Botulinum neurotoxin D-C uses synaptotagmin I and II as receptors, and human synaptotagmin II is not an effective receptor for type B, D-C and G toxins

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Accepted 27 February 2012
Journal of Cell Science 125, 3233–3242

doi: 10.1242/jcs.103564

Summary

Botulinum neurotoxins (BoNTs) are classified into seven types (A–G), but multiple subtype and mosaic toxins exist. These subtype and mosaic toxins share a high sequence identity, and presumably the same receptors and substrates with their parental toxins. Here, we report that a mosaic toxin, type D-C (BoNT/D-C), uses different receptors from its parental toxin BoNT/C. BoNT/D-C, but not BoNT/C, binds directly to the luminal domains of synaptic vesicle proteins synaptotagmin (Syt) I and II, and requires expression of SytI/II to enter neurons. The SytII luminal fragment containing the toxin-binding site can block the entry of BoNT/D-C into neurons and reduce its toxicity in vivo in mice. We also found that gangliosides increase binding of BoNT/D-C to SytI/II and enhance the ability of the SytII luminal fragment to block BoNT/D-C entry into neurons. These data establish SytI/II, in conjunction with gangliosides, as the receptors for BoNT/D-C, and indicate that BoNT/D-C is functionally distinct from BoNT/C. We further found that BoNT/D-C recognizes the same binding site on SytI/II where BoNT/B and G also bind, but utilizes a receptor-binding interface that is distinct from BoNT/B and G. Finally, we also report that human and chimpanzee SytII has diminished binding and function as the receptor for BoNT/B, D-C and G owing to a single residue change from rodent SytII within the toxin binding site, potentially reducing the potency of these BoNTs in humans and chimpanzees.

Key words: Botulinum neurotoxin, Botulinum neurotoxin B, Botulinum toxin, Botulism, Synaptotagmin

Introduction

Botulinum neurotoxins (BoNTs) are a family of protein toxins produced by several distinct anaerobic gram-positive Clostridia (Schiavo et al., 2000; Hill et al., 2007; Montal, 2010). They are the most potent toxins known and cause the disease botulism in humans and animals. BoNTs target neurons and cleave host proteins that are required for synaptic vesicle fusion; the resultant blockage of neurotransmitter release at the neuromuscular junction causes paralysis in animals and humans.

The general population is not immunized against BoNTs as botulism is a rare disease. Because of this lack of immunity and the extreme potency of BoNTs, these toxins are considered to be one of the most dangerous potential bioterrorism agents (Arnon et al., 2001). However, a lack of immunity in the population also enables the medical application of BoNTs in treating a growing list of conditions, ranging from muscle spasm to chronic pain (Dolly et al., 2009).

BoNTs are composed of a light chain (LC, ~50 kDa) and a heavy chain (HC, ~100 kDa), connected by a disulfide bond (Schiavo et al., 2000; Simpson, 2004; Montal, 2010). The heavy chain can be further divided into two functional domains, the C-terminal receptor binding domain (H_C) and the N-terminal membrane translocation domain (H_N). The HC mediates toxin entry into neurons through receptor-mediated endocytosis and subsequent translocation of the LC across endosomal membrane into the cytosol, whereas the LC functions as a protease to cleave target proteins (Schiavo et al., 2000; Simpson, 2004; Montal, 2010).

BoNTs are classified into seven types (BoNT/A–G), on the basis of their lack of cross-recognition by distinct polyclonal antisera. Functionally, members of BoNTs differ substantially in two ways: the specific cleavage of host target proteins by their light chains, and the recognition of receptors by their H_Cs. Since the seminal studies by G. Schiavo and colleagues in the early 1990s (Schiavo et al., 1992), the target specificity for all BoNT LCs has been well established. BoNT/B, D, F and G cleave different sites on a synaptic vesicle protein synaptobrevin (Syb); BoNT/A, C and E cleave different sites on a peripheral membrane protein SNAP-25; and BoNT/C also cleaves a plasma membrane protein syntaxin (Syx) (Schiavo et al., 2000). These three proteins, designated as SNARE proteins, form the minimal machinery that mediates synaptic vesicle exocytosis (Jahn and Scheller, 2006).

The H_C contains two sub-domains, the N-terminal domain (H_{CN}) and the C-terminal domain (H_{CC}). The H_{CN} has been suggested to facilitate the attachment of toxins to plasma membranes by binding to phosphatidylinositol phosphates

(Muraro et al., 2009). The H_{CC} contains the receptor-binding sites. It has been proposed that BoNTs recognize neurons by binding to two components in a 'double receptor' model (Montecucco, 1986). The first toxin-binding component identified was glycosphingolipid gangliosides (Simpson and Rapport, 1971). Recent studies suggest that gangliosides are a shared receptor component for all seven BoNTs (Swaminathan and Eswaramoorthy, 2000; Rummel et al., 2004a; Dong et al., 2007; Rummel et al., 2007; Stenmark et al., 2008; Fu et al., 2009; Rummel et al., 2009; Karalewitz et al., 2010; Strotmeier et al., 2010; Benson et al., 2011; Kroken et al., 2011; Nuemket et al., 2011; Peng et al., 2011; Strotmeier et al., 2011).

Recent studies have also revealed the additional protein receptors for BoNTs. Among the seven BoNTs, BoNT/B and G share the highest sequence identity within their H_{CC}s (50%, supplementary material Fig. S1A,B), and these two toxins share two homologous synaptic vesicle proteins synaptotagmin (Syt) I and II as their protein receptors (Nishiki et al., 1994; Nishiki et al., 1996; Dong et al., 2003; Rummel et al., 2004b; Dong et al., 2007; Rummel et al., 2007; Schmitt et al., 2010; Stenmark et al., 2010). The co-crystal structures of BoNT/B bound to SytII have revealed that BoNT/B recognizes the region of SytII that is adjacent to the membrane through a hydrophobic groove in the H_{CC} (Chai et al., 2006; Jin et al., 2006). The protein receptor for BoNT/A, E and D has also been identified as another synaptic vesicle protein, SV2 (Dong et al., 2006; Mahrhold et al., 2006; Dong et al., 2008; Peng et al., 2011). Although the molecular details of SV2-binding for each toxin remain unsolved, BoNT/A, E and D appear to utilize distinct binding mechanisms to recognize SV2. For instance, only BoNT/A, but not BoNT/E and D, can bind recombinant SV2 that is purified from *Escherichia coli*. Furthermore, abolishing a specific glycosylation site in SV2 disrupts its receptor function for BoNT/E and attenuates its function for BoNT/A, but has no effect on BoNT/D entry. In addition, BoNT/F is also reported to bind SV2 (Fu et al., 2009; Rummel et al., 2009), but it remains to be determined whether SV2 is a functional receptor for BoNT/F, because a lack of SV2 expression did not reduce the sensitivity of cultured hippocampal neurons to BoNT/F (Yeh et al., 2010; Peng et al., 2011).

Recent sequencing studies have expanded the scope of the seven BoNTs (Smith et al., 2005; Hill et al., 2007). First, there are multiple subtypes, which can be recognized by the same anti-serum, but contain substantial protein sequence differences (2.6%–31.6%) (Smith et al., 2005). Second, multiple mosaic toxins exist, which are likely to be derived from the recombination of toxin genes. The sequence differences between subtype and mosaic toxins with their parental BoNTs could be due to genetic drift, with the expectation that the substrate and receptor specificities are preserved. This should be true for most toxin variants, but could there be exceptional cases where the 'drift' leads to changes of substrates and/or receptors from the parental toxins?

Among the known subtype and mosaic toxins, BoNT/D-C (also known as BoNT/D-SA, D/5995 and D/4947) caught our attention (Moriishi et al., 1996; Karalewitz et al., 2010; Nuemket et al., 2011). BoNT/D-C is considered to be a mosaic toxin composed of the LC-H_N of BoNT/D, with an H_C derived from BoNT/C (Fig. 1A). However, mice immunized using the H_C of BoNT/C (C/H_C) are fully protected against BoNT/C, but are only partially protected from BoNT/D-C (Webb et al., 2007), suggesting that the H_Cs between BoNT/D-C and BoNT/C are substantially different.

