The Botulinum Toxin as a Therapeutic Agent: Molecular Structure and Mechanism of Action in Motor and Sensory Systems

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Abstract

Botulinum neurotoxin (BoNT) produced by *Clostridium botulinum* is the most potent molecule known to mankind. Higher potency of BoNT is attributed to several factors, including structural and functional uniqueness, target specificity, and longevity. Although BoNT is an extremely toxic molecule, it is now increasingly used for the treatment of disorders related to muscle hyperactivity and glandular hyperactivity. Weakening of muscles due to peripheral action of BoNT produces a therapeutic effect. Depending on the target tissue, BoNT can block the cholinergic neuromuscular or cholinergic autonomic innervation of exocrine glands and smooth muscles. In recent observations of the analgesic properties of BoNT, the toxin modifies the sensory feedback loop to the central nervous system. Differential effects of BoNT in excitatory and inhibitory neurons provide a unique therapeutic tool. In this review the authors briefly summarize the structure and mechanism of actions of BoNT on motor and sensory neurons to explain its therapeutic effects and future potential.

Keywords
- botulinum neurotoxin
- sensory neurons
- longevity
- SNAP-25
- neurotransmitter

Botulinum neurotoxins (BoNTs), the most potent toxins known to mankind (the lethal dose for humans is 1 µg/kg by the oral, 10–13 ng/kg by the inhalational, and 1–2 ng/kg by the intravenous or intramuscular routes), are metalloproteases that act on nerve–muscle junctions to block exocytosis through a very specific and exclusive endopeptidase activity against soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins of presynaptic vesicle fusion machinery. Botulinum neurotoxins are produced by ubiquitous anaerobic bacteria, *Clostridium botulinum.* Clostridia are gram-positive, spore-forming bacteria widely present in soil, water, and the gastrointestinal (GI) tract of most animals and humans. Out of various species of Clostridia, *C. botulinum* produces a neurotoxin that causes the neuroparalytic disease botulism, a paralytic illness of motor neuron and autonomic nerves.[1] Toxin exposure generally occurs after ingestion of contaminated food with *C. botulinum* spores and colonization of the GI tract (infant botulism), or with preformed toxin (food poisoning). Of the seven known serotypes (A-G) of BoNT, epidemiologically only A, B, E, and F are known to cause human botulism. Food-borne botulism is the most common form of botulism in the United States (15% food borne, 65% infant, and 20% wound),[2] and BoNT-A is the most toxic among different serotypes.[3] Botulinum neurotoxin can be absorbed at one or more sites in the mouth to gut route, but most common is intestinal colonization of the bacteria. Botulinum neurotoxin can be detected in the serum 2 days to 2 weeks after the onset of symptoms.[4]

In 1928, Sommer and Snipe at the University of California isolated BoNT as a stable acid precipitate for the first time.[5] Subsequently, standardized preparations of BoNT and maintenance of rigorous safety standards for its therapeutic use

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were achieved by Edward J. Schantz, Carl Lamanna, and colleagues from the Department of Microbiology and Toxicology at the University of Wisconsin-Madison.[6–8] The first documented use of BoNT for the treatment of disease was in the 1970s, approximately 150 years after Kerner's initial observations about the potential use of BoNT as a therapeutic, when Dr. Alan Scott, an ophthalmologist, locally injected minute doses of BoNT to selectively deactivate muscle spasticity in the strabismus in monkeys.[9] Following the success of a series of clinical studies on humans suffering from strabismus,[10] the U.S. Food and Drug Administration (FDA) in 1989 approved the use of botulinumtoxinA (BoNT-A), BOTOX, manufactured by Allergan, Inc., for the treatment of strabismus, blepharospasm, and hemifacial spasm. Since then, the very lethal botulinum toxins, types A and B, have been extensively used for the treatment of a myriad of dystonic and nondystonic movement disorders and a host of other medical conditions, including axillary hyperhidrosis, spasticity, tremors, pain management, etc. The high efficacy of BoNT-A, coupled with a good safety profile, has prompted its empirical use in a variety of ophthalmological, urological, gastrointestinal, secretory, and dermatological disorders.[11] The list of conditions treated with botulinum toxin is expanding at a brisk rate.

