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Review

Highly specific interactions between botulinum neurotoxins and synaptic vesicle proteins

A. T. Brunger*, R. Jin+ and M. A. Breidenbach++

Howard Hughes Medical Institute and Departments of Molecular and Cellular Physiology, Neurology and Neurological Sciences, Structural Biology, and Photon Science, Stanford University, Stanford, CA 94305 (USA), e-mail: brunger@stanford.edu

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Abstract. Despite its extreme toxicity, botulinum neurotoxin is widely utilized in low doses as a treatment for several neurological disorders; higher doses cause the neuroparalytic syndrome botulism. The toxin blocks neurotransmitter release by preferentially attaching to pre-synaptic membrane receptors at neuromuscular junctions and subsequently delivering a Zn^{2+}-dependent protease component to presynaptic neuronal cytosol. These highly specialized enzymes exclusively hydrolyze peptide bonds within SNARE (soluble *N*-ethylmaleiamide sensitive factor attachment protein receptor) proteins. In this review we discuss the structural basis for botulinum toxin's exquisite specificity for its neuronal cell-surface receptors and intracellular SNARE targets.

Keywords. SNARE, botulism, tetanus, synaptotagmin, metalloprotease, endocytosis, synaptic vesicle, neurotransmission.

Introduction

Clostridial neurotoxins (CNTs) are produced by species of anaerobic, Gram-positive, spore-forming, rod-shaped, bacteria within the genus *Clostridium.* Botulinum neurotoxins (BoNTs), expressed by *Clostridium botulinum*, cause botulism, a severe neurological disease associated with a life-threatening flaccid paralysis affecting both humans and animals [1]. Tetanus neurotoxin (TeNT), expressed by *Clostridium tetani*, causes tetanus, a disease characterized by spastic paralysis that causes opposing skeletal muscles to contract spasmodically [2]. The first scientific observations of the paralytic syndrome botulism were made by Justinus Kerner in 1820 [3] who discovered that the disease can be caused by the intake of contaminated smoked sausages (Latin: *botulus*). Tetanus has been recognized since ancient times and was already described by Hippocrates; in 1867 it was hypothesized that an infectious agent is the cause [1]. There are seven serotypes of BoNTs (termed A, B, C1, D, E, F, G) and one TeNT [2, 4]. BoNTs interfere with the acetylcholine release process itself, but not with acetylcholine storage or Ca^{2+} influx, implying that CNTs block neuronal exocytosis [5]. CNTs block neurotransmitter release by proteolytic cleavage of SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors), proteins that play a key role in Ca^{2+}-triggered neurotransmitter release [6, 7].

* Corresponding author.
+ Present address: Center for Neuroscience, Aging and Stem Cell Research, Burnham Institute for Medical Research, La Jolla, CA 92037 (USA).
++ Present address: Department of Chemistry, University of California at Berkeley, Berkeley, CA 94720 (USA)

