (clone no. 1) were treated with 30 nM BoNT/B that was premixed with the indicated concentration of the syt II 1–267 fragment in the absence (top) or presence (bottom) of 25 μg/ml gangliosides for 48 h. Samples were analyzed by immunoblotting as described in Fig. 3 B. Cleavage of syb II was inhibited by the 1–267 fragment of syt II; inclusion of gangliosides increased the ability of the syt fragment to block cleavage of syb II. Fragment 61–267 had no effect. (C) Experiments were performed as in A, but using the P21 or P21S peptides.
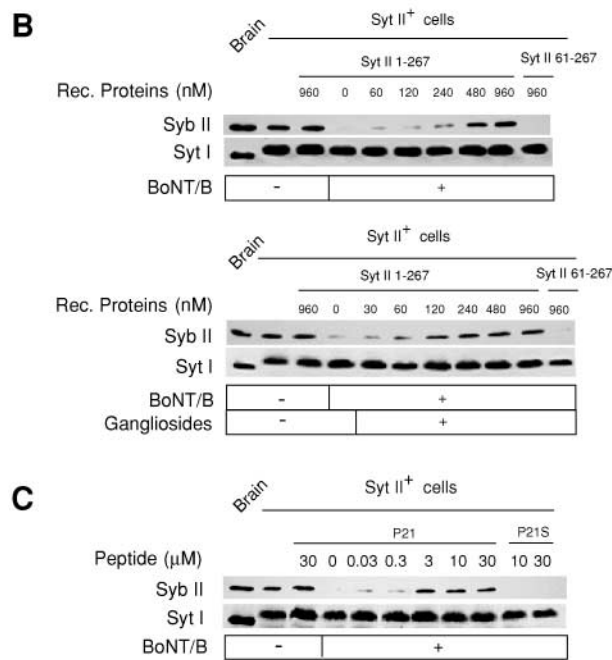
Activity-dependent uptake of BoNT/B into motor nerve terminals

The cause of death from BoNT/B intoxication is asphyxiation due to blockade of neurotransmission at the diaphragm. Therefore, we extended our analyses to explore the mechanism of toxin entry into neurons in this tissue. As shown in Fig. 7 A, stimulation with KCl results in a dramatic increase in the levels of BoNT/B associated with the neuromuscular junction (5.7-fold; Fig. 7 B), and a concomitant loss of syb II immunoreactivity (3.2-fold; Fig. 7 C). The increase in binding of BoNT/B and the loss of syb II were virtually abolished by incubation with the syt II fragment 1–267/gangliosides, but not by a mixture of syt II 61–267/gangliosides (Fig. 7, A–C). These data demonstrate that uptake of BoNT/B is activity dependent at its natural target. Moreover, binding and entry of the toxin can be completely prevented by syt II fragments that contain the toxin-binding site, whereas syt fragments lacking the toxin-binding site have no effect.