The potential use of BoNT-A in aesthetics was first demonstrated in 1987 based on the observation that facial wrinkles were diminished upon treatment with BoNT-A for blepharospasm.[12] Dynamic facial lines and wrinkles are caused by patterns of repetitive muscle contractions or facial expressions. Botulinum toxin injections have revolutionized the cosmetic approach to rejuvenation of an aging face, and are now widely used for several aesthetic procedures, including the treatment of glabella frown lines, forehead furrows, and periorbital wrinkles.[13]

Depending on the target tissue, BoNTs can block the cholinergic neuromuscular or cholinergic autonomic innervations of exocrine glands and smooth muscles. The very ability of the toxin to produce flaccid muscle paralysis through chemical denervation has been put to good use, and these potentially lethal toxins have been licensed to treat an ever-expanding list of medical disorders, and more popularly in the field of aesthetic medicine. Nerve terminal intoxication by BoNTs is completely reversible, and the duration of therapeutic effect of BoNTs varies for different serotypes. Thus, it is both the most potent toxin molecule, and a "wonder drug" against numerous neuromuscular and sensory disorders.

Molecular Structure of Botulinum Neurotoxins

Botulinum neurotoxin is produced as a single polypeptide chain with a molecular mass of approximately 150 kDa that displays low intrinsic activity. This precursor protein is subsequently cleaved by bacterial proteases at an exposed protein-sensitive loop generating a fully active neurotoxin composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) (►**Fig. 1A**). The HC and LC remain linked by noncovalent protein–protein interactions, a conserved interchain disulfide bridge, and a belt that extends from the HC and wraps around the LC.[14] During the intoxication process, the interchain bridge is reduced, a necessary prerequisite for the intracellular action of the toxins.[15] The three-dimensional (3D) structures of BoNTs reveal that they are folded into three distinct domains that are functionally related to their cell intoxication mechanism. The N-terminal domain is the 50 kDa LC, which is a Zn^{2+} dependent endoprotease. The 100 kDa HC contains an N-terminal translocation domain and a C-terminal receptor-binding domain.[14]

Botulinum neurotoxins are secreted from the *Clostridium botulinum* bacteria in the form of multimeric complexes, with a set of nontoxic proteins coded for by genes adjacent to the neurotoxin gene.[16,17] These protein complexes range in size from 300 kDa to 900 kDa and exist in

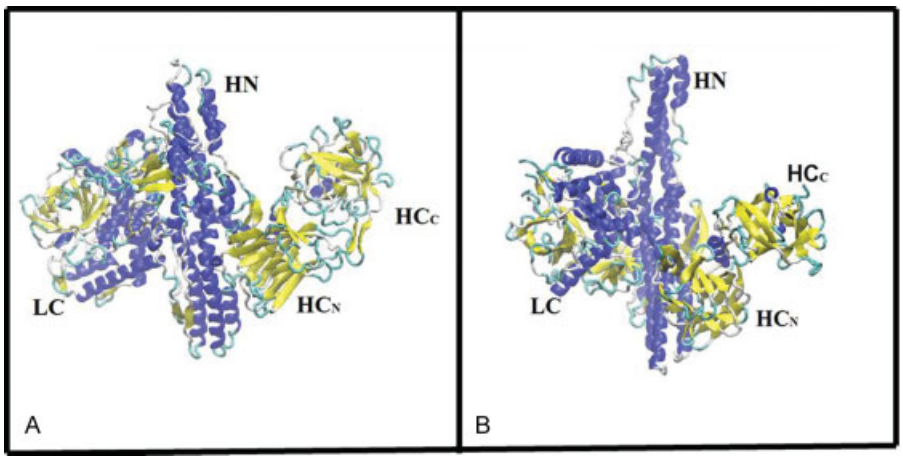

Fig. 1 (**A**) Structure of botulinum neurotoxin (BoNT; PDB ID: 3BTA). Binding domain heavy chain N-terminus (HC_N) and heavy chain C-terminus (HC_C), translocation domain (HN), and catalytic or endopeptidase domain (LC) are shown. Alpha-helix and β sheets are depicted in blue and yellow, respectively. All the BoNT serotypes, except BoNT-E, share similar structural organization of their four domains. (**B**) Structure of nontoxic nonhemagglutinin A (NTNHA; PDB ID: 3VUO). In the crystal structure, domain organization of NTNHA is similar to BoNT-A.

three progenitor toxin forms: M (medium), L (large), and LL (extra large) forms. The M form consists of neurotoxin (150 kDa) and a nontoxic protein component (120 kDa), which is called neurotoxin-binding protein (NBP)[18] or nontoxic nonhemagglutinin component (NTNH)[19] with 12S molecular size (the molecular size of complex forms is expressed as sedimentation equilibrium values). The L form has a molecular weight of approximately 500 kDa and a molecular size of 16S. The LL form is approximately 900 kDa and 19S. The L and LL complexes consist of the 150 kDa neurotoxin moiety and a set of complexing proteins made of a NTNH/NBP and several hemagglutinin proteins (HA). These are referred to as neurotoxin-associated proteins (NAPs), and also as complexing or accessory proteins. Stabilized through noncovalent interactions, NAPs account for up to 70% of the total mass of the BoNT complex.[20,21]

