

# New insights into clostridial neurotoxin–SNARE interactions

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**Botulinum neurotoxin serotype A (BoNT/A) has achieved a dichotomous status in modern medicine; it is both a versatile treatment for several neurological disorders and a lethal poison responsible for causing the neuroparalytic syndrome botulism. The extent of paralysis largely depends on the dosage of toxin received. The toxins block neurotransmitter release by delivering their Zn<sup>2+</sup>-dependent protease components to the presynaptic side of chemical synapses. These highly specialized enzymes exclusively hydrolyze peptide bonds within SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins. Recently, the structural basis for the highly specific interaction between BoNT/A and its target SNARE, SNAP-25 (synaptosomal-associated protein of 25 kDa), was elucidated. New details regarding the nature of the toxin–SNARE interactions could be exploited for novel inhibitor design.**

## Introduction: why are inhibitors necessary?

Low doses of BoNT/A are now widely used for alleviating the symptoms of various disorders, including paralytic strabismus, blepharospasm, cervical dystonia and severe hyperhidrosis [1]. Thus, it is legitimate to question the need for developing inhibitors of this versatile therapeutic compound. It is important to recognize that dosage is the only discriminator between the localized effects of BoNT/A as a therapeutic agent and the more generalized flaccid paralysis associated with botulism. Although rare, incidents of inadvertent botulism have been reported following deliberate intramuscular injection of at least one commercially available brand of the toxin [2,3]. BoNT/A is actually the most potent known biological toxin, with an estimated human lethal intravenous dose of 1–5 ng/kg [4,5]. Owing to its ease of production, dissemination and extreme lethality, the US Centers for Disease Control and Prevention has categorized BoNT/A among the most serious potential biological threats to human health and national security [6]. Furthermore, available antitoxin treatments for the various BoNT serotypes are mired with problems, including a lack of efficacy and substantial risk of allergic reaction [7]. At best, existing antitoxin treatments (available for serotypes A, B and E) can neutralize BoNTs in the circulatory system if

administered early in the clinical course of the disease but they cannot act on molecules that have been internalized into the nervous periphery. Although occurrences of food-borne, wound and infant botulism are both extremely rare and typically treatable, there remains a potential for large-scale outbreaks which could overwhelm existing capacity for proper medical care. Therefore, the development of effective alternative BoNT antagonists remains a priority.

