Review

An Update on the Mechanism of Action of Tetanus and Botulinum Neurotoxins

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Dedicated to the memory of Professor Franc Gubenšek

Abstract

Tetanus and botulinum neurotoxins, produced by anaerobic bacteria of the genus Clostridium, are the most toxic proteins known and are the sole responsible for the pathogenesis of tetanus and botulism. They enter peripheral cholinergic nerve terminals and cleave proteins of the neuroexocytosis apparatus causing a persistent, but reversible, inhibition of neurotransmitter release. Botulinum neurotoxins are used in the therapy of many human syndromes caused by hyperactive cholinergic nerve terminals. Here we focus on the many advances that were recently made on the understanding of their molecular mechanism of action and on their use in human therapy.

Keywords: Tetanus and botulinum neurotoxins; neuromuscular junction; neuroexocytosis; muscle paralysis

1. Introduction

Neurotoxigenic strains of anaerobic and sporigenic bacteria of the genus Clostridium produce one tetanus neurotoxin (TeNT), which causes the spastic paralisis of tetanus, and seven serologically distinct botulinum neurotoxins (BoNT/A to /G) which cause the flaccid paralysis of botulism. Tetanus has killed millions of people before a highly efficacious and inexpensive vaccine was developed, leading to the virtual disappearance of the disease in those countries endowed with an organized system of preventive medicine. Unfortunately, tetanus still takes the life of many children in the form of tetanus neonatorum in less developed regions of the world.[1] Apart from selected human populations with particular feeding habits, botulism has had little impact on human health when compared to tetanus. Contamination with BoNTs has diminished further following improvment in production and preservation of food.[2] Infant and wound botulism are still medically important, the diseases occur through contamination of the intestinal GI tract or from wounds (by tattooing or other procedures) with spores, that then germinate in an anaerobic environment and deliver the neurotoxins.[2–4]

2. Absorption and Toxicity of TeNT and BoNTs

These 150 kDa neurotoxins share similar sequences, suggesting that they originated from an ancestral toxin gene that differentiated then in to the different bacteria to produce TeNT and the BoNTs variants. In both cases, the protein is synthesized in the cytosol and it is released by bacterial autolysis, alone in the case of TeNT and with a variable number of associated, non toxic proteins, in the case of BoNTs. It is believed that these accessory proteins stabilize BoNT during its passage through the stomach and then release BoNT at the neutral pH of the intestine, where the toxin is adsorbed and delivered into the general circulation.[5] Currently there is disagreement among different laboratories whether the accessory proteins participate or not in the trans-epithelial transport of BoNT from the apical to the basolateral side of intestinal epithelial cells.[5–10] In any case, the toxin is delivered in the lymph in its 150 kDa "naked" form.[11] Once in the tissue fluids, it reaches its specific targets which are the peripheral cholinergic nerve terminals.[12–14] Humans are exposed to BoNT/A almost exclusively during treatment of many human syndromes characte-

rized by hyperfunction of peripheral nerve terminals.[15,16] In this case the toxin is injected intramuscularly and shows a striking limited diffusion around the site of injection,[17,18] a property which is of paramount relevance for the clinical use of BoNT/A. Similarly, TeNT enters the general circulation from its site of production, i.e. the infected wounded site, which may even have healed . Mouse LD_{50} is 0.2–0.5 ng kg^{-1} for TeNT and is in the range of 0.1–1 ng kg^{-1} for BoNTs.[19] Toxicity varies among animal species and it is the highest in mammalians with humans and horses being among the most sensitive species.[19–21] This makes them the most potent human poisons known. In terms of molarity, toxins may reach in body fluids femtomolar to picomolar concentrations. Clostridial neurotoxins are practically not toxic in invertebrates, whereas in mammals death by TeNT poisoning is proceeded by the generalized muscle contractures of spastic paralysis and is due to cardiac failure and/or respiratory failure,[1] whilst BoNTs kill by respiratory paralysis; however, it should be noted that if the patient is kept alive by mechanical ventilation he/she will recover completely; i.e. botulism may be fully reversible, and the clinical effects of BoNT/A are reversed over a period of months depending on the dose and the type of nerve affected.[16]