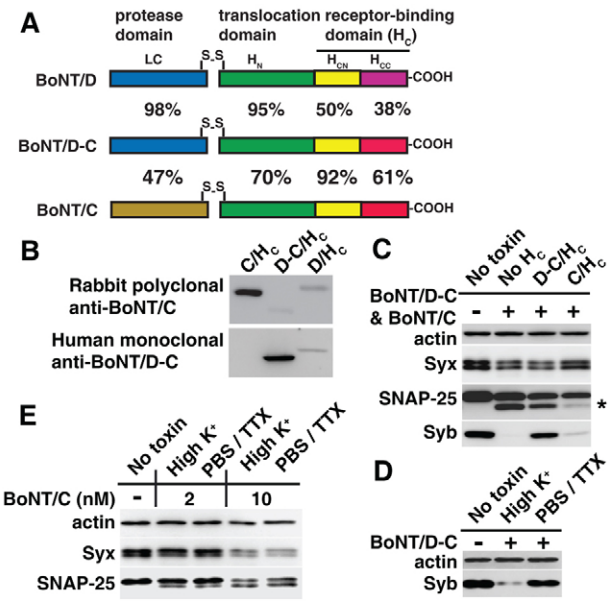


Fig. 1. BoNT/D-C does not share the same receptor with BoNT/C. (**A**) Schematic diagrams showing the protein sequence identity between each functional domain of BoNT/D-C with the corresponding regions in its parental toxins, BoNT/D and BoNT/C. (**B**) Recombinant C/H_C, $D\text{-}C/H_C$, and D/H_C proteins (150 ng) were subjected to immunoblot analysis, using either a rabbit polyclonal BoNT/C antibody generated using the full-length BoNT/C (upper panel), or a human monoclonal BoNT/D-C antibody (clone 8DC2) raised against the full-length BoNT/D-C (lower panel). The polyclonal BoNT/C antibody recognized C/H_C, but not $D\text{-}C/H_C$ and D/H_C. The monoclonal BoNT/D-C antibody recognized $D\text{-}C/H_C$, but not C/H_C and D/H_C. (**C**) Cultured rat hippocampal neurons were exposed to BoNT/C (10 nM) and BoNT/D-C (0.3 nM) simultaneously for 5 minutes in high K^+ buffer, with or without (No H_C) the presence of 1 μM of recombinant $D\text{-}C/H_C$ or C/H_C. Cells were washed and further incubated in toxin-free media for 6 hours. Cell lysates were subjected to immunoblot analysis using antibodies against the proteins that are indicated. Cells that were not exposed to toxins served as a control (No toxin). Actin serves as an internal loading control. Cleavage of Syb by BoNT/D-C results in the loss of its immunoblot signals. BoNT/C cleaves both Syx and SNAP-25. Cleavage of Syx by BoNT/C results in the loss of its immunoblot signals, whereas cleavage of SNAP-25 by BoNT/C generates a smaller fragment indicated by an asterisk. $D\text{-}C/H_C$ reduced the cleavage of Syb by BoNT/D-C, but it did not affect Syx and SNAP-25 cleavage by BoNT/C. Similarly, C/H_C reduced entry of BoNT/C, but had no effect on BoNT/D-C entry. (**D**) Neurons were exposed to BoNT/D-C (0.3 nM) for 5 minutes in conditions that either stimulate synaptic vesicle exocytosis (High K^+) or reduce synaptic vesicle exocytosis (PBS plus TTX). Using high K^+ buffer increased cleavage of Syb. Unless indicated in the figure, high K^+ buffer was used to load toxins into neurons in all toxin-loading experiments. (**E**) Experiments were carried out as described in panel D, except that neurons were exposed to the concentrations of BoNT/C that are indicated. Similar levels of Syx and SNAP-25 cleavage were observed in high K^+ buffer and in PBS plus TTX conditions.

Here, we report that BoNT/D-C does not share the same entry pathway and receptors with its parental toxin BoNT/C. Instead, BoNT/D-C utilizes SytI/II as its protein receptor. It recognizes the same region on SytI/II where BoNT/B and G bind, but through a new receptor-binding site within its H_{CC}. These studies establish BoNT/D-C as a unique toxin in the BoNT family. We also report that human and chimpanzee SytII has diminished binding to BoNT/D-C, B and G, owing to a single residue change within the

toxin-binding site from rodent SytII, leaving SytI as the only high-affinity receptor for BoNT/D-C, B and G in humans and chimpanzees.

Results

BoNT/D-C and BoNT/C recognize distinct receptors

We first compared the sequence of BoNT/D-C with its parental toxins BoNT/D and BoNT/C (Fig. 1A). The LC and H_N sequences of BoNT/D-C are almost identical to BoNT/D (98% and 95% identity, Fig. 1A). The sequence of the H_{CN} sub-domain of BoNT/D-C is 92% identical to that of BoNT/C (Fig. 1A), suggesting that the receptor-binding domains of these two toxins are indeed related. However, their H_{CC} sub-domains share a considerably lower sequence identity (61%, Fig. 1A). Next, we purified the recombinant C/H_C, as well as the H_Cs of BoNT/D (D/H_C) and BoNT/D-C ($D\text{-}C/H_C$) (supplementary material Fig. S2). We found that a polyclonal antibody that recognizes C/H_C did not recognize $D\text{-}C/H_C$, and a monoclonal antibody that recognizes $D\text{-}C/H_C$ failed to detect C/H_C (Fig. 1B). These data indicate that BoNT/D-C and BoNT/C differ substantially in their receptor-binding domains.

Furthermore, we found that excessive amounts of $D\text{-}C/H_C$ diminished entry of BoNT/D-C into cultured hippocampal neurons and reduced cleavage of Syb by BoNT/D-C, but it did not reduce cleavage of Syx and SNAP-25 by BoNT/C (Fig. 1C). Similarly, C/H_C inhibited BoNT/C entry and protected Syx and SNAP-25, but it failed to reduce cleavage of Syb by BoNT/D-C (Fig. 1C). This lack of cross-competition suggests that $D\text{-}C/H_C$ and C/H_C recognize distinct receptors.

We further examined whether BoNT/D-C and BoNT/C share the same entry pathway into neurons. Synaptic vesicle recycling has been utilized by several BoNTs as their main cell entry pathway. We found that stimulating synaptic vesicle exocytosis with high levels of potassium solution (high K^+ buffer) increased the cleavage of Syb by BoNT/D-C compared with conditions when synaptic vesicle exocytosis was inhibited (PBS plus TTX, Fig. 1D), indicating that synaptic vesicle recycling also mediates BoNT/D-C entry into neurons. By contrast, cleavage of Syx and SNAP-25 by BoNT/C were at similar levels under these conditions (Fig. 1E), suggesting that synaptic vesicle recycling is not essential for BoNT/C entry into hippocampal neurons.

The receptor-binding domain of BoNT/D-C binds directly to SytI/II

Because BoNT/D-C enters neurons through synaptic vesicle recycling, we next used $D\text{-}C/H_C$ fused with glutathione *S*-transferase (GST) to pull down interacting synaptic vesicle proteins from rat brain detergent (Triton X-100) extracts. Bound materials were analyzed by western blot, detecting major synaptic vesicle membrane proteins, including SV2, SytI/II, synaptophysin (Syp) and synaptogyrin I (Syg) (Fig. 2A). C/H_C and the H_C of BoNT/B (B/H_C) were assayed in parallel as controls (Fig. 2A). C/H_C did not pull down any major vesicle proteins, consistent with the finding that synaptic vesicle recycling is not essential for BoNT/C entry into neurons (Fig. 1E). As expected, B/H_C pulled down SytI and SytII (Fig. 2A). We found that $D\text{-}C/H_C$ also pulled down substantial amounts of SytI and SytII (Fig. 2A). $D\text{-}C/H_C$ pulled down less SytI/II than B/H_C (Fig. 2A), suggesting that $D\text{-}C/H_C$ has a lower binding affinity to SytI/II than B/H_C.