## The clostridial neurotoxins

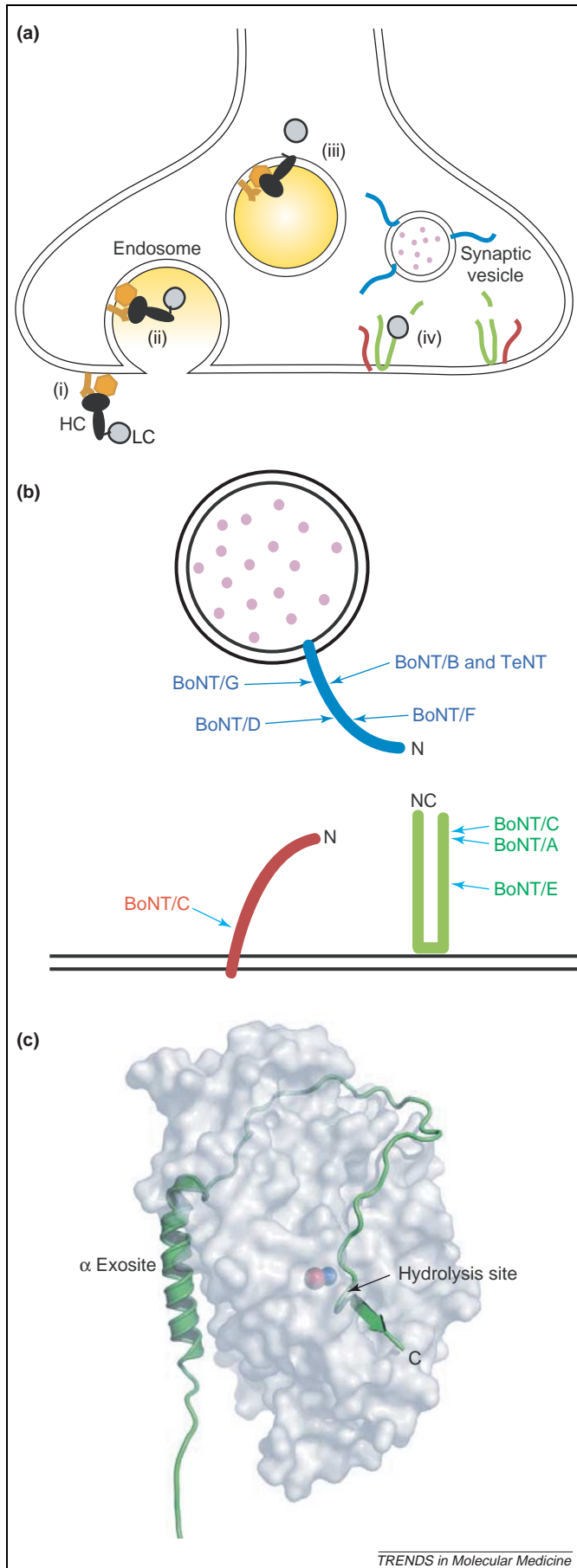
The clostridial neurotoxin (CNT) family of structurally and functionally related toxins includes the BoNT serotypes A–G and tetanus neurotoxin (TeNT). The Zn<sup>2+</sup>-binding His-Glu-X-X-His motif that was identified in CNT primary structures immediately indicated that they might use a Zn<sup>2+</sup>-dependant proteolytic activity in their biochemical mechanism of action [8]. The toxins are synthesized as single-polypeptide chains of ~150 kDa, but must be posttranslationally modified by a bacterial or tissue protease for activation [9,10]. The active form of the toxin consists of a 50-kDa light chain (LC, the protease component that includes the Zn<sup>2+</sup>-binding motif) and a 100-kDa heavy chain (HC) [11]. Although proteolytically nicked, the chains remain covalently and reversibly linked by a disulphide bond until exposed to reducing conditions, such as the nerve cytosol [12]. Each CNT contains three functionally distinct components that participate in the extensively reviewed four-step mechanism of CNT intoxication, involving neurospecific binding, receptor-mediated endocytosis, endosomal translocation and SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteolysis (Figure 1a) [13–15]. Simply stated, the various CNTs can be thought of as well-delivered proteases that specifically cleave sites within the neuronal SNARE proteins synaptobrevin (sb), syntaxin (sx) and SNAP-25, all of which are required for synaptic exocytosis. The toxin HCs largely serve as the delivery systems for their corresponding LC-protease components.

## SNAREs and neurotransmission

Much of our understanding of the crucial role that SNAREs have in neurotransmission can be directly traced to the finding that botulism and tetanus toxins block synaptic-vesicle fusion. At neuromuscular junctions (NMJs), acetylcholine is predominantly secreted through

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Available online 11 July 2005



full-vesicle fusion events rather than by a transient 'kiss-and-run' mechanism, which probably has a more prominent role in the central nervous system [16–18]. A continuous cycle of 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 [19–21]. Here, we focus on the fusion step of docked and primed synaptic vesicles with the presynaptic membrane. As the nerve terminal is depolarized during the arrival of an action potential, a rapid influx of  $\text{Ca}^{2+}$  ions enters the nerve cytosol through voltage-gated  $\text{Ca}^{2+}$  channels, triggering fusion events [16]. 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 together such that phospholipid bilayer reorganization into a fused state becomes energetically favorable [22]. Among the essential proteins for this task are the SNAREs [23,24]. Neuronal SNAREs are membrane bound, either by a single transmembrane region, as for sb and sx [25], or by posttranslational palmitoylation, as in the case of SNAP-25 [26]. 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 [27,28].

