Botulinum Neurotoxins: Mechanism of Action

O. Rossetto, M. Pirazzini, F. Fabris, and C. Montecucco

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Abstract

Botulinum neurotoxins (BoNTs) are a growing family of bacterial protein toxins that cause botulism, a rare but often fatal animal and human disease. They are the most potent toxins known owing to their molecular architecture, which underlies their mechanism of action. BoNTs target peripheral nerve terminals by a unique mode of binding and enter into their cytosol where they cleave SNARE proteins, thus inhibiting the neurotransmitter release. The specificity and rapidity of binding, which limits the anatomical area of its neuroparalytic action, and its reversible action make BoNT a valuable pharmaceutical to treat neurological and

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S. M. Whitcup, M. Hallett (eds.), *Botulinum Toxin Therapy*,
Handbook of Experimental Pharmacology 263, https://doi.org/10.1007/164_2020_355

non-neurological diseases determined by hyperactivity of cholinergic nerve terminals. This review reports the progress on our understanding of how BoNTs cause nerve paralysis highlighting the different steps of their molecular mechanism of action as key aspects to explain their extreme toxicity but also their unique pharmacological properties.

Keywords

Botulinum neurotoxins · Botulism · Neuromuscular junction · Neuroparalysis

1 Introduction

The two proteins most largely used in human therapy are the immunoglobulins (Ig) and the botulinum neurotoxins (BoNTs). Apart from sharing the molecular weight of 150 kDa, they have different structures and mechanisms of action. The Ig act by binding specifically their target molecules present on the cell surface or dispersed in body fluids, whilst the BoNTs bind to cholinergic nerve terminals and then enter the cytosol where they catalytically inactivate by proteolysis three protein, dubbed SNAREs, involved in the release of neurotransmitter, thus causing neuroparalysis. Accordingly, minute doses of BoNTs are capable of counteracting diseases caused by hyperfunctioning nerve terminals, and their action is based on a unique set of molecular properties that will be described in the present chapter.

BoNTs are produced by bacteria of the genus *Clostridium* though other bacteria of different classes and even phyla may harbour the gene encoding for BoNT and BoNT-like proteins. They consist of two chains (L, 50 kDa and H, 100 kDa) linked by a single SS bridge. They are produced in eight different serotypes (indicated by letters: BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F, BoNT/G and BoNT/X) (Rossetto et al. 2014; Dong et al. 2019). Many subtypes of serotypes are known (dubbed with a suffix number: BoNT/A1, BoNT/A2, etc.) plus chimeric neurotoxins (BoNT/CD, BoNT/DC, BoNT/FA) for a total of many dozens of different toxins (Peck et al. 2017). This figure is bound to increase with expanding DNA sequencing of known bacteria and of novel isolates (Doxey et al. 2018, 2019).

The BoNT molecule shown in Fig. 1b is complexed with a homologous protein dubbed NTNHA (in orange in Fig. 1a), devoid of protease activity, forming a heterodimer that is much more stable than BoNT alone to the acidic and proteolytic conditions found in the gastrointestinal tract (Gu et al. 2012). This heterodimer assembles with accessory non-toxic proteins (Fig. 1a) to form progenitor toxin complexes (PTCs-BoNT/A1 $\approx$ 500–900 kDa), more stable at acidic pH. PTCs rapidly dissociate under slightly alkaline physiologic solutions. The accessory non-toxic proteins are believed to mediate the binding of the complex to the intestinal mucus and then to assist the translocation of the BoNT molecule from the intestinal lumen, across the mucus layer and the polarized epithelial monolayer, into the mucosa in the food-borne and infant forms of botulism (Lam and Jin 2015; Sugawara et al. 2010; Rossetto et al. 2014). The free BoNT then diffuses via lymphatic and blood circulations to the entire body and binds preferentially to