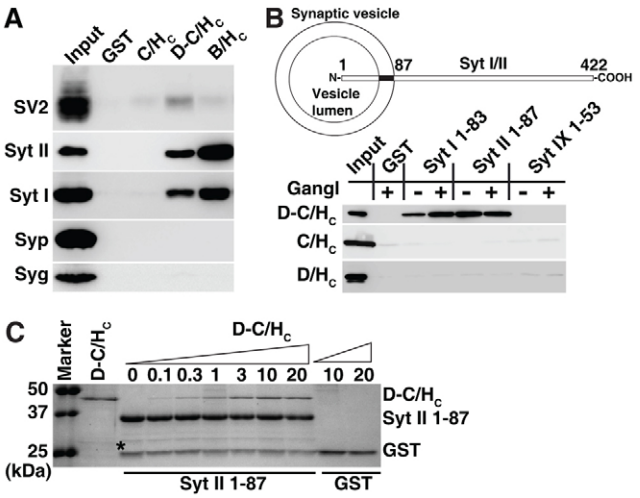


Fig. 2. $D\text{-}C/H_C$ binds directly to the luminal domains of SytI and II. (**A**) Recombinant $D\text{-}C/H_C$, C/H_C and B/H_C were purified as GST fusion proteins and incubated with detergent (Triton X-100) extracts from rat brain. GST protein serves as a control. Pellets were analyzed by immunoblot. (**B**) Upper panel: schematic diagram showing the topology of SytI/II on vesicles. Lower panel: truncations of SytI, II and IX containing the luminal domain and the transmembrane domain were used to pull down soluble $D\text{-}C/H_C$, C/H_C or D/H_C, with (+) or without (−) gangliosides (Gangl). Bound materials were analyzed by immunoblot. (**C**) Pull-down assays were carried out as described in panel B, with indicated concentrations (μM) of $D\text{-}C/H_C$. Bound materials were subjected to SDS-PAGE and visualized by staining with Coomassie blue. The asterisk indicates a degradation band from GST-SytII 1–87. Binding of $D\text{-}C/H_C$ to SytII 1–87 is saturated at high concentrations, suggesting a stoichiometric interaction.

SytI/II are two highly homologous proteins, which localize to vesicles through a single transmembrane domain (Fig. 2B). Their N-terminal luminal domains are the only regions that are exposed to the outside of cells when vesicles fuse to the plasma membrane. Therefore, we constructed and purified SytI/II truncations that contained only the luminal domain plus the transmembrane domain (residues 1–83 in SytI and 1–87 in SytII) as GST fusion proteins and assayed their ability to bind $D\text{-}C/H_C$. A similar region in Syt IX (residues 1–53), a member of the Syt family, as well as GST protein, were assayed in parallel as controls. Including the transmembrane domain enables us to test the effect of lipid co-receptors, gangliosides, which form micelles around the hydrophobic transmembrane domain. These GST fusion proteins were incubated with purified soluble $D\text{-}C/H_C$, C/H_C or D/H_C, with or without gangliosides. We found that $D\text{-}C/H_C$ binds directly to both SytI and SytII (Fig. 2B). $D\text{-}C/H_C$ did not bind Syt IX, neither did C/H_C and D/H_C bind SytI/II (Fig. 2B), demonstrating the specificity of $D\text{-}C/H_C$–SytI/II interactions. Furthermore, binding of $D\text{-}C/H_C$ to SytII 1-87 can be visualized by Coomassie blue staining of SDS-PAGE gels (Fig. 2C). We also found that $D\text{-}C/H_C$ has substantially higher apparent dissociation constants for SytII than BoNT/B under similar assay conditions (330±56 nM versus 8.6±1.2 nM, supplementary material Fig. S3) (Chai et al., 2006), which might explain why $D\text{-}C/H_C$ pulled down less SytI/II than B/H_C (Fig. 2A). The presence of gangliosides enhanced binding of $D\text{-}C/H_C$ to SytI (Fig. 2B), and reduced the apparent dissociation constants for SytII by ~2-fold (supplementary material Fig. S3), indicating that gangliosides facilitate $D\text{-}C/H_C$–SytI/II interactions.

In addition to SytI/II, we also observed weak but appreciable binding of SV2 to D-C/H_C (Fig. 2A). However, we did not detect binding of D-C/H_C to SV2 under current pulldown assay conditions (supplementary material Fig. S4A). Furthermore, lacking SV2 expression in SV2-knockout hippocampal neurons did not reduce entry of BoNT/D-C (supplementary material Fig. S4B). Although it remains to be determined whether SV2 can facilitate Syt-BoNT/D-C binding in a way that can be compensated by other players in SV2-knockout neurons, it is clear that SV2 is not required for BoNT/D-C to enter hippocampal neurons.

SytI/II are required for BoNT/D-C entry into neurons

We next examined whether expression of SytI/II in neurons correlates with the ability of BoNT/D-C to enter neurons. Hippocampal neurons mainly express SytI, but not SytII (Dong et al., 2007), therefore, we tested whether knocking down SytI in hippocampal neurons affects BoNT/D-C entry. Efficient knockdown (KD) of SytI was achieved using an shRNA construct delivered by lentiviral transduction (Fig. 3A). We first characterized SytI-KD neurons using BoNT/A and BoNT/B as controls (Fig. 3B–E). BoNT/A utilizes SV2 as its receptor, and the binding and entry of BoNT/A into neurons was not affected by SytI KD (Fig. 3B,D). These results demonstrate that SytI KD does not substantially affect the synaptic vesicle recycling process. BoNT/B uses SytI/II as its receptors. Binding and entry of BoNT/B was abolished in SytI-KD neurons, and was restored by expression of KD-resistant SytI (Fig. 3C,E). Together, these data validated the efficacy and specificity of our SytI-KD approach.

We found that BoNT/C entry into neurons was not affected by SytI KD because cleavage of Syx and SNAP-25 was at similar levels in SytI-KD neurons and in control neurons (Fig. 3F,G). By contrast, BoNT/D-C failed to enter SytI-KD neurons, as