3. The Structure of BoNTs and TeNT

The inactive single chain 150 kDa clostridial neurotoxins are activated by specific proteolysis within a surface exposed loop subtended by a highly conserved disulfide bridge. Several bacterial and tissue proteinases are able to generate the active neurotoxin,[22,23] whose heavy chain (H, 100 kDa) and light chain (L, 50 kDa) remain associated via non-covalent interactions and via the conserved interchain S-S bond, whose integrity is essential for neurotoxicity (Fig. 1).[24–26] The determination of the structure of BoNT/A[27] was a breakthrough in the field and allowed planning of molecular and cell biology experiments and modelling of the other neurotoxins. These models were largely confirmed by the structure of the other toxins and/or their isolated domains, except for the relative position of domains in BoNT/E (structures deposited in the protein data bank: http://www.rcsb.org/pdb/).[27–29]

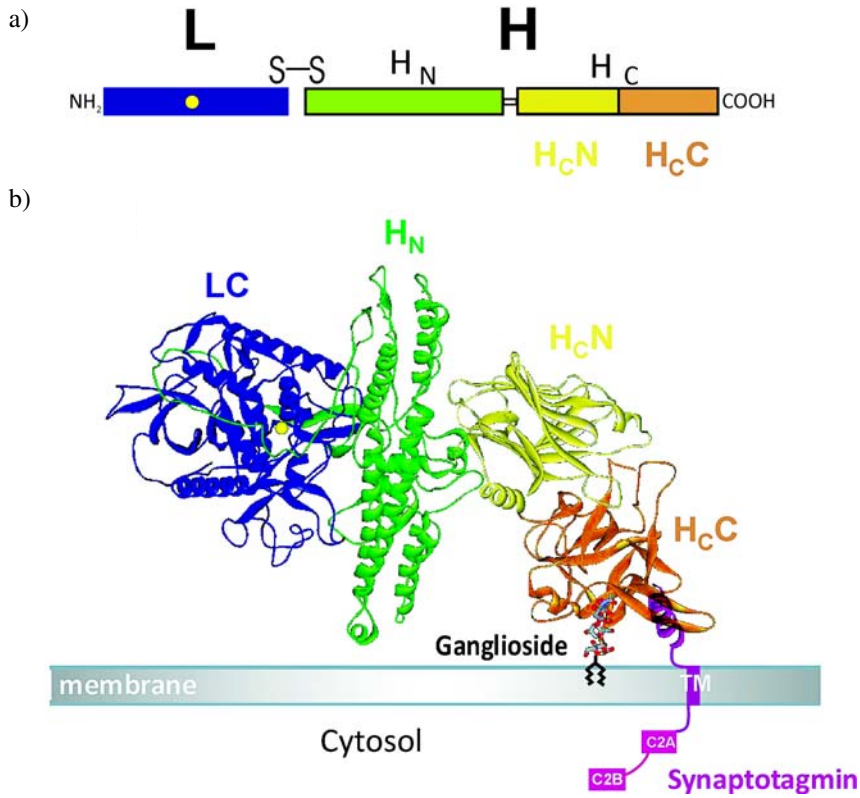

Figure 1. Structure of CNTs. **a**) The neurotoxins are synthesised in the bacterial cytosol as inactive 150 kDa proteins and are activated by specific proteolysis within a surface exposed loop. These toxins are composed of a heavy chain (H, 100 kDa) and a light chain (L, 50 kDa) which remain associated via non-covalent interactions and via the conserved interchain S-S bond. **b**) Tridimensional structure of BoNT/B showing its presynaptic membrane binding. The C-terminal part of the binding domain (H_CC in orange) interacts specifically both with polysialogangliosides and with segment 44–60 of the luminal domain of synaptotagmin (magenta), which adopts an helical conformation upon BoNT/B binding. The remaining part of the synaptotamin molecule is drawn as a transmembrane domain (TM) and two C2 cytoplasmic domains (magenta squares). The light chain (LC), the N-terminal part of the heavy chain (HN), and the two C-terminal subdomains of the heavy chain (HC) are shown in blue, green, yellow and orange, respectively. The yellow sphere represents the atom of zinc of the active site of the L chain metalloprotease.