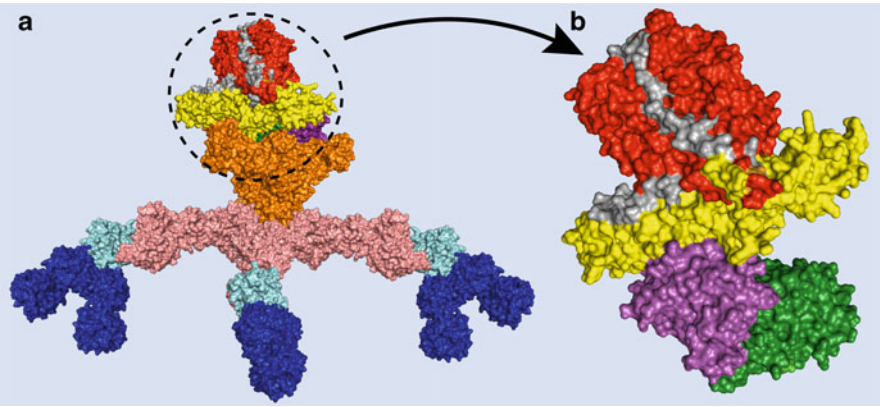

Fig. 1 Molecular structure of BoNT/A1 and its progenitor toxin complex. (**a**) 3D structure of the large progenitor toxin complex (L-PTC) composed of BoNT/A1 on the top, the NTNHA (orange) and the HA complex composed of HA70 (pink), HA17 (cyan) and HA33 (blue). (**b**) Zooming of BoNT/A1 150 kDa molecule showing the organization of the three toxin domains: the neurospecific binding Hc-C sub-domain (green), the lectin-like Hc-N sub-domain (purple), the translocation HN domain (yellow) and the metalloprotease L domain (red). A peptide belt (shown in white) surrounding the L domain and the interchain disulphide bond (orange) linking the L and HN domain are also shown

cholinergic neurons, but do not cross the blood-brain barrier. The intoxication of neurons of the myenteric plexus causes a block of their release of neurotransmitters, thus halting intestinal peristalsis, which is a major symptom in food-borne and in infant botulism (Chatham-Stephens et al. 2018). However, the inhibition of the respiratory function is the major single failure causing death by botulism in humans.

2 Botulism and Toxicity of Botulinum Neurotoxins

Botulism was first described by Kerner in Southern Germany about 200 years ago following episodes of flaccid paralysis and death that afflicted people that had shared contaminated ham and sausages. In 1897 Emile van Ermengem in Belgium demonstrated that the disease was due to a bacterium that produced a powerful poison of peripheral nerves causing the flaccid paralysis characteristic of botulism (Erbguth 2004). Botulism is rare but potentially fatal, and the death rate depends on the capability of identifying the symptoms at hospital admission (Sobel 2005; Fleck-Derderian et al. 2017). The first symptom is the paralysis of cranial nerves with ocular and facial palsy, diplopia and ptosis, dysphagia and dysarthria, followed by a descending flaccid paralysis that includes the neck muscles and the respiratory muscles, which lead the most frequent cause of death: deficient respiration. Additional symptoms are due to paralysis of the autonomic cholinergic nerves with abdominal pain, vomiting, nausea, dry mouth and dizziness. The display of symptoms and the time period intervening between intoxication and development

of overt symptoms vary depending on the amount and type of BoNT and on the toxin route of entry (Sobel 2005; Rossetto et al. 2014; Fleck-Derderian et al. 2017; Chatham-Stephens et al. 2018).

The botulism patient is conscious but cannot operate any muscle. If the disease is rapidly diagnosed and respiration is mechanically assisted, the patient survives and recovers almost completely after a variable period of time, depending on dose and type of BoNT: from the 1–2 weeks of BoNT/E to the several months of BoNT/A1 which is the longest acting BoNT so far known. However, damages can result from prolonged external ventilation and variable levels of permanent fatigue may follow the long periods of paralysis.