Competitive inhibition of syt–BoNT/B interactions neutralizes BoNT/B in vivo

The experiments described above demonstrate that BoNT/B enters PC12 cells and motor nerve terminals through interactions with syts I and II plus gangliosides. To further establish the physiological relevance of our findings, we determined whether syt II fragments that contain the BoNT/B-binding site can neutralize the effects of the toxin in vivo. For these experiments, we used a rapid method to evaluate toxicity in which the i.v. injection of large amounts (10^5–10^6 LD$_{50}$) of BoNT/B into mice results in death on a time scale of minutes to hours, as opposed to
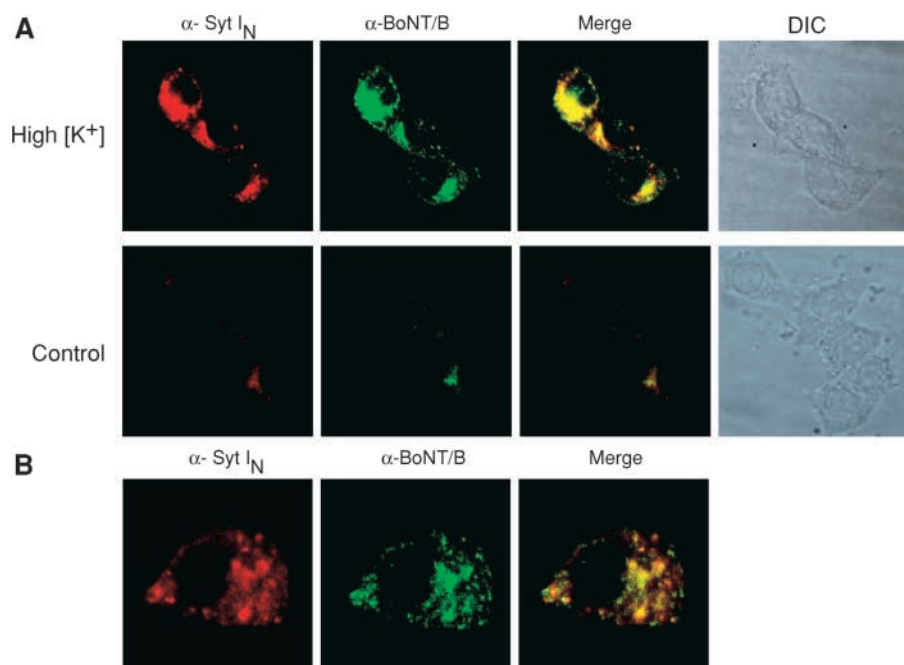
Figure 6. **Activity-dependent internalization of BoNT/B into syt I harboring organelles.** (A) PC12 cells were preloaded with gangliosides and incubated with 50 nM BoNT/B plus 10 μl/ml α-syt I_N antibodies for 10 min at 37°C in either control or high [K⁺] buffers. Cells were then washed, fixed, and permeabilized as described in the Materials and methods section. Uptake of α-syt I_N antibodies was assayed by staining with a rhodamine-conjugated goat anti–mouse secondary antibody; internalized BoNT/B was stained using a rabbit anti-BoNT/B antibody and a FITC-conjugated goat anti–rabbit secondary antibody. Images were generated using a confocal microscope; a single optical section from the middle of the cells is shown. Both α-syt I_N and BoNT/B were internalized, into the same compartment(s), in an activity-dependent manner. (B) An enlarged optical section of a single PC12 cell obtained as described in A. The merged images demonstrate a high degree of colocalization between internalized α-syt I_N antibodies and BoNT/B.

standard 4-d lethality assays (Boroff and Fleck, 1966; Schantz and Kautter, 1978). This assay reduces the amount of time that animals are exposed to the toxin. To this end, we first established a standard curve to relate classically determined LD_{50}/ml values to the time-to-death values that were determined using the rapid assay (Fig. 8 A). This plot was then used to convert the experimentally measured time-to-death to units of apparent LD_{50}/ml. After this conversion, the apparent LD_{50}/ml values were used to calculate the percentage of neutralization of the toxin by syt/ganglioside mixtures.

In contrast to the PC12 cell experiments, the range of [syt II 1–267] that we tested in mice did not afford protection in the absence of gangliosides. This might be due to the fact that the highest affinity receptor is composed of a syt II–ganglioside complex, and that the highest affinity scavenger is needed to compete with toxin binding in vivo. Consistent with this model, syt II fragments 1–267 and 1–87, together with gangliosides, neutralized most of the BoNT/B toxicity in mice (Fig. 8 B). It remains possible that higher doses of syt II 1–267 could provide some degree of protection in vivo.

Syt II 61–267 plus gangliosides did not neutralize the toxin (Fig. 8 B), further establishing the essential role of the luminal domain of syt II for toxin entry in vivo. The potencies of syt II 1–267 and 1–87 were determined (Fig. 8 C); both fragments yielded dose-dependent protection at sub-μM concentrations. Finally, prior i.v. injection with syt II 1–267 or 1–87, mixed with gangliosides, neutralized 70–80% of BoNT/B that was injected 1 min later (Fig. 8 D), indicating that animals can be protected before exposure to toxin. Together, these results support the idea that the physiological receptor for BoNT/B is composed of syt II and gangliosides.

