Botulinum Toxins: Molecular Structures and Synaptic Physiology

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Introduction

Botulinum neurotoxin (BoNT) was identified as the sole cause of botulism over a century ago, after the discovery of the anaerobic and spore-forming bacteria of the genus *Clostridium* [1]. Botulinum neurotoxins are produced by different *C. botulinum* strains, which belong to four phylogenetically distinct groups, and by *C. butyricum* and *C. barati* and are historically classified into seven different serotypes (BoNT/A to /G) based on their immunological properties. Among the seven BoNT serotypes, types A, B, E, and F are associated with botulism in both humans and animals, whereas BoNT/C and /D primarily cause disease in domestic animals. BoNT/G-producing organisms have been isolated from soil but never reported as the cause of botulism. Recently, thanks to the development of next-generation sequencing, many toxin variants named subtypes have been identified within each serotype (distinguished using an alpha-numeric code BoNT/A1, /A2, etc.) and much more are expected to be reported soon [2, 3]. BoNTs bind with high affinity to peripheral cholinergic nerve terminals and enter into their cytosol where they cleave SNARE proteins thus blocking the release of neurotransmitters. The high potency and neurospecificity, the very limited diffusion when locally injected, and the reversibility of action have rendered BoNT/A1 the safest and most efficacious therapeutic for the treatment of a variety of human pathological conditions characterized by hyperfunction of selected nerve terminals. Their clinical use has been continuously expanding since its introduction in the 1970s,

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including treatment of movement disorders such as focal and segmental dystonias and spasticity and cosmetic treatments based on the blockade of hyperactive cholinergic motor nerve terminals [4–8]. The ability of the BoNTs to block the cholinergic autonomic nerve endings innervating salivary and sweat glands provided an effective therapy for hypersalivation and hyperhidrosis [9]. In addition to the blockade of the acetylcholine secretion, animal experiments indicate BoNTs induce blockade of transmitters involved in pain perception, transmission, and processing, and this is on the basis of the expanding pain indications of BoNTs recently explored in humans [10]. This review aims to describe the recent structural and mechanistic studies that have advanced our understanding of BoNT entry and trafficking in nerve cells. These achievements, together with the identification of several toxin variants, can be exploited both to explore new clinical applications and to design novel toxin inhibitors that block a step of intoxication common to the different toxin variants.

Molecular Architecture

BoNTs are produced by bacteria together with nontoxic accessory proteins (NAPs) to form high molecular weight progenitor complexes of various sizes (up to 900 kDa) named PTCs. NAPs include a non-toxic non-hemagglutinin component (NTNHA), which forms with the neurotoxins a hand-in-hand shaped heterodimer, and several hemagglutinin components (HAs) or OrfX proteins. The crystallographic structures of PTCs of some toxin serotypes have been recently defined ([11], [12, 13]) and suggest for NTNHA a protective role of the neurotoxin from the hostile gastrointestinal tract environment after toxin ingestion and from the many proteases present in decaying biological materials where BoNT is produced (for review see [1]). Conversely, HA proteins of PTCs present multiple carbohydrate-binding sites which are likely to act as adhesins binding the intestinal mucus layer and the polarized intestinal epithelial cells of the intestinal wall through which BoNTs enter into the lymphatic circulation and then in the blood circulation [14, 15].

Despite existence of a high number of isoforms, all BoNTs are structurally similar and consist of two chains linked by a unique disulfide bond: a light chain (L, 50 kDa) and a heavy chain (H, 100 kDa). The complete crystallographic structures of three BoNTs (A1, B1, and E1) [16–18] reveal a modular architecture comprising three domains, which are functionally linked to the multi-steps mechanism of neuron intoxication by BoNTs (Fig. 1). The L chain is a zinc-metalloprotease that specifically cleaves the three SNARE proteins necessary for neurotransmitter exocytosis; the N-terminal HN domain assists the translocation of the L chain across the membrane of intraneuronal acidic vesicles into the cytosol; the C-terminal HC domain is responsible for presynaptic binding and endocytosis and consists of two sub-domains (HC-N and HC-C) with different folding and membrane binding properties.