Currently, major BoNT therapeutic products include BoNT-A complex (marketed as BOTOX and Dysport, Galderma Laboratories, L.P.), BoNT-B complex (marketed as MYOBLOC, Solstice Neurosciences, LLC, in the U.S., and NeuroBloc, Eisai Manufacturing Inc., in Europe), and isolated BoNT-A without NAPs (marketed as XEOMIN, Merz North America, Inc.). Although NAPs do not have any therapeutic role, these may play a role in the stability of the BoNT formulation and in diffusion of the injected BoNT for therapeutic purposes.[22,23] In general, BoNT in the complex form is resistant to environmental stress, such as pH, temperature, and proteases. However, commercial products contain additional formulations that may affect the stability of the product. In BoNT-A complex preparations, adding either sodium chloride (BOTOX) or lactose (Dysport) protects the steric conformation of BoNT.[24] Human serum albumin is also added to prevent loss from surface adsorption. The toxin is then dried either with freezing (Dysport) or without freezing (BOTOX).[24] These, as well as the pure BoNT-A product, XEOMIN, are lyophilized products that are reconstituted with saline solution maintained near physiological pH.

The botulinum toxin type B product (MYOBLOC, NeuroBloc) is provided in liquid form at pH 5.6, as opposed to a lyophilized powder that requires reconstitution in saline. It nevertheless is also based on the complex of BoNT-B neurotoxin and NAPs. Botulinum neurotoxin B has shown stability for months when stored appropriately at 2°C to 8°C. However, BoNT-A must be stored at −5°C as a powder and must be used within hours once reconstituted according to the manufacturer's recommendation.[25] The BoNT-B complex appears as a 700 kDa single peak on size-exclusion chromatography (SEC) at pH 5.5, but when exposed to pH 7.8 overnight a small portion of the neurotoxin appears to dissociate from the complex.[26] A similar observation has been made for BoNT-A complex dissolved in 50 mM Tris-HCl, pH 7.6, showing a 569 kDa single peak on a Sephadex G-200 SEC analysis.[27]

A major issue in the literature relates to potential variation in the diffusional behavior of the drug formulation with the size or nature of the BoNT complex. Using radiolabeled BoNT-A complex (900 kDa) and purified BoNT-A (150 kDa), it has been clearly established that there is no significant difference in the diffusion of these reagents at physiological doses.[28,29] In fact, the diffusion was not significant for either of the samples.

It has been pointed out that the composition and perhaps stability of BoNT-A complex depends on the culture and purification conditions.[30] Long-term stabilizing effects of NAPs have been questioned from the stability data of pure 150 kDa BoNT-A preparations used in XEOMIN formulations[31] under temperature conditions of up to 60°C in the presence of human serum albumin and sucrose excipients.

The presence of NAPs in therapeutic products based on BoNT-A complex (BOTOX and Dysport) and BoNT-B complex (MYOBLOC, NeuroBloc) may or may not be needed for stability and biological activity, but are currently present as part of the formulation. The question is whether their presence has any unintended consequences, both positive and negative. This is important to note because BoNT complexes are currently in use as therapeutic drugs, and even if BoNT and NAPs separate either before injection or after injection, nerves and surrounding tissues are exposed to both components. Recent reports on the exposure of neuronal and other cells suggest that there is a massive genomic and cytokine response to the complex, and some of these responses appear to be exclusive to the BoNT and NAPs.[32,33] Because the complex has remained a safe drug for a couple of decades now, it is possible that the cellular responses to NAPs and BoNT may provide a balance in the cellular physiology. Interestingly, the 3D crystal structure of NTNHA and BoNT-A have similar polypeptide foldings (Protein Data Bank [PDB] ID: 3V0A; PDB ID: 3VUO),[34,35] one is catalytically inactive (….. KCLIK…..) and other one is active (…..HELIH…..), respectively (►**Fig. 1B**).

The nontoxic NAPs are believed to protect the neurotoxin from degradation during its passage through the low pH environment of the GI tract.[34] They are also known to assist BoNT translocation across the intestinal mucosal layer.[36,37] The association of NAPs with the toxin is pH dependent; at physiological pH this complex is reported to rapidly dissociate, allowing release of the neurotoxin into the blood stream.[31,38] Assembly and stability of the complex not only depends on pH; it also requires optimal ionic strength. Notably, not only the presence of all the NAPs is needed, but also the proper organization of NAPs and the toxin molecule is needed for the most stable and active molecule. Oral toxicity of BoNT is correlated with the size of the toxin complex between the BoNT and NAPs; the LL complex of BoNT-A is more toxic than the L complex, the L complex is more toxic than the M complex, which is more toxic than isolated 150 kDa toxin.