Discussion

Botulism was first described almost 200 years ago (Kerner, 1817). Among the BoNTs, serotypes A, B, and E are the most common causes of botulism in humans (Hatheway, 1995). To enter neurons, BoNTs first bind, with high affinity and specificity, to presynaptic nerve terminals. Using biochemical methods, a number of toxin-binding proteins have been identified. At present, none of these binding partners have been shown to be important for toxin entry; the identity of the physiological receptors for the CNTs remains an open question. Here, we demonstrate that syts I and II function as receptors that mediate the entry of BoNT/B into cells.

First, we confirmed that BoNT/B binds to syts I and II. This interaction is stoichiometric, highly specific, facilitated by gangliosides, and is mediated by a region of syt that is transiently exposed outside of cells during exocytosis. Using PC12 cells, which are resistant to BoNT/B, we performed experiments to reconstitute entry. These cells have low levels of gangliosides, but express syt I. We found that BoNT/B is able to enter PC12 cells if their plasma membranes are preloaded with sufficient levels of gangliosides. Moreover, entry into ganglioside-loaded cells was strictly dependent on the expression of syt I; entry was abolished when syt I expression was disrupted. Next, we took advantage of our observation that, in contrast to syt I, syt II exhibits significant levels of toxin-binding activity in the absence of gangliosides. Expression of syt II in PC12 cells resulted in the binding and entry of the toxin into cells and overcame the need for preloading the cells with gangliosides. In summary, both loss-of-function (syt I⁻) and gain-of-function experiments (syt II⁺) are consistent with a model in which BoNT/B enters cells via interactions with syt I or syt II.