Botulism is caused solely by the BoNT activity. BoNT is the most poisonous substance known (Rossetto and Montecucco 2019), and this toxicity is due to its neurospecificity and to the neuroparalysis that results from the catalytic action of the metalloprotease L domain in the nerve cytosol. For these reasons, BoNTs are included in the list A of substances with a possible bioterrorist use (Arnon et al. 2001; Bhattacharjee 2011). At the same time, their neurospecific high affinity binding, the reduced spreading of paralysis after injection and the reversibility of the induced neuroparalysis are the basis of the ever-growing therapeutic and aesthetic use of BoNT/A1. In addition, BoNT/B1 has been used in human therapy, and several other BoNTs may follow owing to specific properties useful to treat particular pathologies. Therefore, BoNTs completely fulfil the definition elaborated by Claude Bernard (1866) "Poisons are chemical scalpels to dissect physiological processes" and his prophetic prediction "Powerful poisons will surely become therapeutics, but only after their chemical composition is determined". Indeed BoNT/A1 local injection is the therapy of choice for the treatment of a variety of human pathologies and conditions characterized by hyperfunction of peripheral cholinergic nerve terminals and in plastic surgery (Dressler 2012; Pirazzini et al. 2017; Gart and Gutowski 2016).

Toxicity of botulinum toxins is generally measured as the mouse lethal dose 50% (MLD50), defined as the dose that kills 50% of mice within 4 days, after a single intraperitoneal injection. The MLD50 values vary in the range 0.01–5 ng/kg depending on the BoNT type and in minor proportion on the mice strain. The human lethal dose can be extrapolated from data obtained with primates. For a 70 kg man, the lethal doses are 90–150 ng when injected intravenously, 800–900 ng when inhaled and about 70 μg when introduced orally (Arnon et al. 2001; Rossetto and Montecucco 2019). Recently a large number of in vitro *assays* have been developed to avoid the use of animals in testing BoNT potency and toxicity, and they have been critically discussed by Pellet et al. (2019).

3 The Structural Architecture of Botulinum Neurotoxins

Notwithstanding the large number of serotypes, chimeras and subtypes, BoNTs have a very similar 3D structure, which is strictly linked to their common mechanism of intoxication of nerve terminals. Figure 1b shows the structure of BoNT/A1 (Lacy

et al. 1998; Dong et al. 2019), the toxin predominantly used in human therapy. It is organized in four distinct domains endowed with different functions in nerve terminal intoxication and paralysis (Lacy et al. 1998). The crystal structure of other BoNTs has been determined, and it is very similar apart from a displacement of the third and fourth domains in BoNT/E with respect to A1 (Montal 2010; Swaminathan 2011).

BoNTs display a unique mode of binding to the presynaptic membrane to ensure specificity, high affinity and rapidity of binding (Montecucco 1986; Fogolari et al. 2009). This is achieved by the carboxyl terminal domain (Hc-C, 25 kDa, green in Fig. 1b) which contains one conserved binding site for a polysialoganglioside receptor, which is highly enriched in the nerve presynaptic membrane, and to a second receptors that is present on the luminal side of the membrane of synaptic vesicles (Rummel 2013). Hc-C is linked to a lectin like domain (Hc-N, 25 kDa, purple in Fig. 1b) whose role in binding has not been yet clarified though there is evidence that it binds to microdomains of the membrane (Muraro et al. 2009; Zhang and Varnum 2012).

The Hc-N domain is linked, with little protein-protein interaction, to the HN domain (50 kDa, yellow in Fig. 1b) which in turn is linked to the metalloprotease domain termed light chain (L, 50 kDa, red in Fig. 1b) via a long belt that encircles the L domain and by a unique disulphide bond (grey in Fig. 1b). As Hc-C plus Hc-N plus HN make a single polypeptide chain termed heavy chain (H, 100 kDa), this disulphide is termed interchain, and it is important because it is involved in membrane translocation and it prevents the metalloprotolytic activity of L until it is released in the cytosol upon its reduction (see below in Sect. 4.4).

4 The Conserved Mechanism of Nerve Terminal Paralysis by the Botulinum Neurotoxins

4.1 Neurospecific Binding (Step 1)

Biological evolution of these neurotoxins has led to a structural organization designed to deliver the metalloprotease domain into the cytosol of nerve terminals. This remarkable achievement has been attained by exploiting several physiological functions of nerve terminals. On the basis of the presently available experimental notions, the BoNT mechanism of nerve terminal paralysis consists of the five major steps, depicted in Fig. 2: (1) binding to cholinergic nerve terminals, (2) entry inside recycling synaptic vesicles (SV), (3) crossing of the vesicle membrane by the L domain by exploiting the pH gradient (acid inside) across the membrane, (4) release of L in the cytosol by reduction of the interchain disulphide bond and (5) cleavage of one or more of the three proteins that form the SNARE heterotrimeric complex that is essential for the fusion of synaptic vesicle with the presynaptic membrane, thus releasing their neurotransmitter content (Pantano and Montecucco 2014).