The molecular structure of the complete BoNT-A complex has been recently obtained from X-ray crystal and cryoelectron microscopy, showing a bimodular structure consisting of the BoNT-A and NBP (NTNH) as one module, and the HA-70, HA-17, and HA-33 together as another (►**Fig. 2**).[39] The complete bimodular complex seems to be important for facilitating its intestinal absorption during the toxicoinfection process of the food-poisoning botulism disease.

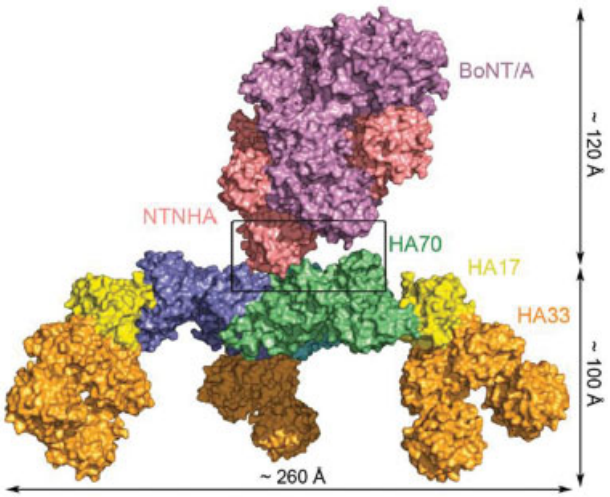

Fig. 2 Molecular structure of botulinum neurotoxin A (BoNT-A) complex reconstructed by fitting the three-dimensional structures of the BoNT-A M complex (BoNT-A and neurotoxin-binding protein) and HA70-HA-17-HA33 complex modules into the electron microscopy image. (From Lee et al, 2013.[39])

Mechanism of Action of Botulinum Neurotoxins

When therapeutic BoNT preparation is injected into the target tissue, it acts as a metalloproteinase that enters peripheral cholinergic nerve terminals and cleaves proteins that are crucial components of the neuroexocytosis apparatus, causing a persistent but reversible inhibition of neurotransmitter release. The exact molecular mechanism of BoNT action still not completely understood, but based on existing experimental evidence, BoNT intoxication is believed to occur through a multistep process involving each of the functional domains of the toxin.[40,41] These steps include binding of the neurotoxin to specific receptors at the presynaptic nerve terminal, internalization of the toxin into the nerve cell and translocation across the endosomal membrane, and intracellular endopeptidase activity against proteins crucial for neurotransmitter release.

Botulinum neurotoxins have high affinity and specificity for their target cells and use two different coreceptors for binding at the neuronal cell surface. The binding of BoNTs to the neuromuscular junction involves a tight association between its receptor binding heavy chain domain and complex polysialogangliosides particularly G1b series, namely GT1b, GD1b, GQ1b, that are known to be enriched in neurons.[42,43] BoNT-A, -B, -E, -F, and -G have a conserved binding pocket for gangliosides, whereas BoNT-C and BoNT-D display two binding sites for gangliosides.

Following binding to the gangliosides, the membrane-bound ganglioside-toxin complex moves to reach the toxin-specific protein receptor. Different BoNT serotypes bind to different protein receptors. Synaptic vesicle glycoprotein 2 (SV2; isoforms A–C), a synaptic vesicle glycoprotein, has been identified as a receptor for BoNT-A, -D, -E, -F, and -G.[44–46] Synaptotagmin, a synaptic vesicle protein, has been identified as the receptor for botulinum neurotoxin (BoNT) types B and G.[47,48] Botulinum neurotoxin G binds to the intraluminal domain of synaptotagmin, whereas BoNT-A, -D, -E, and -F bind to loop 4 of SV2. Botulinum neurotoxin C is the only serotype that does not have a protein receptor identified in neuronal cells. As mentioned above, apart from binding to two gangliosides, BoNT-C has been reported to bind to phosphoinositide-containing liposomes.[49]

Following binding to neuronal cell surface receptors, BoNT is internalized into cellular compartments by receptor-mediated endocytosis.[14,50] After the incorporation of BoNTs within the early endosomes, the acidic environment of the endocytotic vesicles is believed to induce a conformational change in the neurotoxin structure. The heavy chain is inserted into the synaptic vesicle membrane, forming a transmembrane protein conducting channel that translocates the LC into the cytosol.[51]