If syt mediates binding and entry of BoNT/B, then syt ectodomains, which contain the toxin-binding site, should block the action of the toxin. Using syt II⁺ PC12 cells, we
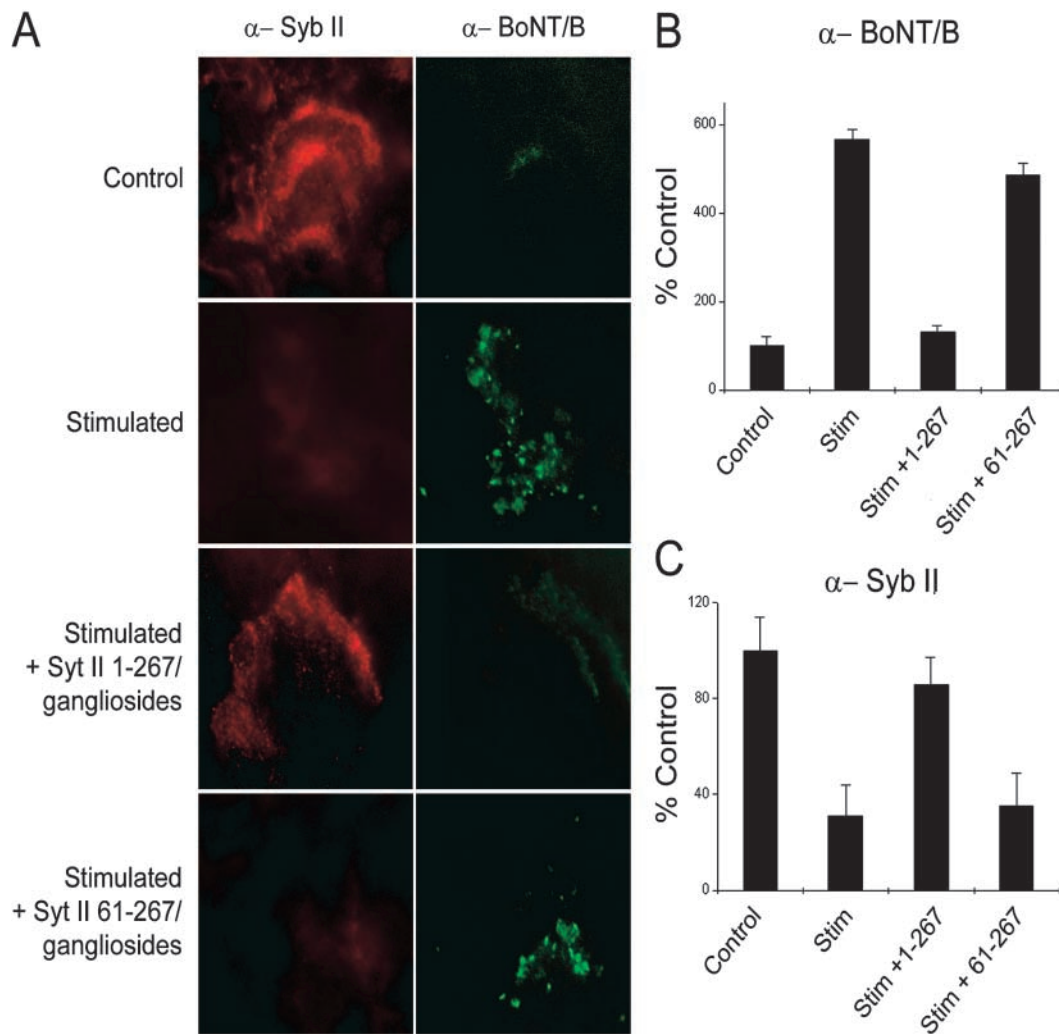
Figure 7.   **Activity-dependent uptake of BoNT/B, followed by cleavage of syb II in rat diaphragm motor nerve terminals.** (A) Rat diaphragm preparations were incubated with 5 nM BoNT/B in mammalian ringer. They were either unstimulated (control), stimulated with high potassium (stimulated), or stimulated in the presence of a mixture of BoNT/B and the protein fragment syt II 1–267 or 61–267 (1 μM) plus gangliosides (25 μg/ml). They were then fixed, permeabilized, and blocked. Control (unstimulated) nerve terminals show bright immunofluorescence for syb II, and very dim labeling of BoNT/B. Stimulation during incubation with BoNT/B resulted in greatly reduced syb II immunofluorescence, whereas BoNT/B levels are markedly enhanced. Stimulation in the presence of both BoNT/B and syt II 1–267/gangliosides resulted in protection of nerve terminals, seen as both preservation of syb II staining, and greatly reduced levels of BoNT/B binding. (B) Quantification of BoNT/B levels under different conditions. Stimulation greatly enhances BoNT/B binding, and this can be blocked by coincubation with syt II 1–267/gangliosides. Syt fragment 61–267 plus gangliosides failed to block binding of BoNT/B. (C) Quantification of syb II levels. Syb II levels show a complementary pattern to those seen with BoNT/B. Levels of immunofluorescence are high in unstimulated tissue, but drop after stimulation. Inclusion of syt II 1–267/gangliosides but not 61–267/gangliosides with BoNT/B protects syb II from cleavage. In B and C, error bars represent the SEM ($n = 15$–22).

observed that was indeed the case; fragments of syt II effectively inhibited the binding of BoNT/B to the surface of cells, and prevented the cleavage of syb II.

We studied the pathway of BoNT/B internalization into PC12 cells using an antibody directed against the NH_2 terminus of syt I. We found that BoNT/B was co-internalized with the antibody in an activity-dependent manner. These results further support our conclusion that syts I and II are functional receptors that mediate BoNT/B entry through recycling of secretory vesicles. This is also likely to be the route of entry of BoNT/B into neurons. In neurons, syts I and II reside on synaptic vesicles where their luminal domains are exposed during cycles of exo-endocytosis (Matteoli et al.,