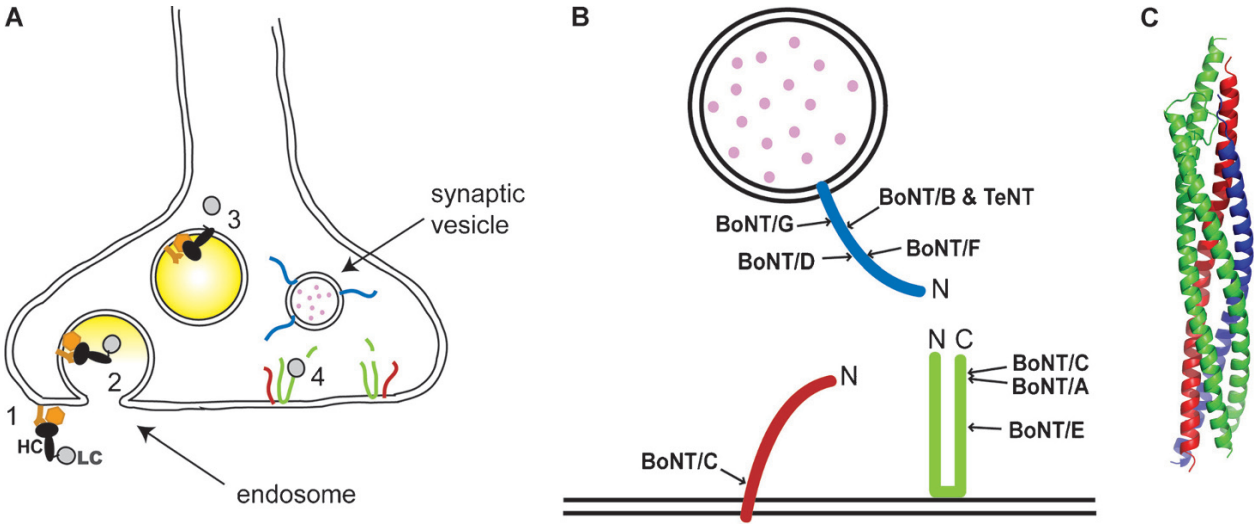

Figure 1. Synaptic SNAREs (soluble *N*-ethylmaleiamide sensitive factor attachment protein receptors) are targeted by clostridial neurotoxins (CNTs) light chains (LCs). (*A*) A four-step model for CNT intoxication includes (1) neurospecific cell-surface binding, (2) receptor-mediated endocytosis, (3) translocation of the LC, and (4) SNARE-specific proteolysis [19, 120, 121]. The toxin heavy chain (HC, black) mediates cell-surface binding with ganglioside and protein receptors (orange). Following endocytosis, the HC also mediates translocation of the LC (gray) if the endosome is acidified. LCs can target the synaptic SNAREs including vesicle-bound synaptobrevin (blue), presynaptic membrane-bound syntaxin (red), and SNAP-25 (green) prior to ternary SNARE complex formation. (*B*) The relative locations of the peptide bonds hydrolyzed by LCs in the core domains of SNARE proteins are shown. The cut sites of the seven botulinum neurotoxin serotypes (BoNT/A–G) and that of tetanus toxin (TeNT) are indicated by arrows. (*C*) Crystal structure of the neuronal SNARE complex [43] consisting of synaptobrevin (blue), syntaxin (red), and SNAP-25 (green) (PDB code 1SFC). This structure represents the post-fusion state of the SNARE complex.

With developments in hygiene and food processing, botulism is no longer a major threat to public health, yet small outbreaks are still reported [8]. In contrast, BoNT/A and BoNT/B in complex with hemagglutinin and pure BoNT/A have become powerful therapeutic agents. For example, controlled application of very low doses of BoNT/A has proven to be an effective treatment for certain neurological disorders associated with an abnormal increase in muscle tone or activity, such as spasticity and focal dystonias [9]. Recently, BoNT/A-containing treatments have also been shown to be beneficial for conditions of achalasia [10], chronic headache [11] and hyperhidrosis [12]. Low dosage is a critical factor in the application of BoNT/A as a therapeutic; its estimated intramuscular or intravenous lethal dose in humans is 1 ng/kg–making BoNT/A approximately 3000-fold more potent than the plant-derived biological toxin ricin [13, 14]. In addition to their role in medicine, BoNTs have become powerful research tools to study the function of SNARE proteins in Ca^{2+}-triggered neurotransmitter release [15]. This review focuses on structural insights of the interactions between CNTs and their proteolytic targets and cell surface receptors.

Modular architecture and mechanism of action

CNTs are synthesized as single polypeptide chains of approximately 150 kDa. This single chain is post-translationally cleaved by certain bacterial and tissue proteases into a 50-kDa light chain (LC) and a 100-kDa heavy chain (HC) [16, 17]. Upon cleavage, the LC and HC of BoNTs remain covalently and reversibly linked by a disulfide bond until being exposed to reducing conditions within the neuronal cytosol [18, 19]. BoNTs associate with non-neurotoxin components such as hemagglutinins that protect the BoNT during the gastrointestinal passage but also enhance production of anti-neurotoxin antibody [20, 21].

All BoNTs employ a similar mechanism of intoxication suggesting that these toxins have a common evolutionary origin [19]. The HCs mediate neuronal cell surface binding, internalization by receptor-mediated endocytosis, and transport of the LC across the membrane into the cytosol (Fig. 1A). Once the LC is released into the cytosol, SNARE targets are proteolyzed by the LC [22, 23].

Primary sequence analyses of LC proteases revealed a conserved His-Glu-X-X-His motif. The His residues in the motif coordinate a Zn^{2+}, while the Glu coordinates a water molecule for hydrolysis in the apo and substrate-bound states; a downstream Glu residue also coordinates the Zn^{2+}. This motif is found