After internalization into the neuronal cytosol, BoNTs exert their toxic effect by virtue of the metalloprotease activity of the LC, which specifically cleaves one of three SNARE proteins that are integral to vesicular trafficking and neurotransmitter release.[14] The specific SNARE protein targets and the site of hydrolytic cleavage varies among the seven BoNT serotypes.[14] The BoNT serotypes A and E specifically cleave SNAP-25 at a unique peptide bond. The BoNT serotypes B, D, F, and G hydrolyze VAMP/synaptobrevin at different single peptide bonds, and BoNT-C cleaves both syntaxin and SNAP-25 (►**Fig. 3**).[14,52]

As mentioned above, the dual receptor model is proposed for receptor-mediated endocytosis. Synaptic vesicle glycoprotein 2C (SV2C) and gangliosides (GT1b/GD1b) are identified as receptors for BoNTs. The SV2C is also expressed in intestinal cells, such as CaCo-2 or m-IC_{el2} cells. Therefore, it is possible that receptors for BoNT in neuronal and intestinal cells are the same. However, BoNT-A Hc binding to intestinal cells is much lower compared with neuronal cells. This may be due to the low affinity of BoNT-A to intestinal receptors, or fewer numbers of receptors for BoNT-A in intestinal cells. The accurate localization in terms of any specialized microtopographical distribution of BoNT receptors in neuronal and intestinal cell membranes is not yet established. However, BoNT-A receptors do not seem to be localized directly on cholesterol-enriched microdomains, whereas SNAREs concentrate in submicrometer sizes.[53–55]

The inhibitory potential of BoNT in involuntary muscle activity makes it a useful therapeutic molecule. Intoxication of the nerve terminal by BoNTs is fully reversible and does not lead to neurodegeneration.[56] During BoNT intoxication, unlike denervation, contact between nerve terminal and muscle fiber is maintained without the loss of motor neurons. The BoNT intoxication process is temporary even though it lasts for a few weeks to months. Histological studies indicated that the recovery process occurs in two stages.[57] Initially, nerve sprouting occurs and new synapses develop, along with an increased vesicle recycling rate. In the second stage, sprout branches of nerve recess and functionality returns to normal. The recovery time varies according to the serotypes and location of intoxication.[58]

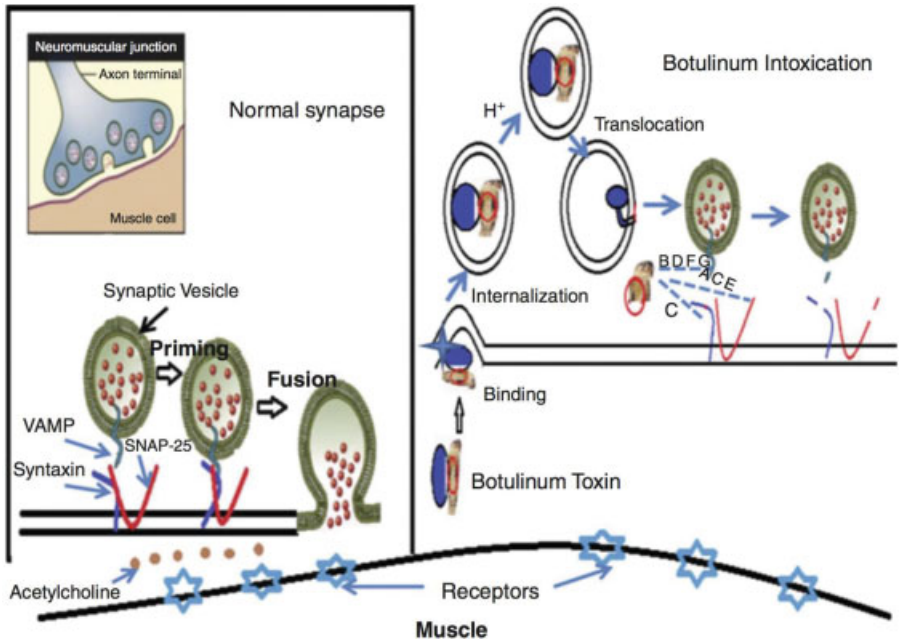

Fig. 3 Mode of action of botulinum neurotoxin (BoNT). (*Left*) Pre- and postsynaptic events in normal synapse. (*Right*) Synaptic events in BoNT-intoxicated synapse. Steps are binding, internalization, translocation, and endopeptidase activity. (From Singh et al, 2014.)