1992; Juzans et al., 1996), providing a means of entry. It is known that cholinergic neurons at the neuromuscular junction express syt II and are the major physiological targets of BoNT/B (Dolly et al., 1984; Juzans et al., 1996). We explored this issue using motor neurons that innervate the diaphragm. Our data unambiguously demonstrate that binding and uptake of BoNT/B is activity dependent and can be blocked by syt fragments in conjunction with gangliosides at the neuromuscular junction (Fig. 7). These results indicate that the mechanism of toxin entry is similar between PC12 cells and motor neurons. Finally, these observations were extended to a whole animal study where we found that syt fragment/ganglioside mixtures neutralized the toxin in mice.
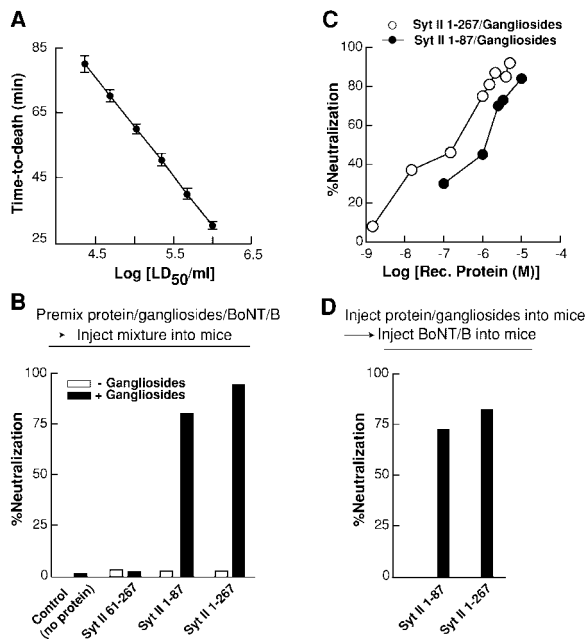
Figure 8. **Protection of mice from BoNT/B toxicity using fragments of syt II.** (A) Specific toxicity of BoNT/B in female mice was determined by an i.v. time-to-death assay. The standard curve was used to convert time-to-death (min) to LD_{50}/ml. The resultant LD_{50}/ml values were used to calculate percentage of neutralization of toxicity using the expression: $1 - [LD_{50}/ml(+ syt II fragment)/LD_{50}/ml(- syt II fragment)] \times 100$, where (+ syt II fragment) refers to samples that contain toxin, gangliosides and recombinant proteins and (− syt II fragment) samples were composed of toxin and gangliosides only. (B) The indicated syt fragments (5 μM) were premixed with gangliosides (250 μg/ml) and BoNT/B concentrations that lie in the linear range of the standard curve in A (i.e., 10^5–10^6 LD_{50}/ml) for 10 min at RT, and injected i.v. (100 μl) into mice. Percentage of neutralization was determined as described in A. In all the in vivo experiments, the indicated concentrations correspond to the initial concentration before i.v. injection; the dilution factor in the circulatory system is ~1:10. (C) Experiments were performed as described in B, but as a function of the syt II 1–267 or 1–87 concentration. (D) Pre-injection of gangliosides (250 μg/ml) plus syt II 1–267 (17 μM) or 1–87 (20 μM) mixtures protects mice from subsequent exposure to BoNT/B. Experiments were performed as in B, except that toxin was injected 1 min after injection of the receptor complex. Note: in B–D, each data point represents the average of at least triplicate determinations; error was within ± 10%.

Our model also indicates that syt I is a less effective receptor, but at high local ganglioside concentrations, syt I may also mediate entry of BoNT/B into neurons that lack syt II. Therefore, the sensitivity of a particular neuron to BoNT/B might depend on the local levels of gangliosides and whether it expresses syt I or syt II.

All of our BoNT/B data are consistent with the double-receptor model for CNTs, as proposed more than 15 years ago (Montecucco, 1986). First, gangliosides facilitate syt–BoNT/B interactions (Fig. 1 A; Nishiki et al., 1996a; Kozaki et al., 1998); second, preloading cells with gangliosides is required for syt I–mediated toxin entry into wild-type PC12 cells (Fig. 3 B); third, preloading cells with gangliosides can facilitate toxin entry that is mediated by syt II (unpublished data); and fourth, gangliosides are required for syt ectodomains to protect mice from BoNT/B (Fig. 8 B). This hy-

pothesis is further supported by the finding that disruption of ganglioside synthesis in mice reduces their sensitivity to CNTs (Kitamura et al., 1999; Bullens et al., 2002).

The simplest explanation for the fact that syt II–ganglioside complexes efficiently block BoNT/B action in PC12 cells, intact hemi-diaphragm preparations, and mice is that syt II plus gangliosides functions as the toxin receptor in vivo. The in vivo experiments establish the physiological significance of our analyses and provide a proof-of-principle that receptor ectodomains might provide a novel means to antagonize the action of bacterial toxins in animals.