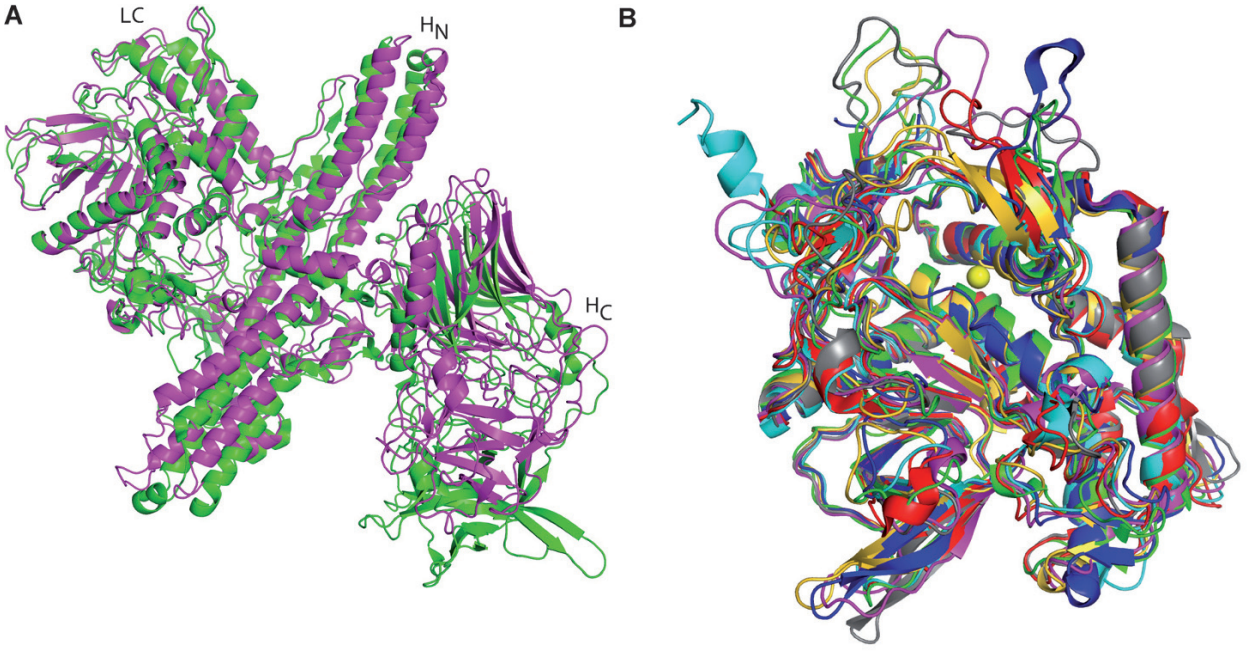

Figure 2. Structures of apo CNTs. (*A*) Apo holotoxin structures: BoNT/A (PDB code 3BTA) [32] (magenta) and BoNT/B (PDB code 1EPW) [33] (green). The LC protease, translocation, and receptor binding domains are indicated. The structures were superimposed using the backbone atoms of the LC protease domain. (*B*) An overall Cα atom superposition of the LC protease structures from all seven serotypes of BoNTs and TeNT: BoNT/A LC (PDB code 2SIG, green) [80], BoNT/B LC (1EPW, magenta, rmsd = 1.6 Å) [33], BoNT/C1 LC (2QN0, gold, rmsd = 2.2 Å) [81], BoNT/D LC (2FPQ, blue, rmsd = 1.3 Å) [82], BoNT/E LC (1T3A, cyan, rmsd = 1.6 Å) [68], BoNT/F LC (2A8A, red, rmsd = 1.9 Å) [83], BoNT/G LC (1ZB7, orange, rmsd = 2.1 Å) [84], and TeNT LC (1Z7H, gray, rmsd = 1.7 Å) [69]. The specified atomic root mean square differences are for all Cα atoms with respect to the BoNT/A LC structure. The Zn^{2+} is shown as a yellow sphere. Despite their different substrate specificities, CNT-LCs display high structural similarity.

in a variety of Zn^{2+}-dependent metalloproteases such as thermolysin, and suggests that LCs may utilize a similar enzymatic mechanism [24, 25]. The CNT-LCs cleave specific peptide bonds within the neuronal SNARE proteins (synaptobrevin, syntaxin, and SNAP-25) (Fig. 1B). BoNT/A and E specifically cleave SNAP-25, while serotypes B, D, F, and G of BoNTs cleave synaptobrevin. BoNT/C1 is unique in that it is able to hydrolyze two substrates: syntaxin [26, 27] and SNAP-25 [28–30].

The seven BoNTs have a high degree of primary sequence conservation, although all are antigenically distinct [31]. Crystal structures of full-length BoNT/A holotoxin [32] and of BoNT/B holotoxin [33] are available (Fig. 2A). Both structures are very similar; they exhibit a modular architecture: the LC protease, translocation (the N-terminal subdomain of HC, H_N) and the receptor binding domains (the C-terminal subdomain of HC, H_C). It is important to note that in these crystal structures the translocation domains are in their water-soluble conformations; the structure of a membrane-inserted translocation domain remains to be elucidated.