Depending on the target tissue, BoNT can block the cholinergic autonomic innervation of the tear, salivary, and sweat glands, or cholinergic neuromuscular innervation of striated and smooth muscles.[59] After intramuscular injection, the dose-dependent paralytic effect of BoNT can be detected within 2 to 3 days. It reaches its maximal effect in less than 2 weeks and gradually begins to decline in a few months due to the ongoing turnover of synapses at the neuromuscular junction.[58] The duration of the effect lasts somewhere between 3 to 6 months; the benefits have been observed with subsequent treatments in terms of increased dosing intervals.[60] There has been no evidence of any long-term or permanent degeneration or atrophy of muscles in patients with repeated injections of BoNTs over an extended period.[58]

Effect of Botulinum Neurotoxin on Active Neurons

Although BoNTs are well known to act on cholinergic nerve presynapses of the motor neurons, resulting in the classic flaccid muscle paralysis of botulism or limited muscle paralysis observed in case of strabismus, blepharospasm, dystonias, etc., it has also been clearly observed that BoNTs bind to and are taken up by sensory neurons of the peripheral nervous system, leading to the blockage of several neurocommunicative molecules such as Substance P and glutamate.[61] Although the basic mechanism of binding with receptors, endocytosis, and intracellular cleavage of SNARE proteins appears to remain the same in both motor and sensory neurons, the pharmacological mechanisms and their consequences vary. Previous studies have mostly focused on the motor neuronal phenomenon; experimental data have now started accumulating on the sensory neuronal events, even as these expand the therapeutic application of BoNTs.

Peripheral versus Central Nervous System Effects of BoNT

The peripheral action of BoNT is a well-established fact, but activity in the central nervous system (CNS) is yet to be clearly established and understood. Also, very little is known about intracellular trafficking of BoNT within the neurons. However, because of its large size (150 kDa) it is difficult for this molecule to pass through the blood–brain barrier, but there are two possibilities for it to reach the CNS when administered in muscles: systemic spread or axonal retrograde/anterograde transport. Lawrence et al[62] suggested that the spread of BoNT-A and BoNT-E within cell bodies and distal neuronal processes may occur by passive diffusion. However, experimental data have not supported the passive diffusion hypothesis[63]; thus axonal transport is the most likely mechanism for distribution and transport of toxin in various regions of axons, and possibly to the CNS as well.[64–67] Various studies have indicated the presence of botulinum toxin in neuronal pathways directed to the CNS, but have not succeeded to establish the transport of active toxin in the CNS.[68,69] However, Restani et al[63] showed a significant amount of SNAP-25 cleavage by BoNT-A in the tectum after delivery into the eyes of a rat model, indicating the strong possibility of anterograde transport and transcytosis of BoNT in axons. Antonucci et al[66] have demonstrated cleavage of SNAP-25 on the facial motor nucleus after peripheral administration, suggesting the possibility of retrograde transport and transcytosis to central neurons and motor neurons. Matak et al[70] successfully demonstrated cleavage of SNAP-25 at distal sites from a low peripheral dose. Truncated SNAP-25 was observed in ipsilateral spinal cord horns after peripheral BoNT-A administration. Colchicine, an axonal transport blocker, prevented SNAP-25 cleavage,[70] indicating trafficking of BoNT-A is through axonal transport. Marino et al[71] have

shown the effect of BoNT-B in reducing plasma extravasation in the hindpaw, dorsal horn SP release, and c-Fos activation in the dorsal root, along with cleavage of VAMP in the dorsal root ganglion. These results provide strong evidence that BoNT is transported from peripheral to central nerve terminals of sensory neurons and attenuate downstream nociceptive processing.

For direct central sensitization, the BoNT molecule needs to go through transcytosis like tetanus toxin. Evidence is building up that BoNT can undergo transcytotic movement in neurons.[66,72] The presence of cleaved SNAREs in dorsal root ganglion, and the possibility of dural extravasation in meningeal afferent neurons after administration of BoNT in somatic afferent neurons, strengthen the hypothesis of transcytosis.[73]

Excitatory versus Inhibitory Neurons

Botulinum neurotoxin molecule has been shown to inhibit the release of serotonin, dopamine, noradrenaline, glutamate, gamma aminobutyric acid (GABA), enkephalin, glycine, substance P, ATP, and calcitonin gene-related peptide (CGRP), somatostatin, and neuronal nitric oxide synthase,[74,75] clearly indicating it can affect both excitatory and inhibitory synapses.