Materials and methods

Cell lines, gangliosides, and toxins
A syt I deficit (Syt I⁻) PC12 cell line was provided by Y. Shoji-Kasai and M. Takahashi (Mitsubishi Institute od Life Sciences, Machida, Japan; Shoji-Kasai et al., 1992). A mixture of bovine brain gangliosides (18% GM_1, 55% GD_{1a}, 10% GT_{1b}, and 2% other gangliosides), hereafter designated as gangliosides, were obtained from Calbiochem. BoNT/A, B, and E were purified as described previously (Dasgupta et al., 1970; Evans et al., 1986; Schmidt and Siegel, 1986).

Antibodies
mAbs directed against syb II (69.1), syt I (α-syt I_N, 604.4; α-syt I_C, 41.1), α/β-SNAP (77.1), and SNAP-25 (71.2) were provided by R. Jahn and S. Engers (Max Planck Institute for Biophysical Chemistry, Gottingen, Germany). Rabbit pAbs directed against syt II were provided by M. Fukuda (RIKEN, Saitama, Japan; Fukuda and Mikoshiba, 2000).

Anti-BoNT/A, B, and E antibodies were generated by immunizing rabbits with formalin-treated purified neurotoxin; antibodies were affinity purified using immobilized neurotoxin.

cDNA and recombinant proteins
cDNA encoding rat syt I (Perin et al., 1990), mouse syt II and IX (Fukuda and Mikoshiba, 2000), and rat syt IV (Vician et al., 1995) were provided by T.C. Sudhof (University of Texas Southwestern Medical Center, Dallas, TX), M. Fukuda, and H. Herschman (University of California, Los Angeles, CA), respectively. Full-length syb II was generated as a GST fusion protein as described previously (Lewis et al., 2001) using a cDNA provided by R. Scheller (Stanford University, Stanford, CA).

To screen for toxin-binding activity, we generated truncated versions of syt I, II, IV, and IX that lacked the C2B domain, but contained all other domains. A number of additional constructs (truncations and chimeras, as indicated in the figures) were also generated by PCR, subcloned into pGEX-2T, and expressed and purified as described previously (Chapman et al., 1996; Lewis et al., 2001). Syt II 1–267 and 61–267 were also subcloned into pTrcHis and purified as NH_2-terminal tagged His_6 fusion proteins as described previously (Chapman et al., 1996).

Pull-down assays
Recombinant proteins were immobilized as GST fusion proteins bound to glutathione-Sepharose beads. Unless otherwise indicated, 10 μg immobilized protein was mixed with the indicated concentrations of BoNT/B, A, or E either with (+; 25 μg/ml) or without (−) gangliosides in 100 μl TBS (20 mM Tris and 150 mM NaCl, pH 7.4) plus 0.5% Triton X-100 for 1 h at 4°C. Beads were washed three times, bound proteins were solubilized by boiling in SDS sample buffer and subjected to SDS-PAGE, and were visualized by staining with Coomassie blue or by immunoblot analysis using anti-toxin antibodies. In all blots, the immunoreactivity for the toxin heavy chain is shown.

A peptide corresponding to residues 40–60 of mouse syt II, P21, and a scrambled version of this peptide, P21S (IKMNDAEFFGKSNFQEKLEKEC), were synthesized (Biotech Center, University of Wisconsin, Madison, Madison, WI) with an added COOH-terminal cys that was used to conjugate them to agarose beads (at 1 mg/ml) using a Sulfolink Kit (Pierce Chemical Co.). 50 μl of the conjugated agarose gel was used in the pull-down assays.

PC12 cell lines and immunoblot analysis
To generate cells that express syt II (syt II⁺), full-length mouse syt II was subcloned into pCDNA3.1(−) (CLONTECH Laboratories, Inc.) and trans-

fected into PC12 cells via electroporation. Transfected cells were selected with 1 mg/ml G418, and several independent monoclonal cell lines were established. Cells were harvested in PBS plus 0.5% Triton X-100, 0.05% SDS, and 5 mM PMSF, and were incubated for 30 min at 4°C on a shaker. Samples were centrifuged at 21,000 g for 10 min, and the concentration of protein in the supernatant was determined using bicinchoninic acid (Pierce Chemical Co.). Samples were subjected to SDS-PAGE and immunoblot analysis; blots were developed using ECL (Pierce Chemical Co.).