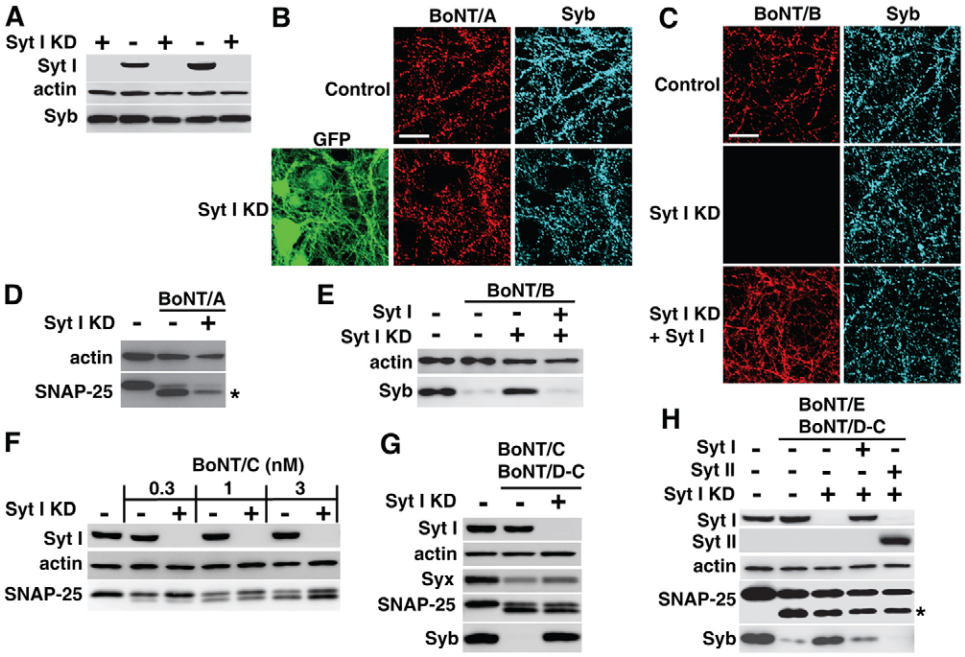


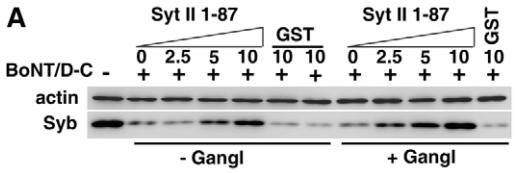
Fig. 3. Expression of SytI/II is required for BoNT/D-C entry into neurons. (**A**) Cultured rat hippocampal neurons were infected with (+) or without (−) SytI KD lentiviruses. Cells were harvested 7 days later and cell lysates were subjected to immunoblot analysis. SytI expression was greatly reduced in SytI KD neurons, demonstrating a high KD efficiency. Expression of Syb was not affected in SytI KD neurons, indicating the specificity of SytI KD. Actin serves as a loading control. (**B**) Control and SytI KD neurons were exposed to BoNT/A (20 nM) for 5 minutes in high K^+ buffer. Cells were washed, fixed and subjected to immunostaining analysis detecting BoNT/A and Syb. GFP was co-expressed with SytI KD shRNA to label infected neurons. The infection efficiency of lentiviruses is close to 100%. Syb serves as a marker for nerve terminals. SytI KD did not affect binding and entry of BoNT/A into nerve terminals. (**C**) Control neurons, SytI KD neurons and KD rescue neurons with the expression of KD resistant SytI were exposed to BoNT/B (20 nM) for 5 minutes in high K^+ buffer. Cells were washed, fixed and subjected to immunostaining analysis. Binding and entry of BoNT/B into nerve terminals was abolished in SytI KD neurons, and was restored by KD resistant SytI. (**D**) Similar levels of SNAP-25 cleavage by BoNT/A (10 nM, 5 minutes exposure, 6 hours incubation) were observed between SytI KD (+) and control neurons (−). Cleavage of SNAP-25 by BoNT/A generates a smaller fragment, indicated by an asterisk. (**E**) Control neurons, SytI KD neurons and KD rescue neurons with the expression of KD resistant SytI were exposed to BoNT/B (20 nM, 5 minutes exposure, 24 hours incubation). Cleavage of Syb was detected through immunoblot analysis. Entry of BoNT/B was blocked in SytI KD neurons and was restored by KD resistant SytI, as evidenced by Syb cleavage. (**F**) SytI KD (+) and control neurons (−) were exposed to indicated concentrations of BoNT/C (5 minutes exposure, 24 hours incubation). Cleavage of SNAP-25 by BoNT/C is similar at all toxin concentrations between control and SytI KD neurons, indicating that SytI is not required for BoNT/C entry into neurons. (**G**) Neurons infected with (+) or without (−) lentiviruses expressing SytI KD shRNA were exposed to BoNT/C (3 nM) and BoNT/D-C (0.3 nM) simultaneously for 5 minutes. Cells were washed and further incubated in toxin-free media for 24 hours. SytI KD blocked entry of BoNT/D-C, but did not affect BoNT/C entry. (**H**) Rescue experiments were carried out for SytI KD neurons, using either KD-resistant SytI or SytII. Neurons were exposed to BoNT/D-C (0.3 nM) and BoNT/E (0.3 nM) simultaneously for 5 minutes. Cells were washed and incubated further for 6 hours. Entry of BoNT/D-C into SytI KD neurons was restored by KD resistant SytI and SytII. Cleavage of SNAP-25 by BoNT/E, which generates a smaller fragment (indicated by an asterisk) is not affected by the expression levels of SytI/II, and serves as an internal control.

evidenced by the lack of Syb cleavage (Fig. 3G). Furthermore, entry of BoNT/D-C was rescued by expression of either KD-resistant SytI or SytII (Fig. 3H). Entry of BoNT/E, which uses SV2 as its receptor, was not affected by either SytI KD or expression of SytII (Fig. 3H), further demonstrating the specificity of SytI/II expression for mediating entry of BoNT/D-C into neurons.

The luminal domain of SytII can block BoNT/D-C entry into neurons in vitro and reduce its toxicity in vivo

We next tested whether the luminal domain of SytII, which binds BoNT/D-C better than that of SytI (Fig. 2B), can compete with BoNT/D-C for binding and entry into neurons in vitro and in vivo. We pre-incubated soluble GST-tagged SytII 1–87 with BoNT/D-C, with and without gangliosides, before exposing the mixtures to cultured hippocampal neurons. Soluble GST protein was assayed in parallel as a control. As shown in Fig. 4A, SytII 1–87 reduced BoNT/D-C entry into neurons in a dose-dependent manner. Gangliosides enhanced the ability of SytII 1–87 to block BoNT/D-C entry, as similar degrees of Syb protection were achieved at lower concentrations of SytII 1–87 in the presence of gangliosides (Fig. 4A). Gangliosides alone, GST alone or the combination of GST and gangliosides did not reduce the entry of BoNT/D-C into neurons (Fig. 4A).

We then carried out a rapid time-to-death assay to evaluate whether SytII 1–87 can reduce the toxicity of BoNT/D-C in vivo. This assay was conducted by injecting high levels of toxin into mice, which results in death within 30 minutes to 1 hour. Within this range of toxin concentrations, the in vivo toxicity can be estimated on the basis of survival time by using a standard curve (Boroff and Fleck, 1966; Dong et al., 2003). A longer time-to-death corresponds to lower effective toxicity in vivo and would suggest that part of the toxin has been neutralized. We pre-mixed either soluble GST or GST-tagged SytII 1–87 with the same amount of BoNT/D-C, with or without gangliosides for 30 minutes. The mixtures were then injected into mice. There were four mice in each group and their time-to-death values are listed in Fig. 4B. GST–SytII 1–87 reduced the effective toxicity of BoNT/D-C to 7.8×10^5 LD_{50}/ml, a 22% reduction when compared with GST (1×10^6 LD_{50}/ml, Fig. 4B). The addition of gangliosides further enhanced the ability of GST–SytII 1–87 to neutralize BoNT/D-C in vivo (4.4×10^5 LD_{50}/ml, a 54% reduction, Fig. 4B), whereas adding gangliosides to GST proteins did not substantially change the toxicity of BoNT/D-C (9.5×10^5 LD_{50}/ml, Fig. 4B). Together, these data demonstrate that SytI/II are the functionally and physiologically relevant protein receptors for BoNT/D-C.



B

BoNT/D-C pre-mix with:	Time-to-death (min)	Effective toxicity (LD_{50}/ml)
GST	30 28 29 30	1.0×10^6
GST-Syt II 1-87	33 35 31 35	7.8×10^5
GST + gangl	31 31 30 32	9.5×10^5
GST-Syt II 1-87 + gangl	44 41 37 37	4.4×10^5

Fig. 4. The luminal domain of SytII can inhibit BoNT/D-C entry into neurons in vitro and reduce the toxicity of BoNT/D-C in vivo. (**A**) BoNT/D-C (0.3 nM) was pre-incubated for 30 minutes with indicated concentrations (μM) of soluble GST-tagged SytII 1–87 or soluble GST protein, with (−) or without (+) gangliosides, in high K^+ buffers. Neurons were then exposed to the mixtures for 5 minutes, washed and incubated further in toxin-free media for 6 hours. SytII 1–87 inhibited entry of BoNT/D-C into neurons in a dose-dependent manner. Gangliosides enhanced the ability of SytII 1–87 to inhibit BoNT/D-C entry. (**B**) The same amount of BoNT/D-C was pre-mixed with either soluble GST or GST–SytII 1–87 proteins, with or without gangliosides for 30 minutes at 4℃. The mixtures were injected into mice and time-to-death of each mouse is listed. The effective toxicity of the mixture in vivo is estimated from an established standard curve reflecting the linear relationship between the time-to-death and the log value of the toxicity (in LD_{50}/ml units) (Boroff and Fleck, 1966; Dong et al., 2003).

BoNT/D-C shares the same binding site on SytI/II with BoNT/B

We then mapped the binding site on SytII for BoNT/D-C by testing a series of deletion mutants within the luminal domain of SytII (Fig. 5A). We found that D-C/H_C binds to SytII 1–61 just as well as it binds to SytII 1–87 (Fig. 5A, middle panel), confirming that the transmembrane domain is not required for toxin binding. Deleting the first 40 residues (SytII 40–267) did not affect D-C/H_C binding, although further deletion to residue 47 (SytII 47–267) reduced toxin binding (Fig. 5A, lower panel). Therefore, the binding site for BoNT/D-C is located within residues 40–61 on SytII.