SNAREs and Ca^{2+}-triggered neurotransmitter release

Much of our understanding of the critical role SNAREs play in neurotransmission can be directly traced to the finding that botulism and tetanus toxins block Ca^{2+}-triggered neurotransmitter release. At neuromuscular junctions (NMJs), acetylcholine is predominantly secreted *via* full vesicle fusion events rather than by a transient "kiss-and-run" mechanism, which likely plays a more prominent role in the central nervous system [34–36]. A continuous cycle of synaptic vesicle formation, delivery, fusion, and local recycling occurs such that a steady supply of vesicles is available for neurotransmitter release when triggered by the arrival of an action potential [37].

As the nerve terminal is depolarized, a rapid influx of Ca^{2+} enters the nerve cytosol through voltage-gated Ca^{2+} channels, triggering fusion events [36]. While tethering complexes hold docked vesicles in close proximity to their target membranes, an additional set of proteins interact to bring the two membranes close enough so that phospholipid bilayer reorganization into a fused state becomes energetically favorable [38]. Among the essential proteins for this task are the SNAREs [39, 40]. Neuronal SNAREs are membrane

bound, either *via* a single transmembrane region as in the cases of synaptobrevin and syntaxin [41], or by post-translational palmitoylation as in the case of SNAP-25 [42]. SNARE proteins contain at least one core domain that can adopt a parallel, coiled-coil conformation when given the opportunity to interact with other SNARE proteins [43] (Fig. 1C).

Intense biochemical and biophysical scrutiny of SNARE proteins has yielded the "zipper model" of membrane fusion [44–47]. The principle of this model is simple: SNAREs protruding from the synaptic vesicle membrane (mainly synaptobrevin) assemble into low energy core complexes with SNAREs anchored to the presynaptic membrane (mainly syntaxin and SNAP-25). The core domains of SNARE proteins are mostly unstructured in the absence of binding partners [48–50], but are entirely helical when the ternary complex is formed [43]. The helices formed by SNARE proteins are amphipathic and the coiled-coil structure is largely stabilized by hydrophobic packing [51]. A notable exception is the conserved "ionic layer" formed at the center of the complex by a network of salt bridges and hydrogen bonds [43]. The resulting structure is remarkably stable, resisting extreme chemical and thermal denaturing conditions [52–54]. The stepwise assembly of these low-energy complexes is thought to counter the energetic penalty of bringing phospholipid headgroups from opposing membranes together at a distance where membrane reorganization into a fusogenic state becomes favorable [55]. SNARE-mediated docking and fusion appears to be a general strategy for combining independent compartments in eukaryotic cells, but SNAREs are not the only factors imparting targeting specificity between intracellular membranes as originally believed. A number of additional proteins form tethering complexes to assist in this process [56, 57]. In addition, SNARE assembly is not inherently Ca^{2+} sensitive; additional factors are required for regulation of synaptic vesicle fusion. Synaptotagmin 1, a Ca^{2+}-binding protein, has been shown to be a sensor for Ca^{2+}-induced fusion events [58, 59]. Other factors such as Munc18 (nSec1), Munc13, and complexin bind to SNAREs and may play a role in regulating SNARE complex assembly [60–63]. The precise sequence of events and role of the various components of Ca^{2+}-triggered neurotransmitter release remain to be elucidated [64].

The crucial role of SNAREs in synaptic exocytosis was illuminated by the discovery that they are the physiological targets of the CNTs; in 1992, Schiavo and colleagues [6] reported that the intracellular proteolytic target of TeNT and BoNT/B is synaptobrevin. The target sites of the other BoNT serotypes are summarized in Figure 1B [6, 7, 27, 65, 66]. Remarkably, all CNT LCs target sites within the core domains of SNARE proteins.

CNT LC proteases

BoNT and TeNT LCs are amongst the most selective proteases known [67]. As mentioned above, primary sequence and structural analysis of LCs suggest that their enzymatic mechanism is related to that of other Zn^{2+} metalloproteases [32, 33, 68–70], but the structural basis of SNARE target selectivity is unusual. Oddly, the LCs do not appear to recognize a consensus site, or even have rigorous requirements for particular side chains flanking the scissile bond [71]. Also, the LCs generally require long stretches of their target SNAREs for optimal efficiency [30, 71, 72–75]. Indeed, point mutations in SNARE regions remote from the scissile bond can dramatically reduce LC efficiency [74, 76–78]. The cleavage-site selectivity of CNT-LCs is remarkable. For example, the scissile bond in SNAP-25 for BoNT/A (Gln197-Arg198) is shifted by exactly one residue compared to that for BoNT/C1 (Arg198-Ala199). BoNT/C1 cleaves only one of two identical neighboring peptide bonds (Lys253-Ala254 and Lys260-Ala261) in syntaxin-1A [27].