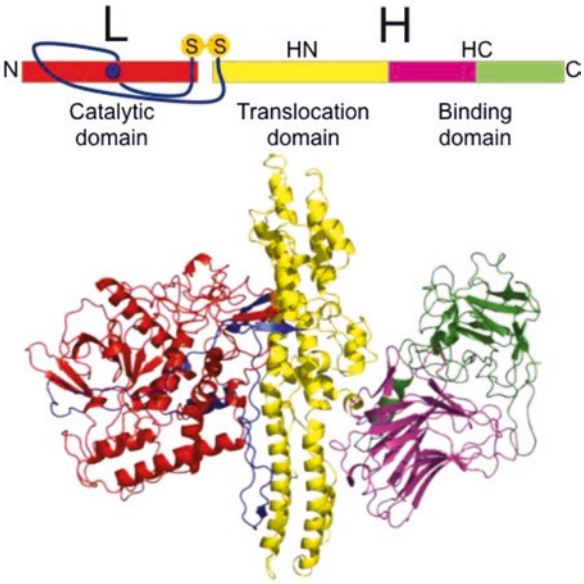

Fig. 1 Molecular structure of BoNT/A1. Schematic drawing and crystal structure of BoNT/A1 (PDB ID: 3BTA) [16] showing the organization of the three toxin domains: the binding domain which consists of the neurospecific binding HC-C sub-domain (*green*) and the lectin-like HC-N sub-domain (*purple*), the translocation HN domain (*yellow*), and the metalloprotease L domain (*red*). A peptide belt (shown in *blue*) surrounding the L domain, the atom of zinc at the center of the catalytic domain (*blue ball*) and the inter-chain disulfide bond (*orange*) linking the L and HN domain are also shown

Mechanism of Action

The structural organization of BoNTs has been designed by the evolution to deliver the catalytic L chain into the host cell cytosol through a mechanism of nerve terminal intoxication which can be conveniently divided into five major steps: (1) binding to nerve terminals, (2) internalization within an endocytic compartment, (3) low pH driven translocation of the L chain across the vesicle membrane, (4) release of the L chain in the cytosol by reduction of the interchain disulfide bond, and (5) proteolytic cleavage of SNARE proteins with ensuing blockade of neurotransmitter release and neuroparalysis (Fig. 2).

Binding to Nerve Terminals

After entering the lymphatic and blood circulations, the C-terminal part of the HC domain (sub-domain HC-C, 25 kDa) mediates the interaction of BoNTs with unmyelinated areas of motor neurons at the neuromuscular junction (NMJ), ensuring a rapid and strong interaction of the toxin with peripheral cholinergic nerve endings