Entry of BoNTs into PC12 cells

In experiments that did not involve preloading, cells were grown to 70% confluence and incubated with BoNT for 48 h. For experiment in which cells were preloaded with gangliosides, cells were grown to 80% confluence followed by incubation in serum-free media plus 250 µg/ml gangliosides. 24 h later, the serum-free/ganglioside media was replaced with complete media, and the cells were incubated with toxin for 48 h. Cells were harvested and entry of CNTs was assayed via immunoblot analysis using antibodies directed against syb II or SNAP-25.

For blocking experiments, syt II 1–267 and syt II 61–267 were generated as His$_6$ fusion proteins; syt II 1–87 was generated as a GST fusion protein that was eluted from beads using 10 mM glutathione plus 0.5% Triton X-100. Protein fragments or peptides were premixed with BoNT/B in 200 µl TBS for 1 h at 4°C before adding into 2 ml cell culture media (per well in a 6-well plate). In some cases, gangliosides were also added in the binding buffer (Fig. 5 B, bottom). The final concentration of BoNT/B was 30 nM, the final concentration of gangliosides was 25 µg/ml, and the final [syt fragment] is indicated in the figure legends.

Binding of BoNT/B to PC12 cells

Cells treated with toxin, plus or minus preincubation with syt fragments, were washed three times with PBS, fixed with 4% PFA (15 min at RT), permeabilized with 0.1% Triton X-100 (for 10 min at RT), and stained with a rabbit anti-BoNT/B primary antibody and an FITC-conjugated goat anti–rabbit secondary antibody (Jackson ImmunoResearch Laboratories). In the syt II fragment competition assays described in Fig. 5, syt II 1–267 and 61–267 fragments form aggregates bound to cells (Bai et al., 2000); these were visualized using a mouse anti-His$_6$ primary antibody (QIAGEN) and a rhodamine-conjugated goat anti–mouse secondary antibody (Jackson ImmunoResearch Laboratories). The fluorescence images were obtained as described for the motor nerve terminal experiments. We note that for these experiments, free detergent was removed from the recombinant syt fragments by washing the immobilized proteins with detergent-free buffers before elution. However, in the case of syt II 1–87, low levels of Triton X-100 were needed to elute the protein from the beads; because of this, experiments using this fragment were performed within 6 h to avoid effects of detergent on the cells.

Antibody and toxin uptake experiments

Cells were treated with either control solution (15 mM Hepes, 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, 0.5 mM ascorbic acid, and 0.1% BSA, pH 7.4), or high [K$^+$] solution (same as control solution but adjusted to 95 mM NaCl and 56 mM KCl), for 10 min at 37°C, in the presence of BoNT/B plus 10 µl mAb against the luminal (α-syt I$_N$; clone 604.4) or cytoplasmic domain of syt I (α-syt 1$_c$; clone 41.1). Cells were washed with culture media, incubated for 30 min at 37°C, fixed, and permeabilized. A rabbit anti-BoNT/B primary antibody was used to stain BoNT/B; staining was visualized using an FITC-conjugated goat anti–rabbit secondary antibody. Rhodamine-conjugated goat anti–mouse secondary antibodies were used to visualize internalized syt I antibodies. Confocal images were collected with a confocal microscope (MRC 1000; Bio-Rad Laboratories; Keck Center for Biological Imaging, University of Wisconsin, Madison, Madison, WI) using a 100× oil immersion objective.