The apo structures of all members of the family of CNT-LCs are now available: BoNT/A LC [32, 78–80], BoNT/B LC [33], BoNT/C1 LC [81], BoNT/D LC [82], BoNT/E LC [68], BoNT/F LC [83], BoNT/G LC [84], and TeNT LC [69, 70] (Fig. 2B). The structural differences among the CNT-LCs are mostly limited to solvent-exposed loops and potential substrate interaction sites. The striking similarity of LC active sites naturally leads to the question of which LC features are determinants of substrate selectivity. Furthermore, none of the LCs efficiently cleave truncated substrate peptides less than 20–30 residues. Rather, unusually long stretches of the substrates are required for optimal cleavage [30, 71, 72,74,75]. In general, long sequences that are located N-terminal of the scissile bonds appear to be important for cleavage, as revealed by mutagenesis studies on synaptobrevin and SNAP-25 [73, 85, 86]. For example, the optimal portion of SNAP-25 required for maximally efficient cleavage by BoNT/A spans residues 146–202 [30, 87]. Other CNTs require 30–60 residue stretches of their substrates for efficient cleavage, regardless of scissile-bond location [72, 73, 75].

The structure of a BoNT/A·SNAP-25 complex [78] finally provided insights into the basis of LC substrate selectivity. To date, this is the only structure of a complex between a CNT-LC and its substrate; a previous report of the structure of a complex between