Residues 40–61 on SytII also compose the binding site for BoNT/B (Dong et al., 2003). Consistently, we found that D-C/H_C, but not C/H_C, was able to reduce BoNT/B binding to SytII in a dose-dependent manner (Fig. 5B). Excessive amounts of D-C/H_C also blocked BoNT/B binding and entry into hippocampal neurons (Fig. 5C). D-C/H_C and BoNT/B have distinct binding preferences for different complex gangliosides (Karalewitz et al., 2010). It is likely that D-C/H_C competes with BoNT/B for binding to the protein receptor in neurons. Furthermore, binding and entry of BoNT/A to neurons was not affected by D-C/H_C, neither did C/H_C affect BoNT/B binding and entry (Fig. 5C), demonstrating the specificity of the competition between D-C/H_C and BoNT/B in neurons.

Defining the binding interface between BoNT/D-C and SytII through mutagenesis studies

Co-crystal structures of BoNT/B–SytII have revealed that the toxin-binding site of SytII forms an amphiphatic helix with its hydrophobic side binding to BoNT/B (Chai et al., 2006; Jin et al., 2006). To determine whether BoNT/D-C recognizes the same binding interface on SytII, we mutated key hydrophobic residues in the SytII helix that contribute to BoNT/B binding. As shown in Fig. 6A, the F54A mutation completely abolished the binding of both D-C/H_C and BoNT/B in pull-down assays. Mutations F47A and F55A, respectively, also substantially reduced the binding affinity of both toxins to SytII, as their binding becomes more reliant on gangliosides (Fig. 6A).

We further examined whether mutations within the toxin-binding site of SytI/II have equal effects for BoNT/D-C and BoNT/B in neurons. We tested two SytI mutants (SytI mut.: F46A, M47A, E49K and SytI del.: Δ39–49 of SytI) previously known to disrupt the binding and entry of BoNT/B into neurons (Dong et al., 2007), as well as a SytII mutant (F54A) (Fig. 6A). These three

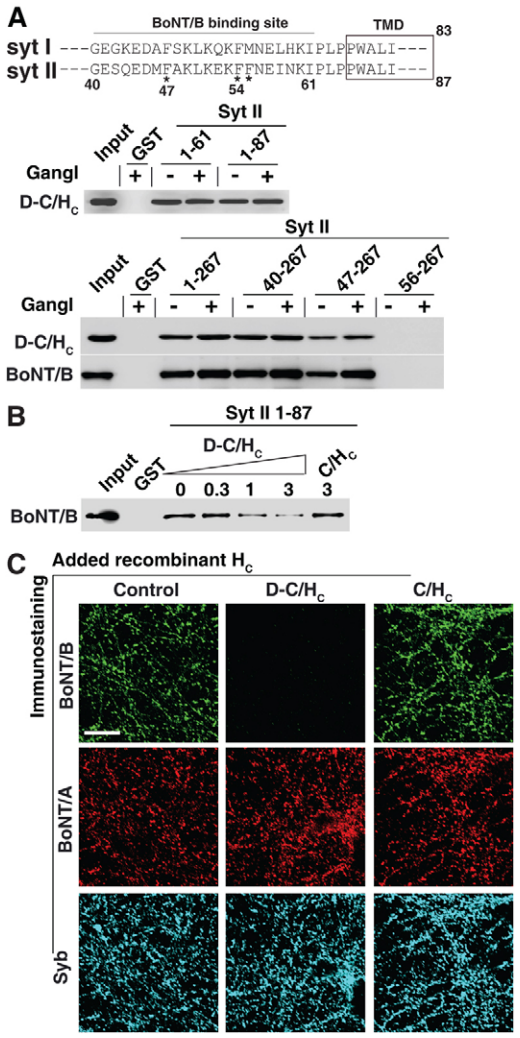


Fig. 5. BoNT/D-C shares the same binding site on SytII with BoNT/B. (**A**) Upper panel: sequence alignment of the membrane adjacent regions and (partial) transmembrane domains (TMD) of SytI (rat) and SytII (mouse). The BoNT/B-binding site is labeled. Pull-down assays were carried out as described in Fig. 2B, using either immobilized SytII 1–61 and 1–87 (middle panel), or the SytII luminal domain truncations indicated (lower panel). To facilitate protein purification, all SytII luminal domain truncations end at residue 267. (**B**) Immobilized SytII 1–87 was used to pull down BoNT/B (100 nM), in the presence of indicated concentrations (μM) of D-C/H_C or C/H_C. (**C**) Neurons were exposed to BoNT/B (20 nM) and BoNT/A (20 nM) simultaneously, with the presence of 3 μM D-C/H_C or C/H_C for 5 minutes. Control cells were exposed to BoNT/B and A without H_Cs. Cells were washed and fixed. Binding and entry of BoNT/B and BoNT/A into nerve terminals were detected using their specific antibodies through immunostaining. Syb was detected as a marker for nerve terminals.

mutants were all localized to nerve terminals and were capable of taking up antibodies that recognized their luminal domains upon synaptic vesicle exocytosis (supplementary material Fig. S5). Therefore, these mutants were localized correctly, with their luminal domains that were exposed to the outside of neurons during vesicle recycling, yet all three mutants failed to mediate entry of either BoNT/D-C or BoNT/B into neurons (Fig. 6B). As a control, deleting residues outside the toxin-binding site, such as residues 63–65 of SytII, did not affect the ability of SytII to mediate the entry of BoNT/B and BoNT/D-C (Fig. 6B). Together, these data suggest that BoNT/D-C shares a similar binding interface on SytII with BoNT/B and recognizes the hydrophobic side of the SytII helix.

To gain further insight into how BoNT/D-C, but not the closely related BoNT/C, evolved to bind SytII, we compared the crystal structures of D-C/H_C, C/H_C, and the BoNT/B–SytII complex, and made a structure-based sequence alignment (Fig. 6C; supplementary material Fig. S6). There are substantial structural differences in the area where SytII binds BoNT/B in these three toxins (Fig. 6C). In particular, the hydrophobic groove in BoNT/B where SytII binds is not conserved in BoNT/D-C, suggesting that BoNT/D-C uses a binding interface that is distinct from BoNT/B (Fig. 6C, upper panel).

On the basis of assumptions that hydrophobic interactions are important for binding SytII (Fig. 6A,B) and that the receptor-binding site is located within the H_{CC}, we identified a putative Syt-binding site in BoNT/D-C (Fig. 6D, magenta ellipse). This potential Syt-binding site partially overlaps with the area analogous to the Syt-binding site in BoNT/B (Fig. 6D, black ellipse), but extends to a hydrophobic patch centered on residue L1226 (Fig. 6D, magenta ellipse). This hydrophobic patch does not exist in either BoNT/C or BoNT/B (Fig. 6C,D).

To experimentally test whether this putative binding site is involved in Syt binding, we carried out targeted mutagenesis by replacing residues in BoNT/D-C with aligned residues in BoNT/C (Fig. 6E; supplementary material Fig. S6). In addition, we also examined a double mutation P1182S, S1183Y, in which case the mutated residues were chosen to disrupt potential protein interactions. These mutant D-C/H_Cs were purified as GST-tagged proteins to pull down SytI/II from rat brain detergent extracts. We found that mutations Y1180K, I1264Q and P1182S/S1183Y reduced the binding of both SytI and SytII (Fig. 6E). Mutations L1196R and L1226K, however, selectively diminished binding of SytII, without substantially affecting SytI binding (Fig. 6E). The finding that L1196R and L1226K retained SytI binding demonstrates that these mutant proteins are properly folded. Therefore, their loss of SytII binding strongly suggests that L1196R and L1226K are in direct contact with SytII. Together, these data suggest that BoNT/D-C utilizes a novel Syt binding site (Fig. 6D, magenta ellipse), which is oriented at a different angle compared with the established Syt binding site in BoNT/B.