Botulinum neurotoxin is more efficient in blocking the neurotransmitter release from excitatory neurons compared with inhibitory neurons.[76] Although both types of neurons efficiently internalize the BoNT molecule, the low level of SNAP-25 at the inhibitory terminals,[77,78] or negative regulation by cleaved SNARE protein may be responsible for lower efficiency for BoNT effect on inhibitory neurons.[76,79,80] Grumelli et al[81] and Verderio et al[78] showed that reducing calcium concentration increases the sensitivity of BoNT-A toxin to inhibitory neurons. It is possible that SNAP-25 or truncated SNAP-25 or both regulate calcium dynamics. The SNAP-25 level is higher in excitatory neurons, and SNAP-25 is a negative regulator of calcium channels,[82] making BoNT-A more sensitive to excitatory neurons. Alternatively, other isoforms of SNAP-25 may be responsible for vesicle fusion in inhibitory neurons.[76]

Effect on Sensory Neurons

Although BoNT is effective in blocking acetylcholine release at the synapse, the intradermal injection of BoNT-A reduces calcitonin gene-related peptide or CGRP,[83] which plays a role in nociception. Based on several in vitro experiments, the induction of nociceptive action of BoNT might be due to the blockage or the reduction of expression of neuropeptide transmitters like substance P and CGRP from the primary sensory neurons.[84–86] Botulinum neurotoxin has been used "off label" in several forms of chronic pain. It was observed that BoNT-A reduces pain in some conditions resulting from excessive muscle contraction, like in the painful dystonias,[87] but also in pain states not associated with muscle hypercontraction, such as migraine,[88] trigeminal neuralgia,[89] neuropathic pain,[90] refractory joint pain,[91] and low-back pain.[92] There are two components that may play a role in BoNT efficacy in pain modulation: impaired neurotransmitter release from the peripheral sensory nerve, and a neuromodulatory effect on receptors and ion channels. The fusion of synaptic vesicles with the plasma membrane carries various receptors, including receptors for pain, to the plasma membrane. The peripheral administration of toxin disrupts the transfer of receptors, such as TRPV1 and TRPA1, to the synaptic membranes.[93,94] Another possibility could be the involvement of BoNT in another endogenous system, such as the opioid system.[95]

In the case of migraine, which has both central and peripheral sensitization, BoNT can be used as an effective therapeutic tool. Apart from peripheral effects, reduction in neurotransmitter release, the peripheral administration of BoNT reduces c-FOS expression.[96] Pain-induced c-Fos activation in distinct brain areas is intimately linked with nociceptive neurotransmission and the initiation and integration of central stress responses.[97] In mechanociception, BoNT-A inhibited C-fibers, not the Aδ nociceptor. In other words, BoNT possibly interferes with the function of high-threshold mechanosensitive ion channels.[93]

Interestingly, BoNT did not affect the normal pain threshold, and is believed to affect only chronic or hypersensitive pain, not acute pain.[98] The lack of effect upon acute nociception indicates and substantiates the arguments that BoNT's effect on nociception is more than a simple block of the afferent terminal release. As in other medical treatments involving BoNT, pain treatment also has tolerable and little side effects. Nevertheless, botulinum treatments evoke antigen response that hinders its long-term use as a medication.[99,100] Apart from antigen response, BoNT administration also significantly increases inflammatory cytokine levels.[101]

In summary, BoNT acts on sensory neurons in the following ways: (1) reduces release of key neurotransmitters at the nerve terminals, (2) indirectly affects upstream pathways, and (3) has a direct effect on expression of ion channel receptors on the neuronal membrane surface.

Alternative Mechanisms of BoNT Action

Until recently it was widely believed that the toxic and therapeutic action of BoNT-A is because of SNAP-25 cleavage. The BoNT molecule stays active inside the cells for a long time (weeks to months), and therefore it is possible that it directly affects other cellular pathways or it can indirectly trigger/affect pathways though physiological consequences of the SNAP-25 cleavage. Ray et al. demonstrated that treatment of PC-12 cells with BoNT-A reduced the K^+-stimulated acetylcholine and arachidonic acid release.[102] RhoB signaling pathway affects actin reorganization and regulates various cellular functions, including acetylcholine release induced by lysophosphatidic acid.[103] Botulinum neurotoxin A also prevented neurotransmitter release evoked by phospholipids[103] through degradation of RhoB.