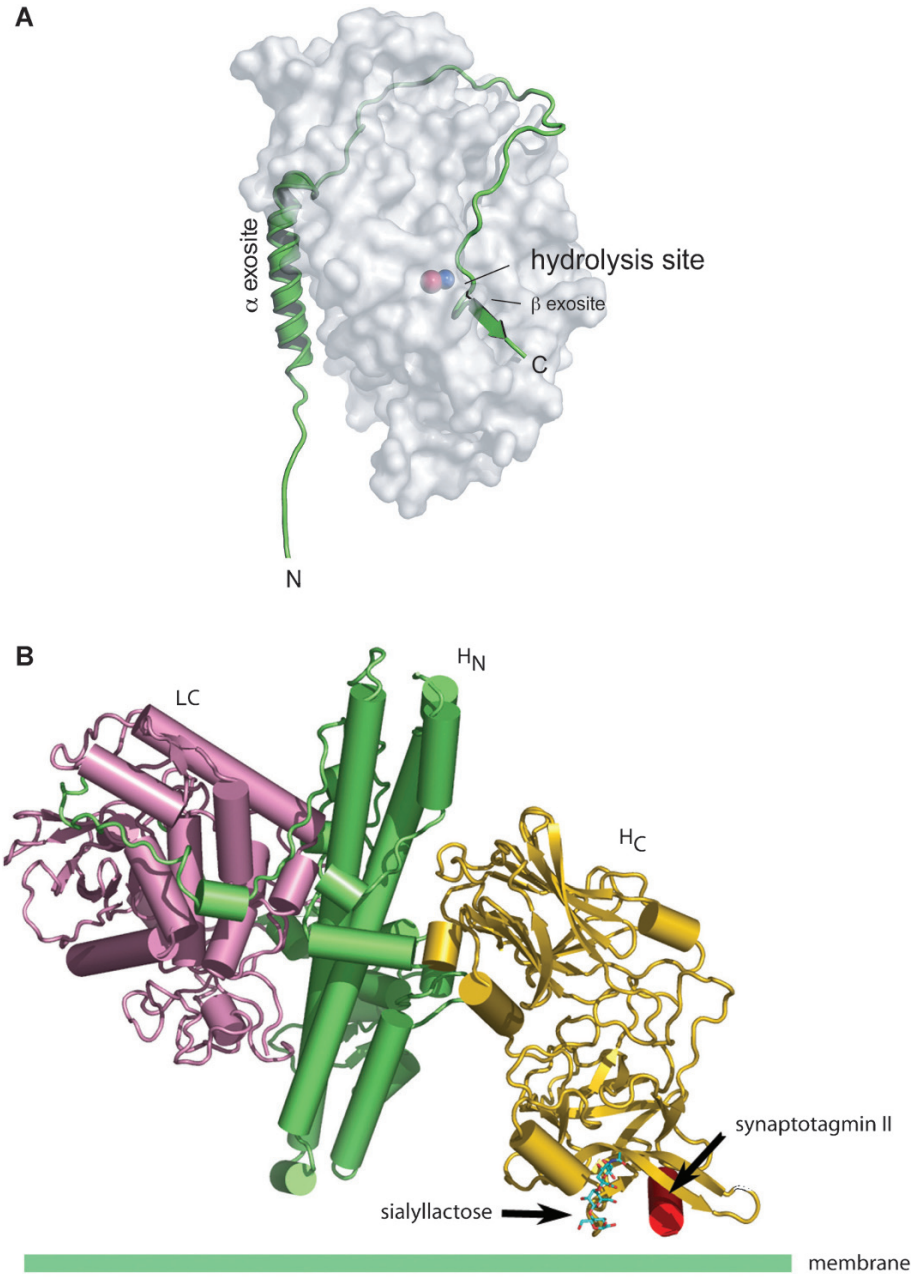

Figure 3. Interactions between BoNTs and synaptic vesicle proteins. (*A*) Structure of the BoNT/A·LC-SNAP-25 complex. The protease component of BoNT/A (gray) forms an extended interface with the C-terminal core domain of SNAP-25 (green). Multiple sites of enzyme-substrate interaction remote from the catalytic Zn^{2+} (magenta sphere) and associated nucleophile (blue sphere) extend around most of the toxin's circumference, imparting the protease with exquisite specificity. SNAP-25 is unstructured in the absence of a binding partner but adopts a mix of α-helix, β-sheet, and extended conformations when complexed with BoNT/A. (*B*) Proposed binding mode of BoNT/B on the membrane surface. The structure of a sialyllactose-bound BoNT/B (PDB code 1F31) was superimposed on that of the complex of BoNT/B-H_C and synaptotagmin II (PDB code 1NM1) using the coordinates of the H_C subdomain for the alignment. The LC, the N-terminal part of the heavy chain (H_N), and the C-terminal domain of the heavy chain (H_C) are shown in pink, green and gold, respectively.

BoNT/B-LC and synaptobrevin 2 [88] is not supported by the experimental data [78, 89]. Remarkably, SNAP-25 wraps around most of the circumference of the LC; the extensive interface between the enzyme and its substrate is not restricted to the active site (Fig. 3A). Moreover, in contrast to the contiguous helical conformation observed in the ternary SNARE complex [43], SNAP-25 adopts three distinct types of secondary structure upon binding to BoNT/A. The N-terminal residues of SNAP-25 (147–167) form an α-helix, the C-terminal residues (201–204) form a distorted β-strand, and residues in between are mostly extended [78]. Mutagenesis and kinetics experiments demonstrated that the N-terminal α-helix and the C-terminal β-sheet are critical for an efficient substrate binding and cleavage, and are termed α- and β-exosites, respectively. The structure confirmed the existence of such exosites, which had been postulated before based on biochemical experiments [76, 90].

The highly unusual extended enzyme–substrate interface used by BoNT/A serves to properly orient its conformationally variable SNARE target such that the scissile peptide bond is placed within close proximity of the catalytic motif of the enzyme. Notably, many of the interactions that impart substrate specificity occur on the face of the protease that

is opposite to its active site (α-exosite), and the C terminus of the substrate (β-exosite) induces a conformational change in the active site pocket, probably rendering the protease competent for catalysis. The multi-site binding strategy used by BoNT/A accounts for the extreme selectivity of this enzyme. The structure of the BoNT/A·SNAP-25 complex vividly illustrates the extent of substrate that must be available for efficient proteolysis to occur. SNAREs exhibit considerable conformational variability; they can exist as monomeric components with little secondary structure, as partially structured SNARE complexes or subcomplexes, or in complex with regulatory factors [91]. Thus, BoNT/A probably cannot efficiently hydrolyze SNAP-25 if any portion of the C-terminal core domain is already incorporated into a ternary SNARE complex (*cf.* Fig. 1C) or bound to a regulatory factor.

Structural and biochemical studies of the BoNT/C1-LC have provided further information regarding the toxin-substrate interaction [81]. BoNT/C1-LC is unique among all BoNTs in that it exhibits dual specificity toward both syntaxin and SNAP-25. Interestingly, while both BoNT/A and BoNT/C1 cleave SNAP-25, the scissile bond is shifted by only a single residue (Gln197-Arg198 for BoNT/A and Arg198-Ala199 for BoNT/C1). Structural modeling revealed that the remote α-exosite that was previously identified in the complex of BoNT/A-LC and SNAP-25 is structurally conserved in BoNT/C1. Single site mutations in the predicted α-exosite of BoNT/C1 had a significant but less severe effect on SNAP-25 cleavage in comparison to that of BoNT/A, suggesting that this region plays a less stringent role in substrate discrimination. Such a "promiscuous" substrate-binding strategy by the α-exosite could account for its dual substrate specificity. As a crucial supplement to the function of the remote α-exosite, the scissile-bond proximal exosites probably ensure the correct register for hydrolysis. This includes the β-exosite as observed on BoNT/A and key residues surrounding the scissile peptide bond. A small, distinct pocket (S1') near the active site of BoNT/C1 was found that potentially ensures the correct register for the cleavage site by only allowing Ala as the P1' residue for both SNAP-25 and syntaxin. Mutations of this SNAP-25 residue dramatically reduced enzymatic activity of BoNT/C1 [81]. The S1' pocket is significantly larger in BoNT/A LC allowing it to accommodate the Arg residue as the P1' residue as revealed by the crystal structures of the inhibitor L-arginine hydroxamate (ArgHX) with wild-type BoNT/A-LC [92] as well as an inactive double-mutant of BoNT/A LC [93].