Clostridial neurotoxins consist of four domains: a) a N-terminal metalloprotease domain (L chain), b) an intermediate membrane translocation domain (H_N 50 kDa), c) the N-terminal domain of the binding part, termed H_CN, and d) its C-terminal domain (H_CC) (see Fig. 1).[27,29–31] This multi-domain structure is shared by many bacterial exotoxins with intracellular targets, which enter host cells passing through an acidic intracellular compartment. Cell intoxication by these bacterial toxins can be conveniently and functionally divided into four steps:[32,33] 1) binding, 2) internalization, 3) membrane translocation and 4) enzymatic target modification.

4. Binding to the Presynaptic Membrane

This is the first step of cell intoxication and it is largely mediated by the H_CC domain, which is poorly conserved among clostridial neurotoxins and harbours two defined binding sites, termed here W lactose and R sialic binding sites (fig. 1).[34–36] The W lactose site binds the oligosaccharide portion of polysialogangliosides in TeNT and all BoNTs. A large body of evidence indicates that the R sialic site binds a segment of the lumenal domain of a synaptic vesicle (SV) protein synaptotagmin in the case of BoNT/B and /G[37–39] and SV2 in the case of BoNT/A and /E.[40–42] This conclusion fits the model of a double receptor (ganglioside plus a membrane protein) proposed to account for the neurospecificity of these neurotoxins and for their subsequent entry into nerve terminals.[43,12] This model also accounts for the different trafficking of TeNT and BoNTs because it was posited that TeNT protein receptor is responsible for its entry into vesicles destined to retroaxonal transport toward the CNS, whilst BoNTs protein receptors are responsible for the entry into endocytic vesicles of the peripheral nerve terminals. The demonstration of the binding of BoNT/A, /B, E and /G to the lumenal part of SV identified these vesicles as the "Trojan horses" responsible for their entry.[14] However, evidence for the binding of a second polysialoganglioside molecule to the R sialic site of TeNT, BoNT/C and /D were recently provided.[34,44–46] Other data indicate that SV2 is the protein receptor of TeNT, BoNT/D and /F.[44,47,48] Clearly, there are some inconsistencies here, as the double polysialoganglioside binding only of TeNT and BoNT does not explain their different intracellular trafficking, and the SV2 binding of TeNT would drive this toxin to exert some peripheral action, which has not been detected in clinical tetanus. Moreover, it is difficult to envisage the driving of BoNT/C and /D inside the SV lumen only via lipid binding. On the basis of the double receptor model, one would predict that different glycoprotein receptors bind TeNT and BoNT/C and /D and are responsible for their endocytosis inside different vesicles with different intraneuronal routing: the hypothetical peripheral receptor of TeNT will address it versus the CNS, whilst the protein receptors of BoNT/C and /D are SV proteins. An intense research is currently addressing this specific point and it is expected to clarify this important issue in the next future. Recently, computational modelling has shown that BoNT binding to the presynaptic membrane may be diffusion-controlled only, which would make binding very rapid once the toxin has reached the synaptic cleft.[49] In fact, these neurotoxins possess an electric dipole with the positive pole localized on the receptor binding domain HcC; molecular modelling shows that, while approaching the negatively charged presynaptic membrane, the toxins would reorient themselves and make contact with the membrane surface with HcC, thus maximizing the chance of binding since the PSG binding site is located on the tip of HcC.