Co-immunoprecipitation

Recombinant syt I 1–265 GST was purified as described earlier in the Materials and methods section and was and cleaved from the GST tag using thrombin. 5 µl of mAb α-syt I$_N$ (604.4) was incubated with 300 nM BoNT/B, with or without 1.5 µM Syt I 1–265, in 100 µl TBS plus 0.5% Triton X-100 and 25 µg/ml gangliosides, for 1 h at 4°C. 30 µl Protein G fast flow beads (Amersham Biosciences) was added, samples were mixed for 1 h, beads were washed three times in binding buffer, and bound material was analyzed by SDS-PAGE and immunoblotting using an anti-BoNT/B pAb and α–syt I$_N$ (604.4).

Rat hemi-diaphragm experiments

Rat hemi-diaphragms were placed in an ice-cold ringer (in mM: NaCl 138.8, KCl 4, NaHCO$_3$ 12, KH$_2$PO$_4$ 1, MgCl$_2$ 2, CaCl$_2$ 2, and glucose 11),

gassed with 95% CO$_2$/5% O$_2$. Stimulation was performed with a similar solution where [KCl] was increased to 45 mM, and the NaCl appropriately reduced. Hemi-diaphrams were incubated with high potassium ringer containing 5 nM BoNT/B for 10 min at RT. In some experiments, the BoNT/B was premixed with either the syt II fragment 1–267, or fragment 61–267, both mixed with 25 µg/ml gangliosides. At the end of the stimulation/incubation period, the preparations were fixed (4% PFA), permeabilized (0.3% Triton X-100), and blocked in goat serum before immunolabeling with a rabbit anti-BoNT/B antibody and a monoclonal anti-Syb II antibody. Immunofluorescence was visualized using a FITC-conjugated anti–rabbit antibody, and a TRITC-conjugated anti–mouse antibody. A region of muscle adjacent to the site of nerve entry (where a large number of surface nerve terminals are to be found) was placed in a viewing chamber with a glass bottom comprising a single coverslip. Immunofluorescence images were obtained using a microscope (model TE300; Nikon), with a cooled CCD camera (MicroMAX; Princeton Instruments) controlled by MetaMorph® software (Universal Imaging Corp.). Fluorescence intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Neutralization of BoNT/B activity in vivo

For each batch of BoNT/B, the LD$_{50}$ value for mice (20–22 g; Institute of Cancer Research strain; ICR, Philadelphia, PA) was determined using standard methods (Schantz and Kautter, 1978). The LD$_{50}$ corresponds to the amount of toxin, introduced via i.p. injection, that results in 50% death after 4 d. Our preparations of BoNT/B had activities of ~10^8 LD$_{50}$/mg. For toxin neutralization experiments, we made use of the more rapid i.v. time-to-death assay (Boroff and Fleck, 1966). First, we generated a standard curve in which the relationship between time-to-death of mice injected, i.v., with 100 µl BoNT/B (expressed in min) is plotted versus the specific toxicity of BoNT/B that was determined using the standard method described earlier in the Materials and methods section (log [LD$_{50}$/ml]). Within the linear range, 10^4–10^6 LD$_{50}$/ml, this plot was used to convert experimentally determined time-to-death, from i.v. injection of relatively large doses of toxin, to LD$_{50}$/ml values. For the toxin neutralization experiments, BoNT/B was premixed with gangliosides alone (250 µg/ml) or gangliosides plus the indicated syt II fragments for 10 min at RT and then injected i.v. into mice. In all experiments, the total injection volume was always 100 µl. Neutralization of the toxin is indicated by an extension in the time-to-death of mice injected with toxin alone versus injection with toxin that had been pre-mixed wit syt fragments/gangliosides. The increase in the time-to-death was converted into a decrease in the apparent [LD$_{50}$/ml] using the standard curve, and the percentage of neutralization was calculated using the expression: $1- $ [LD$_{50}$/ml (+ syt II fragment)/LD$_{50}$/ml (− syt II fragment)] × 100, where (+ syt II fragment) refers to samples that contain toxin, gangliosides, and recombinant proteins, and (− syt II fragment) samples were composed of toxin and gangliosides only.

Online supplemental material

Fig. S1 shows the mapping of the BoNT/B-binding site within the luminal domain of syt I. Fig. S2 demonstrates the simultaneous and specific internalization of syt I luminal domain antibodies and BoNT/B into PC12 cells. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200305098/DC1.

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