The crystal structure of the BoNT/A-LC·SNAP-25 complex revealed a small loop (residues 183–190) that detaches from the surface of BoNT/A-LC and separates the α-exosite from the active site. This loop may be able to accommodate the necessary "slack" for the cleavage-site register shift between BoNT/A and BoNT/C1 while maintaining the approximate position of the α-exosite. Consistent with this notion, there is little effect on substrate cleavage upon insertion of up to three extra residues in this loop [81]. The divided roles for substrate discrimination among different exosites could provide some flexibility of the precise scissile bond position while ensuring high overall substrate specificity.

Receptor interactions

Prior to BoNT internalization and SNARE proteolysis, CNTs must selectively bind to the surfaces of peripheral nerve cells. Complex gangliosides, a class of glycosphingolipids which are particularly abundant in the outer leaflet of nerve cell membranes, have long been recognized to function as receptors for CNTs. Later, the existence of two classes of binding sites distinguished by different affinities and protease sensitivities [94, 95] led to a dual-receptor concept: complex gangliosides first accumulate CNTs on the plasma membrane before protein receptors subsequently mediate their endocytosis, with a different protein receptor being recognized by each BoNT [22, 96, 97]. Such a dual-receptor binding process could account for the extraordinary binding affinity and specificity of CNTs.

Ganglioside-binding sites have been identified for several CNTs [33, 98–101]. The structure of a complex between BoNT/B and sialyllactose revealed a conserved binding pocket [33], which was also shown to be essential for ganglioside recognition of BoNT/A, BoNT/B and TeNT [99, 100]. The amino acids that form this binding site are conserved among all CNTs except BoNT/D. The trisialoganglioside GT1b was found to interact with the receptor binding domains of BoNT/A, BoNT/B and TeNT [99, 100]. At present, the only protein receptors to have been identified are synaptotagmin I and synaptotagmin II for BoNT/B and BoNT/G, respectively, and synaptic vesicle protein SV2 (isoforms A, B and C) for BoNT/A [102–106]. Furthermore, BoNT/A and B were observed to bind synaptic vesicle protein complexes in synaptosome lysates [107]. The complexes comprised several proteins including synaptotagmin I, SV2, synaptophysin, VAMP2, and the vacuolar proton pump. However, it is unknown if any of these proteins play a role in the toxin binding and endocytosis processes in addition to synaptotagmins and SV2. In contrast to CNTs, TeNT has two ganglioside binding sites but no

protein receptor has yet been found in neuronal cells [99].

The BoNT protein receptors SV2 and synaptotagmins I and II are localized to synaptic vesicles. The luminal domains of these protein receptors become exposed to the extracellular space when synaptic vesicles fuse with the presynaptic membrane upon depolarization of the pre-synaptic terminal. This is likely to be the temporal window in which BoNTs interact with their specific receptors. Similarly, it is probably during this period that passive neutralizing antitoxins can act [108].

Synaptotagmins are a family of transmembrane proteins that trigger Ca^{2+}-dependent neurotransmitter release. Synaptotagmins I and II are essential for synaptic transmission in neuromuscular junctions [109]. BoNT/B and BoNT/G bind to the luminal domains of synaptotagmins I and II when they are exposed on the neuronal cell surface. The 25-kDa C-terminal portions of the heavy chains (H_C subdomains) of BoNT/B and BoNT/G are solely responsible for specific binding with the luminal domains of synaptotagmins I and II [104]. The luminal domain of synaptotagmin II is unstructured in solution [110]. Upon binding to BoNT/B, it adopts an α-helical conformation, which binds at the distal tip of the H_C subdomain of BoNT/B in a saddle-shaped crevice on the surface (Fig. 3B) [110, 111]. The extensive intermolecular interface has a buried surface area of about 1200 $Å^2$, involving mostly hydrophobic residues and complementary salt bridges.

The toxin-receptor interactions are highly specific. Mutations in the synaptotagmin binding cleft greatly reduced the toxicity of BoNT/B by up to 1000-fold and are more significant than mutations in the ganglioside-binding pocket [110]. The structure of the BoNT/B synaptotagmin II complex also sheds light on the interaction of BoNT/G with its receptor. BoNT/G is the closest homologue to BoNT/B and also binds to the membrane-proximal region of synaptotagmin I and II [104]. Primary sequence analysis revealed that the synaptotagmin binding site is conserved among BoNT/B and BoNT/G, but not in other toxin family members. Mutations of some of the BoNT/G residues that are equivalent to the synaptotagmin-interacting residues on BoNT/B significantly decrease the binding affinities between synaptotagmins and BoNT/G [112]. Taken together, BoNT/B and G likely employ the same strategy for receptor binding.