Intense biochemical and biophysical scrutiny of SNARE proteins has yielded the 'zipper model' of membrane fusion [29–32]. The principle of this model is simple: SNAREs protruding from the synaptic vesicle membrane (mainly sb) assemble into low-energy core complexes with SNAREs anchored to the presynaptic membrane (mainly sx and SNAP-25). The core domains of SNARE proteins are mostly unstructured in the absence of binding partners [33–35], but are entirely helical when the ternary complex is formed [28]. The helices formed by SNARE proteins are amphipathic and the coiled-coil structure is largely stabilized by hydrophobic packing [36]. A notable exception is the conserved 'ionic layer' formed at the center of the complex by a network of salt bridges and hydrogen bonds [28]. The resulting structure is remarkably stable, resisting extreme chemical and thermal denaturing conditions [20,37,38]. The stepwise assembly of these low-energy complexes is thought to counter the energetic penalty of bringing phospholipid

**Figure 1.** Synaptic SNAREs are targeted by CNT light chains. (a) A four-step model for CNT intoxication includes (i) neurospecific cell-surface binding, (ii) receptor-mediated endocytosis, (iii) translocation of the light chain and (iv) SNARE-specific proteolysis [13–15]. The toxin heavy chain (HC, black) mediates cell-surface binding with ganglioside and glycoprotein receptors (orange). Following endocytosis, the HC also mediates translocation of the light chain (LC, gray) if the endosome is acidified. LCs can target the synaptic SNAREs, including vesicle-bound sb (blue), presynaptic membrane-bound sx (red) and SNAP-25 (green), before ternary SNARE-complex formation. (b) The relative locations of the peptide bonds in SNARE core domains hydrolyzed by LCs in the core domains of SNARE proteins are shown. The cut sites of the seven botulinum neurotoxin serotypes and that of tetanus toxin (TeNT) are indicated by the arrows. (c) 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  $\text{Zn}^{2+}$  (magenta sphere) and associated nucleophile (blue sphere) extend around most of the circumference of the toxin, imparting the protease with exquisite specificity. SNAP-25 is unstructured in the absence of a binding partner but adopts a mix of  $\alpha$ ,  $\beta$  and extended conformations when complexed with BoNT/A.

head-groups from opposing membranes together at a distance in which membrane reorganization into a fusogenic state becomes favorable [39]. SNARE-mediated docking and fusion appears to be a general strategy for combining independent compartments in eukaryotic cells, but SNARE assembly might not be the only factor imparting targeting specificity between intracellular membranes, as was originally believed, and other factors such as tethering complexes are probably required [40,41]. In addition, SNARE assembly does not impart  $\text{Ca}^{2+}$  sensitivity; therefore, additional factors are required for the regulation of synaptic-vesicle fusion. Synaptotagmin 1, a  $\text{Ca}^{2+}$ -binding protein, is a sensor for  $\text{Ca}^{2+}$ -induced fusion events [42,43]. Additional regulators, such as nSec1, appear to function by binding to SNAREs to regulate the assembly of the core complex [44]. The precise sequence of events and the molecular mechanism of SNARE-complex assembly and synaptotagmin-mediated  $\text{Ca}^{2+}$  triggering remain to be elucidated [45].

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 [46] reported that the intracellular proteolytic target of TeNT and BoNT/B is sb. A plethora of additional research identified target sites of the other BoNT serotypes, summarized in Figure 1b [46–51]. Remarkably, all CNT LCs target sites within the core domains of SNARE proteins.

### Unusual biochemical aspects of CNT–SNARE interactions

BoNT and TeNT LCs are the most selective proteases known [6]. The available crystal structures of LCs suggest that they use a catalytic strategy similar to the general  $\text{Zn}^{2+}$  metalloprotease thermolysin because their primary catalytic residues adopt a similar geometry [52–56], but the structural basis of SNARE selectivity has remained elusive. Oddly, the LCs do not appear to recognize a consensus site or even have rigorous requirements for particular side-chains flanking the scissile bond [57]. Furthermore, the LCs generally require long stretches of their target SNAREs for optimal efficiency [50,57–61]. Point mutations in SNARE regions remote from the scissile bond can dramatically reduce LC efficiency [60,62–64]. Recent findings, including the structure of a BoNT/A–SNAP-25 complex [64], finally provided some insight into the basis of LC substrate selectivity.