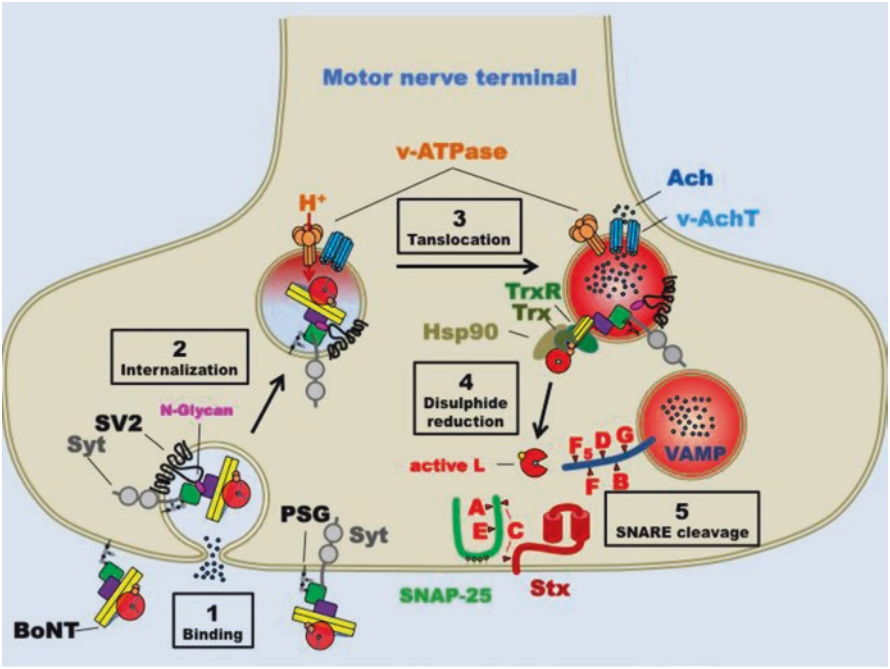

Fig. 2 Multi-steps mechanism of nerve terminal intoxication by botulinum neurotoxins. The first step (1) is the binding of the HC domain (*green*) to a polysialoganglioside (PSG) receptor of the presynaptic membrane (*gray* and *black*), followed by binding to a protein receptor. The currently known protein receptors are: (a) synaptotagmin (Syt, *gray*) for BoNT/B1, /DC, and /G; (b) glycosylated SV2 (*black* with its attached N-glycan in fuchsia) for BoNT/A1 and /E1. Syt may be located either within the exocytosed synaptic vesicle or on the presynaptic membrane. The BoNT is then internalized inside SVs. The acidification of the vesicle, operated by the v-ATPase proton pump (*orange*), drives the accumulation of neurotransmitter (*blue dots*) via the vesicular acetylcholine transporter (*light blue*). The protonation of BoNT leads to the membrane translocation of the L chain into the cytosol (3), which is assisted by the HN domain (*yellow*). The L chain (*red*) is released from the HN domain by the action of the thioredoxin reductase–thioredoxin system (TrxR–Trx, *green*) and Hsp90 (*mud color*), which reduces the inter-chain disulfide bond (*dark yellow*) and assists the refolding of the protease respectively (4). In the cytosol, the L chain displays its metalloprotease activity: BoNT/B, /D, /F, /G cleave VAMP (*blue*), BoNT/A and BoNT/E cleave SNAP-25 (*green*), and BoNT/C cleaves both SNAP-25 and syntaxin (Stx, *dark red*) (5). Each of these proteolytic events is sufficient to cause a prolonged inhibition of neurotransmitter release with consequent neuroparalysis

[1, 19, 20]. HC-C is responsible for the neurospecific binding to a polysialoganglioside and to the luminal domain of a synaptic vesicle protein [19, 21]. The latter has been defined in molecular details for BoNT/B1, BoNT/G, and the hybrid BoNT/DC which bind segment 40–60 of the luminal domain of the synaptic vesicle protein synaptotagmin (Syt) and for BoNT/A1 and BoNT/E1, which in contrast bind specifically to two different segments of the fourth luminal loop of the synaptic vesicle glycoprotein SV2 (for a complete list of references see [1, 21]). It has been also recently shown that BoNT/A1 binding to neuronal glycosylated SV2C involves a protein–protein and a protein-N-glycan interaction [22]. Biophysical, cellular, and functional studies

demonstrated that SV2 glycans are essential for BoNT/A1 binding to neurons and its extreme toxicity at the motor nerve terminals. The knowledge of the molecular details of BoNT/A-SV2 drives the development of high-affinity peptides to interfere with toxin binding and therefore to counteract BoNT/A intoxications [22].

Such a dual receptor binding to polysialogangliosides and to a protein receptor ensures a higher binding affinity and is required for the ensuing internalization and trafficking of the toxin within endocytic compartments, which is initiated by synaptic vesicles (SV) retrieval after the release of their neurotransmitter content [19, 21].