The dual-receptor hypothesis for BoNTs was proposed more than 20 years ago [96], but the spatial and functional relationship between these two receptors had been unclear. Crystal structures now offer clues about this relationship: the luminal domain of synaptotagmin II and a sialyllactose carbohydrate moiety occupy two adjacent but non-overlapping binding sites (Fig. 3B) [33, 98, 100, 110]. Ganglioside or synaptotagmin binding does not cause significant structural changes in the H_C subdomain. However, they appear to act synergistically; the dissociation constant between the receptor-binding domain of BoNT/B and the luminal domain of synaptotagmin II in solution is more than 100-fold larger than that measured between BoNT/B and full-length synaptotagmin II (including the transmembrane region) in the presence of gangliosides and micelles [102]. Deletion of the transmembrane domain of synaptotagmin I abolishes ganglioside-dependent binding [113]. Clearly, further experiments are needed to characterize potential intramembrane interactions between the two receptors. Nevertheless, toxin-receptor interactions may be different for other members of the CNT family. As mentioned above, two carbohydrate-binding sites in the H_C subdomain of TeNT are required for its function [99]. These different mechanisms of cell-surface recognition may explain the differences in CNT trafficking in peripheral neurons. Characterization of both the protein and carbohydrate receptor sites could provide an approach to retarget BoNTs to different cell types by site-directed mutagenesis. Such modified BoNTs could possibly be used as drug delivery systems [114].

For CNTs, proper orientation on the membrane surface is important for efficient endocytosis and subsequent translocation of the LC to the cytosol [115, 116]. In the case of BoNT/B, the simultaneous attachment of synaptotagmin and ganglioside ligands imposes geometric restrictions on the orientation of BoNT/B with respect to the membrane surface (Fig. 3B). Two strongly negative-charged molecular surfaces, which remain charged even in an acidic endosomal lumen, further restrict the orientation of BoNT/B on the membrane surface. In addition, four solvent-exposed lysine residues are conserved in the luminal domain of both synaptotagmins I and II, which may interact with phospholipid headgroups. The interactions between the toxin's HC and nearby negatively charged phospholipids appear to stabilize the toxin on membranes [22]. Interestingly, the receptor binding region, especially around the synaptotagmin II binding site, were recognized by mouse anti-BoNT/B antibodies [117].

It is conceivable that CNTs first interact with the oligosaccharide portion of polysialogangliosides, which are highly enriched at nerve terminals, causing the CNT to adhere to the neuronal cell surface. Upon binding to exposed gangliosides, the toxins will be constrained to the plasma membrane sur-

face, thereby significantly increasing localized toxin concentration [118]. The toxin–ganglioside complex could then diffuse laterally before binding to a second, less abundant, protein receptor. The abundance of polysialogangliosides ensures high trapping efficiency while enhanced specificity is conferred by the protein receptor.

Conclusions

The unrivaled potency of the BoNTs undoubtedly arises from the combined high specificities of both multiple receptor-mediated HC interactions with neuronal cell surfaces and multifaceted LC interactions with intracellular SNAREs. Remarkably, BoNT/A and BoNT/B bind to one component of the synaptic vesicle fusion machinery (SV2 and synaptotagmin, respectively) and then cleave another upon entry (SNAP-25 and synaptobrevin, respectively). The extraordinary specificity of the BoNT-LC proteases is attributed to the existence of multiple substrate-binding sites including exosites that are remote from the scissile bond. Clearly, more structures of BoNTs in complex with their substrates and receptors are needed to determine if the receptor and substrate recognition mechanisms are conserved among BoNTs and to provide starting points for structure-based inhibitor development.

Precise delivery of the most discriminating proteases known ensures their efficacy. While we now have an excellent understanding for how some BoNTs identify their targeted cell type and how their protease components identify their targeted substrates, very limited information is available regarding the means by which LCs are translocated through an endosomal membrane into the neuronal cytosol. While the N-terminal part of the HC (the translocation domain, Fig. 2A) is known to mediate this process, its mechanism remains an active area of research. The N terminus of the translocation domain, known as the "belt" region, might act as a surrogate pseudosubstrate inhibitor of the LC protease or as a chaperone during the translocation step [119]. A better molecular understanding of the CNT translocation mechanism CNTs has the potential to yield new clinical applications, such as using BoNTs as delivery systems for drugs into neuronal cytosol. The extent to which an LC sequence can be modified and yet successfully translocated remains to be determined.

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