Neurite sprouting at the neuromuscular junctions treated with BoNT has been suggested to be related to SNAP-25 cleavage. However, Coffield and Yan[104] demonstrated that the sprouting phenomenon is dependent of toxin doses. At lower doses, BoNT showed a dose-dependent increase in sprouting. However, at higher doses BoNT suppressed sprouting,[104] indicating that sprouting is dependent on SNAP-25 cleavage at lower doses, but at higher doses toxin is possibly

acting on other pathways related to neuritogenesis. In cell cultures, BoNT-A is shown to increase caspase 3/7, indicative of antiproliferative activity in nonneuronal cells.[105,106] In contrast, Kumar et al demonstrated reduced caspase 3/7 activity in neuroblastoma cells.[107] In another study with human dermal fibroblasts, BoNT is hypothesized to stimulate the extracellular matrix,[108] and showed upregulation of collagen synthesis and reduction in the production of MMPs (matrix metalloproteinase). Notably, fibroblasts do not express SNAP-25. BoNT-A is shown to effect gene expression in nonneuronal cells (lacking SNAP-25).[32] Astrocytes do not express SNAP-25 (although synaptobrevin II is present in these cells), but glutamate release is affected by BoNT-A and BoNT-C.[109] HEK293 do not carry SNAP-25, but calcium current in these cells is affected by BoNT.[110] Thus, experimental evidence suggests that even without SNAP-25 cleavage, BoNT is able to exert significant effects on cellular physiology. Even in neuronal cells, BoNT-A treatment is shown to affect several genes related to neurite outgrowth, Ca^{2+} sensitization, proteosomal degradation pathways, and inflammatory pathways.[32]

Longevity of BoNT Action

One of the major advantages of BoNT as a therapeutic agent is its long-lasting effects on muscle relaxation (paralysis) through its intracellular effects on presynaptic nerve endings. For example, BoNT-A has consistently shown long-lasting paralysis from 3 months to about a year compared with that of BoNT-E, which lasts for about 4 weeks, both in animal studies as well as in human therapeutics.[111,112] Some studies have indicated that longevity may arise from differential persistence of the endopeptidase activities of respective serotypes.[113,114] However, another study indicated that the lifetime of SNAP-25 cleaved by BoNT-A (SNAP-25A) and by BoNT-E (SNAP-25E), or their further degraded/digested products due to host-cellular clearance mechanisms, correlated to the duration of paralysis exhibited by BoNT-A and BoNT-E, respectively.[115] The localization of BoNT-A LC is near the plasma membrane, whereas cytosolic localization of BoNT-E has also been proposed to be a reason behind their different half-lives.[116] But co-localization of the toxins and SNAP-25 within the same cells has not been shown. Reduced susceptibility to ubiquitin-dependent proteolysis, and/or the presence of di-leucine motif in the BoNT-A LC, underlies yet another proposed mechanism contributing to neuroparalytic longevity.[117,118]

Differences in longevity of the toxic action indicate the possibility of the structural variability in the LC domain of BoNT inside the neurons. One possible source of structural variations may be through posttranslational modifications that include phosphorylation, palmitoylation, and ubiquitination.[119] The nonreceptor tyrosine kinases c-Src and PYK2 are abundant in neuronal and neuroendocrine cells, indicating phosphorylation of BoNT that might modulate LC activity within the neurons.[120] In a study with PC-12 cells and Tat-His tagged BoNTA LC, it was shown that cleavage of cellular SNAP-25 was reduced when the c-Src kinase activity was inhibited with specific antagonists,[121] implying the role of BoNT-A LC phosphorylation in its intracellular endopeptidase activity. Recent work by Toth et al[122] showed phosphorylation of all serotypes of BoNT LC, except BoNT-F LC, by c-Src-kinase under in vitro conditions, and its effect on the stability of LCs against autocatalytic cleavage. As BoNT LC exerts its catalytic action on synaptosomal proteins and survives within the eukaryotic neurons for an extended period,[112,113] it is possible that it gets phosphorylated in the neurons.[122]

In summary, the longevity of BoNT paralytic action, though very important for its therapeutic use, is a phenomenon that still needs molecular, cellular, and physiological explanation.

Concluding Remarks

Here we have briefly described the structural and functional relevance of botulinum toxins in their biological function. BoNT toxins emerged from nature as a sophisticated toxin with high specificity, and structural and functional uniqueness; they offer an excellent alternative to available therapeutics for many uncommon diseases. Considering the fact that BoNT is more active in excitatory neurons than inhibitory neurons, it may be a useful therapeutic candidate in the treatment of pathologies characterized by an imbalance of these two signals, such as epilepsy. Although BoNT action is very specific, it can influence several cellular processes due to its intracellular longevity. The possibility of retrograde transport and transcytosis of the BoNT molecule open a whole new possibility for BoNT as a therapeutic agent. Careful study of the structural and functional aspects of botulinum toxin is needed to unravel several cellular and functional mechanisms associated with BoNT action on motor and sensory neurons. Knowledge acquired from these studies will provide us with additional therapeutic tools, and the possibility of novel fundamental scientific knowledge.

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