After entering the lymphatic and blood circulations, following intestinal absorption or inspiration or injection, the BoNTs rapidly gain access to the perineuronal

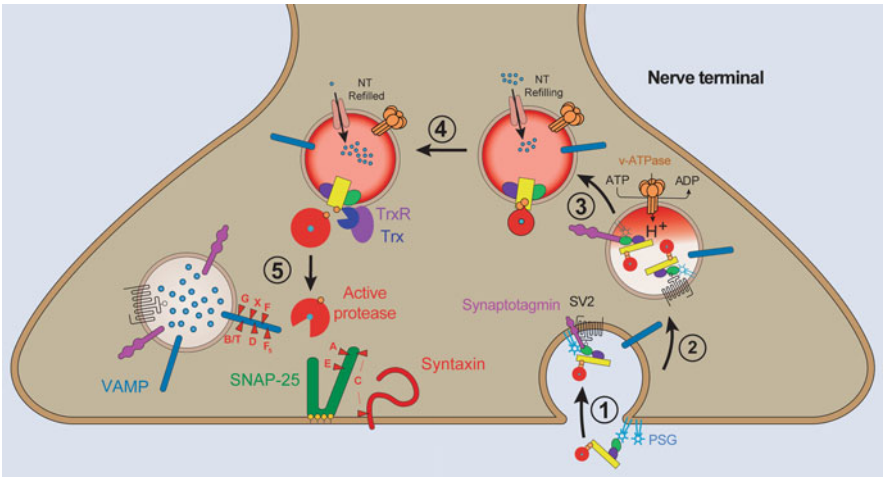

Fig. 2 Mechanism of botulinum neurotoxins entry and paralysis of nerve terminals. The paralysis of nerve terminals by botulinum neurotoxins is a multistep process. The first step (1) is the binding of the HC-C domain (green) to a polysialoganglioside (PSG, light blue) receptor of the presynaptic membrane, followed by binding to a protein receptor of the lumen of synaptic vesicles. The currently known protein receptors are (i) synaptotagmin (magenta) for BoNT/B1, BoNT/DC and BoNT/G; (ii) glycosylated SV2 (black with its attached N-glycan depicted as a hexagon) for BoNT/A1 and BoNT/E1. The BoNT is then internalized inside SVs (step 2). Step 3 begins with the acidification (red) of the vesicle lumen caused by the v-ATPase (orange), which generates a pH gradient that drives the accumulation of neurotransmitter (blue dots) via the vesicular neurotransmitter transporter (pink). The protonation of BoNT leads to the membrane translocation of the L chain into the cytosol which ends step 3. This process is assisted in an unknown way by the HN domain (yellow). Step 4 is the release of the L chain (red with a central light blue dot that represents the active site Zn^{2+} atom) from the HN domain by the action of the thioredoxin reductase – thioredoxin system (TrxR-Trx, violet and dark blue, respectively) which reduces the interchain disulphide bond (orange dots representing the two sulphur atoms). In the cytosol, the L chain displays its metalloprotease activity (step 5): BoNT/B, BoNT/D, BoNT/F, BoNT/G and BoNT/X cleave VAMP (blue); BoNT/A and BoNT/E cleave SNAP-25 (green); and BoNT/C cleaves both SNAP-25 and syntaxin (red). Each of these proteolytic events is sufficient by itself to cause a prolonged inhibition of neurotransmitter release with consequent neuroparalysis. This notion proves that VAMP, SNAP-25 and syntaxin are the core of the nanomachine that drives the release of neurotransmitters within the synaptic cleft