### New structural details of clostridial light chains

Crystal structures of several substrate-free CNT proteases have been determined, including those of BoNT/A [52,65], BoNT/B [53,66], BoNT/E [54] and TeNT [55,56]. In spite of targeting different SNARE sites (with the exception of BoNT/B and TeNT), the LCs share the same overall fold with differences primarily limited to surface features. The LCs all contain conserved catalytic cores that are structurally related to the general metalloprotease thermolysin [55]. However, the striking overall similarity of the LCs, especially their active sites, provided little insight into their ability for target discrimination. The first X-ray structure of a CNT–substrate complex,

that of the BoNT/A–SNAP-25 complex, has recently revealed that BoNT/A depends on an extensive array of exosites (substrate-binding sites remote from the active site) to provide a substrate-specific boost to catalytic efficiency (Figure 1c) [64]. 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 (Figure 1c), and the C terminus of the substrate 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 identification of novel substrate binding sites on the surface of BoNT/A, such as the  $\alpha$ -exosite (Figure 1c), presents new opportunities for the structure-based design of specific inhibitors of BoNT/A proteolytic activity. Based on the high structural and sequence similarity amongst the clostridial LCs, we speculate that other serotypes use similar mechanisms for substrate recognition [55].

Notably, the structure of the BoNT/A–SNAP-25 complex illustrates the extent of substrate that must be available for efficient proteolysis to occur. In spite of the fact that BoNT/A cleaves only nine residues from the C-terminus of SNAP-25, numerous exosite-binding residues span nearly sixty residues of the SNAP-25 C-terminal core domain (Figure 1c). 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 or bound to a regulatory factor. Interestingly, CNT proteolysis of SNAP-25 might not affect the initial stages of SNARE complex formation; recent results indicate that only the N-terminal core domain is involved in the formation of a  $\text{Ca}^{2+}$ -sensitive binary complex with syntaxin before synaptobrevin binding [67]. The extent of SNARE interactions with other CNT serotypes remains to be determined, but kinetic analyses indicate a similar behavior, in which other CNTs require 30–60-residue stretches of their substrate, regardless of scissile-bond location [50,58,59].

### Initial prospects for light-chain inhibitors

Small-molecule and peptidomimetic inhibitors of CNT LCs are currently being developed. High-throughput random-library screening has recently uncovered several non-peptidic compounds that inhibit BoNT/A-metalloprotease activity with  $K_i$  values in the low micromolar range [5,68]. Substrate-analog inhibitors of BoNT/A have been refined to a  $K_i$  of 330 nM [69,70]. Similar approaches have yielded promising leads for BoNT/B protease inhibitors [6,71]. Although several low- $K_i$  compounds have been identified, in many cases their specificities for their intended targets have yet to be verified. Furthermore, substrate-analog-inhibitor design has largely focused on the peptide sequences that are proximal to the SNARE scissile peptide bonds. The design of peptidic, peptidomimetic or small-molecule compounds that bind to

substrate-recognition exosites could be an attractive alternative strategy.

Compounds that target LC exosites, rather than active sites, might have the advantage of enhanced specificity. By contrast, inhibitors that act at LC active sites might also turn out to be effective inhibitors of other  $Zn^{2+}$ -dependant metalloproteases that use the same  $Zn^{2+}$ -binding motif. Furthermore, inhibitors that bind to specific toxin exosites enable the possibility of selectively blocking activities of individual CNT serotypes. It remains to be determined how and where the current small-molecule inhibitors identified through random screens are actually binding to the LCs. It is desirable to obtain additional high-resolution structures of CNTs in complex with their substrates to assist in the development of novel compounds that block CNT activity by targeting substrate-recognition exosites. Similarly, structural analyses of CNT proteases in complex with presently available small-molecule inhibitors might prove valuable for suggesting chemical modifications to improve their  $K_i$  values and specificity.

### Concluding remarks

The remarkable specificity of the CNT endopeptidases is now known to be attributable to the existence of multiple substrate binding sites that are remote from their catalytic pockets. Although the effects of CNT intoxication are temporary and fatality can typically be avoided with proper respiratory support and the administration of antitoxin, compounds that directly inhibit the action of CNT proteases might prove to be a faster, less care-intensive method for treating botulism and tetanus. Furthermore, novel small-molecule and/or peptidic inhibitors might function where antitoxins cannot, such as within the nerve cytosol, and with less risk of allergic reaction. A detailed understanding of the interactions between CNT proteases and their substrates provides an important step towards a more rational approach to design potent and specific inhibitors.

### Acknowledgements

Support by the National Institutes of Health is acknowledged (1-RO1-MH063105-01 to ATB).

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