Human SytII is not an effective receptor for BoNT/D-C, B and G

Our finding that SytII (F54A) loses the ability to mediate the entry of BoNT/D-C and B (Fig. 6B) confirmed that F54 is a key residue for toxin binding (Chai et al., 2006; Jin et al., 2006). Sequence alignments have revealed that this residue is highly conserved in both SytI and SytII across vertebrates, including platypus, fish, rodents and monkeys (Craxton, 2010). Interestingly, human and chimpanzee SytII are unique in that they contain an L residue at this position (Craxton, 2010), which is the only difference between human and mouse SytII within the toxin-binding site (residue 51 in human SytII, Fig. 7A). Because BoNT/D-C is commonly associated with botulism in animals (Nakamura et al., 2010), but has not been found in human cases (Lindström and Korkeala, 2006), we examined whether this residue change in human SytII reduces the potency of BoNT/D-C

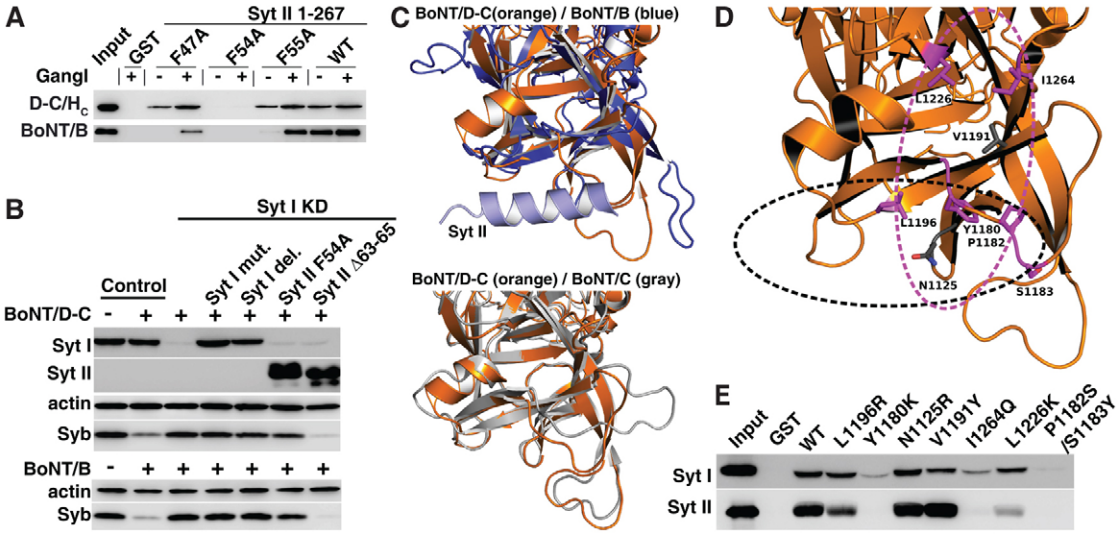


Fig. 6. Defining the binding interface between BoNT/D-C and SytI/II through targeted mutagenesis. (**A**) Pull-down assays were carried out as described in Fig. 2B, using the indicated SytII point mutations to pull down D-C/H_C or BoNT/B. (**B**) The following SytI/II mutants were expressed in SytI KD neurons through lentiviral transduction: SytI mutation (SytI mut.: F46A, M47A, E49K), SytI deletion (SytI del.: Δ39–49 of rat SytI), SytII mutation (F54A of mouse SytII), and SytII deletion (Δ63–65 of mouse SytII). SytI mutants were based on a KD-resistant SytI sequence. Neurons were then exposed to either BoNT/D-C (0.3 nM) or BoNT/B (20 nM) for 5 minutes. Cells were washed and further incubated for either 6 hours (BoNT/D-C, upper panel) or 24 hours (BoNT/B, lower panel). Cell lysates were analyzed by immunoblot. (**C**) Upper panel: superimposed structures of D-C/H_{CC} (orange) versus the BoNT/B (blue)–SytII (light blue helix) complex. Lower panel: comparison of D-C/H_{CC} (orange) versus C/H_{CC} (gray). (**D**) Targeted mutagenesis sites in D-C/H_{CC} are marked as sticks (magenta, loss of Syt binding; black, no binding difference, see panel E). The black ellipse highlights the region corresponding to where SytI/II bind BoNT/B. The magenta ellipse indicates the proposed SytI/II binding site in BoNT/D-C. (**E**) Wild type (WT) and indicated D-C/H_C mutants were purified as GST fusion proteins and incubated with detergent extracts from rat brain. Bound materials were subjected to immunoblot analysis detecting SytI and SytII.

in humans. We first created a mouse SytII 1–87 F54L mutant that mimics human SytII, and examined its ability to bind BoNT/D-C in pull-down assays. BoNT/B and G were also assayed as controls. BoNT/B is a major toxin associated with human botulism, thus, we had expected it to bind human SytII considering that F to L is a rather conservative change. To our surprise, SytII F54L has greatly diminished binding not only to BoNT/D-C, but also failed to bind BoNT/B and G in pull-down assays (Fig. 7A). Furthermore, full-length SytII F54L, when expressed through lentiviral transduction in SytI KD neurons, only mediated low levels of BoNT/B binding to neurons as compared with WT SytII, and was substantially less efficient in mediating the entry of BoNT/D-C, BoNT/B and BoNT/G into SytI KD neurons compared with wild type mouse SytII (Fig. 7C).

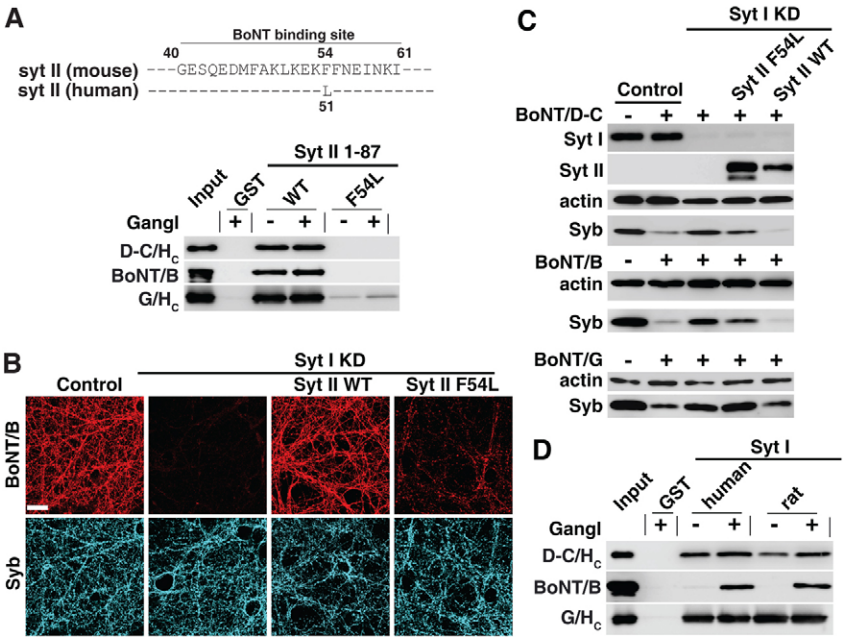


Fig. 7. Human SytII is not an effective receptor for BoNT/D-C, B and G. (**A**) Upper panel: human SytII differs from mouse SytII by one residue within the toxin-binding site (residue 54 in mouse SytII, 51 in human SytII). Lower panel: immobilized mouse SytII 1–87 and a mutant SytII 1–87 mimicking human SytII (F54L) were used to pull down D-C/H_C, BoNT/B or G/H_C. (**B**) Full-length mouse SytII WT and SytII (F54L) were expressed in SytI KD neurons. Binding and entry of BoNT/B into neurons was detected through immunostaining as described in Fig. 5C. (**C**) Full-length mouse SytII WT and SytII (F54L) were expressed in SytI KD neurons. Neurons were exposed to BoNT/D-C (0.3 nM, 5 minutes exposure, 6 hours incubation), BoNT/B (20 nM, 5 minutes exposure, 24 hours incubation) or BoNT/G (40 nM, 5 minutes exposure, 24 hours incubation). SytII (F54L) is less efficient than WT SytII in mediating entry of these three toxins. (**D**) Rat SytI 1-83 and human SytI 1–80 were purified as GST fusion proteins and used to pull down soluble D-C/H_C, BoNT/B and G/H_C. For all three toxins, human SytI mediated similar levels of toxin binding to rat SytI.