fluid compartment, without crossing the blood-brain barrier (Simpson 2013). The local intramuscular injection of very small doses (few MLD50s) of BoNT/A1 leads to a local paralysis, a property of high therapeutic value (Eleopra et al. 2004; Carli et al. 2009). The BoNTs bind very rapidly and with high affinity the presynaptic plasma membrane of skeletal and autonomic cholinergic nerve terminals. The high affinity is due to a double receptor binding: (a) to the oligosaccharide portion of a polysialoganglioside and (b) to the intra-vesicular domain of synaptic vesicle proteins (SV2 for BoNT/A, BoNT/E and BoNT/F or synaptotagmin for BoNT/B, BoNT/DC and BoNT/G). The neurospecific binding of BoNT/C and BoNT/D is not well characterized, but there is evidence that oligosaccharides of glycolipids or

protein-linked N-glycans are involved together with a hydrophobic loop of the toxin that inserts in the lipid bilayer (Nuemket et al. 2011; Rossetto et al. 2014; Zhang and Varnum 2012; Stern et al. 2018; Dong et al. 2019). Once injected, particularly in the case of superficial injections or when small muscle are treated, a competition may be envisaged among entry into the blood or lymphatic circulation via capillaries and binding to the cholinergic terminals followed by the irreversible entry into nerve terminals. If this is the case, the BoNTs shall be capable of rapid membrane binding. In this respect, it is noteworthy that the BoNTs are electrical dipoles with the positive end very close to the PSG binding site and that the PSGs have a strongly negative oligosaccharide head that projects out of the presynaptic membrane like an antenna. In addition, the presynaptic membrane is negatively charged, and this will reorient the BoNT dipole whilst approaching the negatively charged membrane rendering almost any hit with the PSG binding productive (Fogolari et al. 2009). Once this has occurred, the BoNT-PSG complex may move laterally on the membrane to find the second receptor.

4.2 Entry into Nerve Terminals (Step 2)

The internalization of BoNTs is driven by the binding of BoNTs to their second receptor which is localized on the luminal side of the membrane of synaptic vesicles (SV). This binding occurs after the fusion of SV with the presynaptic membrane (Dong et al. 2019). This leads to the exposure of the SV lumen to the plasma membrane surface, whilst BoNT is already bound to PSG; in the case of BoNT/DC and BoNT/B, the hydrophobic loop present between the PSG and SV receptors binding sites additionally contributes to the nerve surface binding (Nuemket et al. 2011; Zhang et al. 2017; Stern et al. 2018).

After intramuscular injection without electrical stimulation, one/two molecules of BoNT/A1 are rapidly taken up and found by electron microscopy inside the lumen of SV at the neuromuscular junction and in neurons in culture (Colasante et al. 2013; Harper et al. 2016). SV exocytosis is strictly coupled to endocytosis, and this explains the fact that BoNT/A1 paralyses is faster in a synaptic terminals stimulated electrically or by exercise, whilst the lowering of synaptic activity prolongs the time of paralysis development (Hughes and Whaler 1962). Recent findings indicate that high activity levels of SV neurotransmitter release leads to SV fusion with incorporation of the SV membrane into the presynaptic membrane (Chanaday et al. 2019). In turn, this would result in an increased extent of exposure of the BoNT luminal SV receptors with a consequent increase of the internalized BoNT (not shown in Fig. 2). This BoNT would end in the lumen of a bulk endosome, rather than in the SV lumen, but SV will form rapidly by clathrin-mediated budding of SV from endosomes. Clearly, the recent novel findings on endocytosis at nerve terminals call for further studies to clarify the different forms of vesicular/endosomal trafficking of the different BoNTs into the nerve terminal. Such studies could lead to improved modes of delivery of BoNT to patients.

4.3 Synaptic Vesicle Membrane Translocation (Step 3)

SV are used as "Trojan horses" by BoNTs to enter inside nerve endings (Fig. 2). However, BoNTs have to exploit another physiological function of the synapse in order to perform the third step of intoxication leading to neuroparalysis. Indeed, BoNT parasitizes the refilling of neurotransmitter inside empty vesicles powered by the action of an ATPase proton pump present on the SV membrane which injects protons inside to create a transmembrane pH gradient that drives the uptake of neurotransmitter from the cytosol into the lumen (Fig. 2). The acidic pH also induces a structural change of BoNT, which falls on the membrane surface and then, in an unknown mode requiring the H chain, leads to the L domain crossing the SV membrane to the cytosolic surface where it remains attached via the SS bond. For more information on step 3, the reader is referred to Pirazzini et al. (2016).