Internalization and Trafficking

The BoNT binding to the luminal domain of SV membrane proteins and their synaptic activity-dependent uptake strongly suggest that most of them are endocytosed at nerve terminals inside these organelles. Indeed, after intramuscular injection, BoNT/A1 is rapidly internalized and found in the average number of one–two molecules of toxin inside the lumen of SV within the neuromuscular junction [23]. Therefore, BoNT/A, and probably also the other toxin serotypes, use SV as "Trojan horses" to enter motor neuron terminals in vivo. In fact, during neurotransmitter (NT) release, the lumen of the SV is transiently opened (exposing the luminal domains of the BoNT protein receptors to the outside) and a complex cascade of protein–protein and protein–lipid interactions trigger the recruitment of clathrin and adaptor proteins to the inner leaflet of the plasma membrane, which marks the onset of the endocytic process and thus the SV retrieval [24]. After internalization and uncoating, SV is refilled with NT, a process driven by the electrochemical proton gradient that is generated by the vesicular ATPase proton pump, and the next cycle of neurotransmission begins. Although BoNT activity is mainly restricted to distal synapses and the role of the synaptic vesicle cycle in BoNT/A1 internalization is unquestionable, many recent lines of evidence suggest that synaptic activity-independent, "alternative" pathways also contribute to BoNT/A1 internalization and direct the neurotoxin through retroaxonal transport mechanisms to the central nervous system (CNS). Recently, internalization of BoNT/A1 in a subpopulation of non-recyclable synaptic vesicles whose fate could be to generate retrograde carriers has also been proposed [25]. A more detailed understanding of these direct effects of BoNTs on central circuits will provide valuable information for present and future uses of these neurotoxins in clinical practice [26].

Toxin Translocation

In order to reach their intracellular targets in the cytosol of nerve cells, the catalytically active L domain must be translocated from the SV lumen into the cytosol. The low pH inside the SV lumen induces a structural change of the HN domain leading to its insertion into the membrane and thus an ion translocation channel is formed

that assists the passage of the partially unfolded L from the lumenal to the cytosolic side of the SV membrane [1, 27, 28]. The disulfide bridge that links the heavy and light chain must remain intact on the luminal side of the vesicle until the last stage of L translocation [29]. Once it has reached the cytosolic face of SV membrane, the L chain has to reacquire the native structure in order to cleave its substrate and it has been recently shown that the host chaperone heat shock protein 90 (Hsp90) assists the refolding of the L chain after vesicle membrane translocation as already demonstrated for other bacterial toxins such as diphtheria toxin [30, 31]. L remains attached to the SV until the interchain disulfide bond is reduced in the reducing environment of the cytosol, a crucial step for productive release of the L catalytic subunit, which is common to all the BoNT variants [32].

Disulfide Bond Reduction

Host cells possess several redox systems and it was recently found that thioredoxin reductase-thioredoxin (TrxR-Trx) system is responsible for the reduction of disulfide bond of all BoNTs and that it physically interacts with the Hsp90 chaperone on the cytosolic surface of SV, which is the site of toxin translocation [30, 32]. Indeed, inhibitors of the TrxR-Trx redox system prevent the intoxication by BoNTs of neurons in culture and more importantly, largely prevent the BoNT-induced paralysis in mice in vivo, regardless of the serotype involved [32–34]. Moreover, the synergistic effect of Hsp90-specific inhibitor geldanamycin with PX-12, an inhibitor of thioredoxin, indicates that this TrxR-Trx-Hsp90 chaperone-redox machinery, which is exploited by all BoNTs to deliver their catalytic domain into the cytosol, can be considered as a target for drug discovery to prevent and treat botulism, regardless of the serotype (or the subtype) causing the intoxication [30].