Thus, human SytII is not an effective toxin receptor for all three BoNTs in neurons.

Next, we examined human SytI, which also has a single residue change from rat SytI (Q44E), but this position is located on the outside of the Syt helix away from the toxin binding interface (Chai et al., 2006; Jin et al., 2006). As expected, the luminal domain of human SytI pulled down all three toxins just as well as rat SytI (Fig. 7D). Therefore, the effective protein receptor for BoNT/B, D-C and G is restricted to SytI in humans and chimpanzees.

Discussion

Sequencing studies have revealed multiple subtype and mosaic BoNTs. Presumably, subtype and mosaic toxins share the same receptors and substrates with their parental toxins. This assumption is challenged by our findings that a mosaic toxin, BoNT/D-C, does not share a protein receptor with BoNT/C, the parental toxin of its receptor-binding domain. We found that BoNT/D-C and BoNT/C enter neurons through distinct entry pathways. BoNT/D-C binds directly to SytI/II, and expression of SytI or SytII in neurons is required for functional entry of BoNT/D-C. Furthermore, the recombinant luminal domain of SytII inhibited BoNT/D-C entry into neurons and reduced the toxicity of BoNT/D-C in vivo in mice. These data established SytI/II as the protein receptors for BoNT/D-C. By contrast, BoNT/C does not rely on synaptic vesicle recycling to enter neurons, it does not bind SytI/II, and its entry is not affected in SytI-KD neurons. These findings expand our current understanding of the diversity of BoNTs and indicate the importance of characterizing the receptors and substrates for major BoNT variants in addition to their serological properties and apparent sequence differences.

Mapping the binding site for BoNT/D-C to residues 40–61 of SytII is also surprising, because this is the same region where BoNT/B and G bind. BoNT/B and G share the highest sequence identity (50%) with each other among all BoNT H_{CC}s. BoNT/D-C, on the other hand, shares only ~27–28% sequence identity to either BoNT/B or G within the H_{CC} (supplementary material Fig. S1A). Furthermore, there are substantial structural differences between BoNT/B and BoNT/D-C in the region where BoNT/B binds SytII. Although the molecular details of BoNT/D-C–Syt interactions remain to be determined, our mutagenesis studies suggest that BoNT/D-C probably uses a new receptor-binding site for recognizing SytI/II.

The observation that BoNT/D-C pulled down low levels of SV2 from rat brain detergent extracts is puzzling. SV2 is known to form a complex with SytI/II (Bennett et al., 1992; Schivell et al., 1996; Lazzell et al., 2004; Baldwin and Barbieri, 2007; Yao et al., 2010), but BoNT/B, which pulled down more SytI/II than BoNT/D-C, apparently pulled down less SV2 (Fig. 2A). Because a lack of SV2 did not affect the sensitivity of neurons to BoNT/D-C (supplementary material Fig. S4B), SV2 is not an essential component for BoNT/D-C binding and entry into neurons. However, it is possible that SV2 contributes to BoNT/D-C binding in a way that can be compensated by other proteins. For instance, structures in the complex glycan of SV2 might contribute to BoNT/D-C binding and could be replaced by similar structures in other proteins. Such an interaction can substantially enhance the binding affinity of BoNT/D-C to neuronal surfaces, and the lack of such co-factors might explain the relatively high apparent dissociation constant between D-C/H_C and recombinant SytII in test tubes. Because SV2 serves as the receptor for BoNT/A, E, D and potentially F (Fu et al., 2009; Rummel et al., 2009) and tetanus neurotoxin (Yeh et al., 2010), whereas SytI/II are the receptors for BoNT/B, D-C and G, the SV2–SytI/II complex has emerged as the central receptor hub for this family of toxins. Whether the complex of SV2–SytI/II serves as the high-affinity toxin-binding site on neuronal surfaces, with one component as the essential binding site and the other as a replaceable facilitator, remains a key question to be examined further.

We also found that gangliosides increase BoNT/D-C binding to SytI/II and enhance the ability of SytII to neutralize BoNT/D-C in vitro and in vivo. These findings are consistent with recent studies on BoNT/D-C binding to gangliosides and the potential ganglioside-binding sites within D-C/H_C (Karalewitz et al., 2010; Nuemket et al., 2011). It is possible that the binding affinity of BoNT/D-C to gangliosides is considerable, because it has been shown that D-C/H_C can bind to neuronal surfaces at 4℃, a condition that inhibits synaptic vesicle exocytosis (Karalewitz et al., 2010). This binding is likely to be mediated by interactions with surface gangliosides, although it is also possible that low levels of vesicle proteins are exposed on neuron surfaces under resting conditions. It was proposed that BoNT/D-C contains both a ganglioside-binding site and a second ganglioside-binding loop, as mutations within both regions abolish ganglioside binding (Nuemket et al., 2011). However, the possibility that mutations in the proposed ganglioside-binding loop disrupt binding indirectly through structural changes has not been excluded. Although it remains to be determined whether BoNT/D-C contains more than one ganglioside-binding site, our findings that BoNT/D-C at sub-nanomolar levels cannot enter neurons lacking SytI/II demonstrate that gangliosides alone cannot form the high-affinity receptor for BoNT/D-C. Thus, we suggest that the high affinity receptors are composed of both gangliosides and SytI/II, supporting a ‘double receptor’ model for BoNT/D-C.

Finally, we also report that human SytII is not an effective BoNT receptor owing to a unique amino acid change from rodent SytII within the toxin-binding site. This conservative change from phenylalanine to leucine at position 54 (mouse SytII) substantially diminished the receptor function of human SytII. Intriguingly, this residue change appears to be recent in evolution because only humans and chimpanzees contain leucine at this position, whereas the majority of vertebrates including many primates, such as Rhesus macaque, maintain the conserved phenylalanine. Although further studies are needed to determine the physiological meaning of this residue change and the potential role of BoNT exposure in human evolution, our findings certainly indicate that this single-residue change could bear substantial functional consequences.

It has been reported that SytII is the dominant isoform that is expressed in rodent motor neurons, whereas SytI is only co-expressed in a subset of motor nerve terminals (Pang et al., 2006). If human motor neurons share the same SytI/II expression pattern as mice, BoNT/B, D-C and G might only target effectively a subset of human motor nerve terminals. Interestingly, a much higher dose of BoNT/B than BoNT/A is usually needed to achieve similar levels of paralytic effects in patients (Brin et al., 1999; Pappert and Germanson, 2008). Although there could be multiple reasons for observed dose differences in humans between these two therapeutic toxins, the potential receptor restriction for BoNT/B in humans can be a contributing factor. If so, it might be desirable to modify the BoNT/B receptor-binding

domain to achieve effective binding to human SytII, as a way of improving the therapeutic efficacy of BoNT/B. Therefore, the distribution of SytI/II in human motor nerve terminals, their contribution to BoNT/B sensitivity in vivo and the potential polymorphism within the toxin-binding site of human SytI/II warrant further studies.

Materials and Methods

Antibodies, toxins and other materials

Mouse monoclonal antibodies against Syb (Cl69.1), SNAP-25 (Cl71.2), Syx (HPC-1), Syp (Cl7.2), SytI ($SytI_N$ Ab: 604.4; cytoplasmic domain: Cl41.1) and SV2 (pan-SV2) were generously provided by Edwin Chapman (Madison, WI). Rabbit polyclonal antibodies against BoNT/B and C were generated in Eric Johnson's lab. Human monoclonal antibodies against BoNT/D-C (8DC2) and BoNT/A (Raz-1) were generously provided by Jianlong Lou and James Marks (San Francisco, CA). Rabbit polyclonal antibody against Syg was generously provided by Roger Janz (Houston, TX). The following antibodies were purchased: mouse monoclonal anti-HA (16B12, Covance), mouse monoclonal anti-SytII (BD transduction), rabbit polyclonal anti-synapsin (Millipore), mouse monoclonal anti-actin and anti-FLAG (Sigma) antibodies.