4.4 Reduction of the Disulphide Interchain Bond (Step 4)

The SS interchain bond is exposed to the cytosol after translocation of the L domain, and it is specifically reduced by the NADPH-thioredoxin reductase-thioredoxin redox system (Trx-Tx), bound to the cytosolic surface of SV (Pirazzini et al. 2014). As shown in Fig. 2, this releases the metalloprotease L domain, which then exerts its catalytic activity on the three SNARE proteins that are essential for the SV fusion followed by neurotransmitter release. This notion led to an important translational potential application because inhibitors of the Trx-Tx redox system were found to prevent botulism acting on all BoNTs independently on the serotype and the subtype (Pirazzini et al. 2014; Zanetti et al. 2015; Rossetto et al. 2019). In fact, the different antigenicity of the many known BoNTs speaks against the possibility of using BoNT-specific antibodies to prevent botulism because too many human monoclonal antibodies should be generated. Inhibitors of Trx-Tx do prevent the BoNT-induced nerve terminal paralysis in vivo and are strong candidates for the prevention of botulism in humans and for the treatment of infant botulism and intestinal botulism which imply a continuous production of novel toxin molecules in the intestine (Rossetto et al. 2019).

4.5 SNARE Protein Cleavage (Step 5)

Once released from the cytosolic face of the SV membrane, the L domain is ready to display its Zn^{2+}-dependent proteolytic activity specifically directed to three target proteins: VAMP, SNAP-25 and syntaxin, as shown in Fig. 2. VAMP (vesicle-associated membrane protein, blue) is a protein spanning the SV membrane of and other vesicular cell organelles depending on the isoforms. The two isoforms principally involved in neurotransmitter release are VAMP-1 and VAMP-2. SNAP-25 (synaptosomal nerve-associated protein of 25 kDa, green) is mainly localized on the cytosolic face of the presynaptic membrane via a quartet of Cys residues located in

the middle of the protein esterified by four palmitoyl chains acting as hydrophobic anchors. Syntaxin (red in Fig. 2) is present in a number of isoforms spanning the plasma membrane and projecting its mass in the cytosol. These three proteins include a coil domain termed SNARE domain and upon coiling form a heterotrimeric SNARE complex, which is essential for the process of neurotransmitter release (Sutton et al. 1998; Jahn and Scheller 2006; Sudhof and Rothman 2009; Pantano and Montecucco 2014). The formation of the SNARE complex brings the SV close to the active zones of neurotransmitter release, ready to fuse when the [Ca^{2+}] trigger is elicited following the opening of the presynaptic membrane voltage-gated Ca^{2+} channels. BoNT/B, BoNT/D, BoNT/F, BoNT/G and BoNT/X cleave VAMP at single and different sites within the coiling domain. BoNT/C cleaves both syntaxin and SNAP-25, whilst BoNT/A and BoNT/E cleave SNAP-25 at different sites. These cleavages prevent the formation and/or function of the SNARE complex and, consequently, of neurotransmitter release; for detailed information on the specificity of BoNTs for the various isoforms of the three SNARE proteins and the cleaved peptide bonds, please see Pirazzini et al. (2017) and Dong et al. (2019). One amazing aspect of the hydrolytic activity of the BoNT metalloproteases is their absolute specificity for the three SNARE proteins which provided the first and strongest evidence of the essential role of these proteins in neurotransmitter release and vesicular trafficking within cells. This is based on a multiple substrate recognitions chain of the substrate molecule by the L chain which include the peptide bond to be cleaved and several sites located at a distance (Rossetto et al. 1994; Brunger and Rummel 2009; Pantano and Montecucco 2014). So far no other protein substrates of the BoNT L chains have been identified, but it cannot be excluded that ancestral forms of BoNT might cleave other essential cell proteins whose cleavage may provide an evolutionary advantage to the toxin-producing organism.