Proteolysis of SNARE Proteins

The L chains of BoNTs are metalloproteases with an atom of Zn^{2+} bound to the motif HExxH at the center of the molecule that once released in the cytosol of the nerve terminal, cleave one of the three SNARE proteins: the vesicle-associated membrane protein VAMP, or the presynaptic membrane proteins SNAP-25 or Syntaxin (for a review see [1]). These three proteins form a heterotrimeric coil-coiled SNARE complex, which represents the core of the neuroexocytosis apparatus [35]. The BoNT proteolytic activity is highly specific and directed toward unique peptide bonds within the sequence of their respective SNARE protein targets. BoNT/B, /D, /F, /G cleave VAMP, BoNT/A and BoNT/E cleave SNAP-25, and BoNT/C cleaves both SNAP-25 and syntaxin. In most cases, BoNT cleavage results in the loss of a large part of the cytosolic portion of SNARE proteins, thus preventing the formation of the SNARE complex. In contrast, in the case of BoNT/A and

BoNT/C, the truncated SNAP25 proteins retain most of their sequences (197 and 198 of 206 amino acid residues, respectively) and are capable of forming stable, though non-functional, SNARE complexes. In any cases, the proteolysis of one SNARE protein prevents the formation of a functional SNARE complex and, consequently, the release of neurotransmitter with ensuing neuroparalysis [36, 37]. All subtypes characterized so far share the same cleavage site of the parent serotype with the exception of BoNT/F5 and a chimeric toxin BoNT/FA, which cleave a different peptide bond of VAMP with respect to BoNT/F1 [38, 39]. However, the available evidence suggests that BoNT/A subtypes have different enzymatic rate [40, 41] and it is, therefore, possible that individual subtypes are highly variable in their potency, onset, and duration of action. The exquisite target specificity of botulinum neurotoxins is due to the unique mode of recognition of VAMP, SNAP-25, or syntaxin by the L chain, which involves multiple interactions of the metalloprotease with its substrate including the cleavage site as well as exosites located along the sequence both before and after the hydrolyzed peptide bond [42, 43]. Different SNARE isoforms coexist within the same cell [44], but only some of them are susceptible to proteolysis by the BoNTs and it has been shown that resistance is associated with mutations in the cleavage site or/and at substrate/enzyme binding exosites [45]. The substrate/enzyme co-crystal structures revealed an extensive interface between protease and the SNARE protein and indicate that the multiple interactions sites remote from the L chain active site bring the cleavage region of the substrate close to the L active site [46, 47]. Structural studies using different length substrates or peptidomimetic inhibitors have demonstrated that the BoNT active site has a high degree of plasticity and will adopt different conformations in response to different substrates or to diverse peptide-based inhibitors [48, 49]. The dynamic nature of BoNT active site and the peculiar mode of binding with extensive enzyme–substrate interface explain why long peptide substrates are needed to test the proteolytic activity of the L chain in vitro and also the current lack of specific and high-affinity inhibitors of their metalloprotease activity [43].

Reversibility of Neuroparalysis Induced by BoNTs and Neuromuscular Junction Plasticity

An important feature of BoNT intoxication is its reversibility in vivo. Indeed, the toxin cleaves a SNARE protein as long as it remains intact in the nerve cytosol, but this activity causes neither neuronal cell death nor axonal degeneration in the intoxicated animal, though the animal may die of respiratory failure. Indeed if a botulism patient is kept under mechanical ventilation and appropriate pharmacological treatments, eventually he/she recovers completely, following the inactivation of the toxin and the replacement of the cleaved SNARE [50]. The duration of the BoNT inhibitory effect varies with serotypes [51] and it contributes to determining the severity of human botulism (type A > type B ≫ type E) [50, 52].