Bovine mixed brain gangliosides were purchased from Matreya LLC (Pleasant Gap, PA) and were reconstituted in Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl) as described previously (Peng et al., 2011).

BoNT/A (Hall-A), BoNT/B (Okra), BoNT/C (Brazil), BoNT/D-C (D5995) and BoNT/E (Alaska) were purified in E. Johnson's lab from indicated strains. BoNT/G (G89) was purchased from Metabiologics (Madison, WI). The quality and specific neurotoxicity of each BoNT preparation were determined using mouse lethality assays and antibody neutralization assays. We note that commercially available BoNT/D from Metabiologics is actually BoNT/D-C. It is from the same D5995 strain in E. Johnson's culture collection. The standard BoNT/D is from strain D1873, which was used in our previous studies on identifying SV2 as the BoNT/D receptor (Peng et al., 2011). These two toxins can be validated by their distinct antibody neutralization profiles: D1873 is completely neutralized by CDC type D anti-serum, whereas D5995 is only partially neutralized by the same anti-serum.

cDNA, constructs and protein purifications

The following cDNAs were generously provided: B/H_C (Joseph Barbieri, Milwaukee, WI) (Baldwin et al., 2008), rat SytI (Thomas Sudhof, Palo Alto, CA), mouse SytII (Mitsunori Fukuda, Ibaraki, Japan) and human SytI (R. Bryan Sutton, Lubbock, TX). The cDNAs encoding the H_Cs of BoNT/C (residues 862–1291, GenBank: X53751.1) and BoNT/D-C (residues 859–1285, GenBank: AB461915.1) were synthesized by GenScript Inc. (New Brunswick, NJ) with the codon optimized for *E.coli* expression.

Recombinant G/H_C was generously provided by J. Barbieri and has been previously described (Baldwin et al., 2008). HA tagged D/H_C was described previously (Peng et al., 2011), and HA tagged $D\text{-}C/H_C$ was constructed in the same way. GST tagged SytI/II fragments, SytI/II mutations and SV2 fragments were described previously (Dong et al., 2003; Dong et al., 2006). $D\text{-}C/H_C$ and C/H_C were subcloned into both pGEX2T and pET28a vectors to produce GST-tagged and His6-tagged proteins, respectively. Protein purification was carried out as previously described (Peng et al., 2011). The purity of His6-tagged H_C proteins is shown in supplementary material Fig. S2.

Neuron culture, lentiviral transduction, SytI KD, toxin loading and antibody uptake experiments

Rat hippocampal neurons were prepared from E18–19 embryos as described previously (Peng et al., 2011). A Lox-Syn-Syn lentivirus vector was used for all lentiviral constructs in neurons as described previously (Peng et al., 2011). This vector contains two separate neuronal-specific synapsin promoters. A Lox-U6-Syn lentiviral vector was generated by replacing one synapsin promoter in Lox-Syn-Syn with a U6 promoter. A SytI shRNA KD construct was generated using the following primers: 5′-GATCCGTGCAAGTGGTGGTAACTGGCTCGAGGCA-GTTACCACCACTTGCACTTTTTTA-3′, targeting base pairs 1063–1081 of rat SytI. KD-resistant SytI was generated by changing GTGGTGGTA to GTAGTAGTG within the targeting site. Lentiviruses were added to neurons at DIV5 (days in vitro). Experiments were carried out on DIV12–14.

Toxin loading into hippocampal neurons was carried out using high K^+ buffer (the same as PBS, but adjusted to 56 mM KCl and 87 mM NaCl plus 1 mM $CaCl_2$) as previously described (Peng et al., 2011). PBS buffer was also used to load toxins in Fig. 1D–E, with the addition of TTX (5 μM), a potent Na^+-channel blocker. Antibody uptake experiments were carried out using either $SytI_N$ Ab (Cl604.4, 1:200) or anti-HA (16B12, 1:100) in high K^+ buffers for 5 minutes. Cells were subsequently washed, fixed and permeabilized for immunostaining analysis as previously described (Peng et al., 2011). Fluorescence images were captured by using a Leica TCS SP5 confocal microscope with a 40× oil immersion objective.

Brain detergent extracts, GST pull-down assay and immunoblot analysis

Detergent extracts from rat brain were prepared as previously described (Peng et al., 2011). GST pull-down assays were carried out using GST fusion proteins or GST protein immobilized on glutathione-Sepharose beads (GE Bioscience, Piscataway, NJ). In Fig. 2A and Fig. 6E, 15 μg of GST-H_Cs or GST were incubated with 0.5 ml detergent extracts from rat brain for 1 hour at 4℃. In other GST pull-down assays, 2 μg of indicated Syt fragments or GST were incubated with indicated H_Cs (0.5 μM) or BoNT/B (100 nM) in 100 μl TBS buffer plus 0.5% Triton X-100, with or without gangliosides (60 μg/ml), for 1 hour at 4℃. Beads were washed three times using TBS buffer plus 0.5% Triton X-100. A total of 10% of bound materials were subjected to SDS-PAGE followed by either Coomassie blue staining (Fig. 2C) or immunoblot analysis using the enhanced chemiluminescence (ECL) method (Pierce, Rockford, IL).

Toxin neutralization assay in vitro and in vivo

GST tagged SytII 1–87 and GST proteins were eluted from beads using 20 mM Glutathione in elution buffer (50 mM Tris, 375 mM NaCl, 0.1% Triton X-100). Eluted proteins were dialyzed in PBS buffer. Indicated concentrations of SytII 1–87 or GST were pre-incubated with BoNT/D-C (0.3 nM) for 30 minutes at 4℃ in 200 μl high K^+ buffers, with or without gangliosides (60 μg/ml). Neurons were then exposed to the mixtures for 5 minutes, washed three times, and further incubated in toxin-free media for 6 hours. Cells were then harvested for immunoblot analysis.

Toxin neutralization in vivo was carried out using a rapid time-to-death assay described previously (Boroff and Fleck, 1966; Dong et al., 2003). Briefly, the same amount of BoNT/D-C was pre-incubated with either GST or GST-tagged SytII 1–87 (90 μM), with or without gangliosides (120 μg/ml), for 30 minutes at 4℃. The mixtures were then injected into mice intravenously (into the lateral tail vein) and the survival time of each mouse was recorded. The dilution factor in mice is ~1:10, therefore, the final concentration of GST and GST–SytII 1–87 in mice is ~9 μM. The remaining effective toxicity of BoNT/D-C in each mixture was estimated using a standard curve as described previously (Boroff and Fleck, 1966; Dong et al., 2003).

The procedure of this rapid time-to-death assay was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Wisconsin – Madison (protocol #M2319). It was justified as it reduces the number of mice that are needed to estimate the toxicity by ~5-fold compared with the standard mouse intraperitoneal lethality assay, as described previously (Boroff and Fleck, 1966).

Structure analysis and sequence alignment

The structure-based alignment of BoNT/D-C, B, C, D and G (PDB codes 3N7L, 1Z0H, 3N7K, 3N7J and 2VXR, respectively) was performed using SSM with default settings. The final figure of the alignment was prepared using ESPript. Structure figures were prepared using PyMol.

Acknowledgements

We are indebted to Edwin Chapman (Madison, WI) for his generous support of this work. We thank the Biomolecule Production Core at the New England Regional Center for Excellence (NERCE), NIAID U54 AI057159, for their assistance on protein purification. L.P., R.P.-A.B, P.S. and M.D. designed and performed experiments, analyzed data and wrote the paper; W.H.T., R.M.P. and E.A.J. performed experiments or contributed key reagents. Authors declare no conflict of interest.

Funding

This study was supported by the National Institutes of Health (National Center for Research Resources) [grant numbers RR000168 and 8P51OD011103-51 to the New England Primate Research Center], as well as grants from the Swedish Research Council [grant number 2010–5200 to P.S.], the Wenner-Gren Foundations and the Swedish Foundation for Strategic Research.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.103564/-/DC1

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