5 The Different Duration of Action of Botulinum Neurotoxins

The BoNTs do not kill the intoxicated neurons, but they paralyse them, and this paralysis is reversible with time. The duration of paralysis depends on the type of BoNT, on the dose and on the animal species. The proteins present in cells turn over with a half-life time which is characteristic of each protein. Similarly, the BoNT L chains are degraded in the cytosol, and the half-lives of the different BoNT serotypes and subtypes are different. This degradation is considered to be the main determinant of the duration of the BoNT-induced neuroparalysis because the progressive disappearance of the L chain allows for the renewal of its substrate with ensuing neurotransmission recovery. The duration of the BoNT/A1-induced neuroparalysis is the longest among BoNTs (3–4 months for human skeletal terminals, 12–-15 months for autonomic cholinergic nerve terminals), whilst the L chain of BoNT/E1 is the shortest living one (paralysis of skeletal terminal lasting about 2–4 weeks). The duration of action of BoNT/A1 in humans is of major importance because it determines the duration of its therapeutic effects. The exceptional length

of the paralysis exerted by BoNT/A1 is likely to be supported by effects additional to the L chain degradation. Indeed, there is evidence that the BoNT/A-cleaved SNAP-25 (SNAP-25$^{\#}$), which retains 197 over 206 amino acid residues, is still capable of forming a SNARE heterotrimer with VAMP and syntaxin, which is non-functional in neuroexocytosis but prevents the function of the normal SNARE complex. In other word, SNAP-25$^{\#}$ acts as a dominant negative that causes by itself neuroparalysis as long as it is present inside nerve terminals (Pantano and Montecucco 2014).

6 Long-Distance Effects of Botulinum Neurotoxins

Generalized peripheral neuroparalysis is the most evident symptom of botulism. However, indirect evidence that these neurotoxins could act at a distance from the injection site, i.e. within spinal cord and brain neuronal circuits, was reported long ago. Later on it was experimentally shown that retroaxonal transport of BoNTs does take place, similarly to tetanus neurotoxin (Mazzocchio and Caleo 2015). Compelling evidence of BoNT/A1 retrotransport to the central nervous system (CNS) was provided by tracing the cleavage of SNAP-25 within CNS neurons after peripheral injection of the toxin, using an antibody very specific for the novel epitope generated by the BoNT/A1 cleavage of SNAP-25 (Antonucci et al. 2008; Restani et al. 2012). BoNT/A1 retrograde transport can occur also via sensory neurons, as shown by the injection in the whisker pad which induces the appearance of truncated SNAP-25 in the trigeminal nucleus caudalis (Antonucci et al. 2008; Matak et al. 2011). These long-distance effects are mediated by an active retro-axonal transport of catalytically competent toxins inside motor axons or sensory neurons, and not by passive spread of BoNT/A1 or of SNAP-25$^{\#}$. Moreover, BoNT/A1 can undergo subsequent events of transcytosis and transport, remaining catalytically active (Antonucci et al. 2008; Matak et al. 2011; Restani et al. 2011). The spinal cord contains several cholinergic interneurons whose neurotransmitter release could be inhibited by the L chains of BoNTs (Miles et al. 2007; Zagoraiou et al. 2009; Ramírez-Jarquín and Tapia 2018) with the results of (1) a further peripheral paralytic effect and (2) alteration of the locomotor activity.

7 Future Directions

Although the major aspects of the cellular and molecular mechanism of action of BoNTs have been elucidated, some aspects are not completely understood and are matters of debate. One of the most intriguing topics is the discovery of genes encoding for many novel BoNTs and BoNT-like toxins. Genetic and bioinformatic methods are providing the tools to expand our understanding of the mechanisms underlying this diversity, but the biologic significance of such a large and growing number of BoNTs has not been explained yet. Therefore, ad hoc investigation should be performed in order to answer the fundamental question of the origin and possible

role(s) these toxins may have for the producing bacteria within their environments (Montecucco and Rasotto 2015).

Besides the evolutionary significance, the definition of the molecular, cellular, tissue, and pharmacological properties of the many novel botulinum toxins that are being discovered is not fulfilled yet. This is an important goal as, together with engineering of novel BoNTs endowed with specific properties and specificities, it will allow the development of novel therapeutics and protocols that will expand the medical uses of BoNTs. Another issue that deserves attention is that of the long-distance effects of BoNT/A1 consequent to its retro-axonal transport to the central nervous system.

Acknowledgements Work in the author's laboratory is supported by University of Padova, CNR and the Ministry of Defence research project RI.PA.NE.

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