The main determinant of the duration of neuroparalysis is the L chain lifetime within the terminal and it appears that BoNT/A1 L chain, which has a very remarkable persistence, has a longer lifetime than that of BoNT/E1 because BoNT/E1 L chain is ubiquitinated and targeted to the ubiquitin-proteasome system, whilst BoNT/A1 L chain escapes the action of the cell degradation system by recruiting de-ubiquitinases, i.e. specialized enzymes that remove polyubiquitin chains [53, 54]. In addition to the protease persistence in the cytosol, other factors come into play to determine the duration of action, including the maintenance of truncated SNARE proteins within motor neurons. In fact, the longer duration of the effects of BoNT/A1 and /C1 is also explained, as anticipated above, by the inhibitory action of the truncated SNAP-25 which persist for long time periods at the synapse [36, 55] and the shorter duration of BoNT/E1 is likely determined by the rapid turnover of its truncated SNAP25 [56–58]. New understanding of the mechanisms by which these remarkable toxins or their proteolytic products persist within their motor neuron targets will help to develop, on one hand, BoNT-based therapeutics with improved persistence properties and therefore produce a longer clinical benefit, and on the other hand, BoNT-antidotes which accelerate the toxin degradation and therefore reverse BoNT intoxication.

In addition to the type of BoNT and to the product of SNARE proteolysis, the duration of the paralysis depends also on the dose, the animal species, the mode of administration, and the type of nerve terminal. Regarding the latter, it is known that the local injection of BoNT/A1 and BoNT/B1 at the human autonomic cholinergic nerve terminals induced a longer duration of neuroparalysis (even more than a year) with respect to the shorter duration of action in skeletal nerve terminals (3/4 months in humans) [9]. Since the BoNT poisoned NMJ undergoes a profound remodeling in which Schwann cells play a crucial and active role, the duration of paralysis is also determined by the response of peripheral Schwann cells (which are not present at the autonomic nerve terminals) to the blockade of neuroexocytosis. Indeed, under the effect of growth factors released by Schwann cells and muscles, the motor end plate enlarges and sprouts develop from the unmyelinated motor axon terminal and from the first node of Ranvier [59, 60]. These nerve sprouts follow projections that emerge from perisynaptic Schwann cells, which multiply and migrate from the original NMJ to other sites of the sarcolemma soon after inactivation of the motor axon terminal. New contacts with the muscle fiber are formed. The number of motor end plates on single muscle fibers increases as well as the number of fibers innervated by a single motor axon. Moreover, in the muscle, BoNTs induce alterations similar to those documented in other forms of denervation with fiber atrophy appreciable already in the first 2 weeks after BoNT injection. The new synapses, though immature, can sustain vesicle recycling [60, 61], but are poorly efficient in ACh release [62], providing a limited contribution to the recovery of the neurotransmission from nerve to the muscle fiber. Once a certain level of functionality is re-established at the original site, terminal and nodal sprouts are pruned, and the newly formed synaptic specializations are eliminated [50].

Concluding Remarks and Future Perspective

Botulinum neurotoxins combine potency and specificity with full reversibility at the cellular level, and these unique properties are on the basis of their clinical use. The recent understanding of their detailed modular structure and of their multi-step molecular mechanism of neuron intoxication together with advances in the techniques for production of recombinant proteins, have opened up the opportunity to modify the binding specificity, affinity, and nerve terminal persistence in order to improve their properties in terms of cell targeting and duration of action [63–65]. The duration of BoNTs activity assumes a paramount significance with respect to their therapeutic use because long-lasting BoNTs require fewer injections and lower doses, limiting the possibility of immunization. Moreover, the recent identification of many new toxin variants foster researchers to characterize their biological activity and it is likely that novel BoNTs with improved and/or different therapeutic targets/properties in terms of potency and duration of action will be discovered in the near future and will constitute potential goldmine to be exploited for new clinical applications.

Eventually, the recent identification of inhibitory molecules, which block common steps of nerve intoxication mechanism such as translocation or the reduction of the interchain disulfide bond can be considered as lead compounds for the development of pan-inhibitors of BoNTs regardless of the toxin variant causing intoxication [30, 32, 34].

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