5. Internalization Into Nerve Terminals

After binding to the neuronal plasma membrane, BoNTs and TeNT are targeted towards distinct region of motorneurons. BoNTs very rapidly appear in the nerve terminals and are located inside the lumen of synaptic vesicles.[50] These vesicles are filled with neurotransmitter following a transport driven by the electrochemical proton gradient generated by a vacuolar-type ATPase proton pump, that acidifies the pH lumen of SV by pumping protons from the cytosol inside the SV (internal pH around 5.6).[51,52] This pump is selectively inhibited by bafilomycin A1 and, in its presence, fluorescent BoNTs accumulate inside nerve terminals, but are prevented from reaching the cytosol and performing their action.[53,54] Synaptic vesicle dock on the presynaptic membrane and are juxtaposed by the coil-coiling of three SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins: VAMP/synaptobrevin of the synaptic vesicle membrane and SNAP-25 and syntaxin of the presynaptic membrane. They form the so called SNARE complex, which juxtapose the SV on the cytosolic face of the membrane[55] and it is likely that the two cis monolayers (one from the synaptic vesicle and one from the plasma membrane) are fused whilst the trans monolayers are not.[56] Such vesicle is ready to release its neurotransmitter content but cannot because it is clamped by a protein termed complexin. The entry of Ca^{2+} ions via the Ca^{2+} channel, opened by depolarization, activates synaptotagmin and releases the complexin clamp to promote a very rapid fusion.[55,57]

Differently from BoNTs, after binding to the NMJ, TeNT is internalized in vesicles that migrate retroaxonally inside the motor neuron and reach the spinal cord.[58–60] These retrograde organelles are not acidified during the transport, a condition necessary to retain TeNT within their lumen. The toxin is then released in the intersynaptic space between peripheral motor neurons and inhibitory interneurons (Renshaw cell), and enters SV when they ex-

pose their lumen to the outside following fusion with the presynaptic membrane and release of the inhibitor neurotransmitter.[47,61–63]

6. Membrane Translocation

The low pH of SV lumen triggers a conformational change of the toxin molecule which exposes hydrophobic residues of the H_N domain, allowing its penetration into the membrane with formation of a trans-membrane protein conducting channel. This process can be studied by electrophysiological techniques that revealed that the H chain acts as a transmembrane chaperone which prevents aggregation of the LC in the acidic vesicle interior, maintains the LC in a partial unfolded conformation during translocation, and releases the LC after its refolding at the neutral cytosolic pH.[31,64,65] An intact inter-chain disulfide bond is a strict requirement for the low pH-induced conformational change of tetanus and botulinum H_N domain which leads to the translocation of the L chain into the cytosol.[24,25,66,67] An intact disulfide bond is therefore a crucial aspect of the clostridial neurotoxins toxicity and is required for chaperone function, acting as a hinge and principal determinant for L chain translocation and release. The presence of reductants and neutral pH in the cytosol promotes release of the LC from the HC after completion of translocation.[68]

7. Enzymatic Target Modification

The crystal structures of the seven BoNT and of TeNT LCs have been determined (for a complete list of references see[31]) and revealed a structural similarity despite the different substrate specificity, with an active site resembling that of the Zn^{2+}–metalloprotease thermolysin. The L chains of BoNTs and TeNT are highly specific proteases that recognize and cleave unique components of the synaptic vesicle docking-fusion complex called SNARE complex. TeNT, BoNT/B, /D, /F and /G cleave VAMP/synaptobrevin, a protein of the SV membrane, at different single peptide bonds whereas BoNT/A and /E cleave SNAP-25 at distinct sites within the COOH-terminus. BoNT/C is unique among the BoNTs since it cleaves both SNAP-25 and syntaxin, another SNARE protein of the presynaptic membrane; (reviewed in[13,21,31,69]). Cleavage of nerve terminal SNAREs results in the inhibition of neurotransmitter release into the synaptic cleft.

A peculiarity of CNT L chains which distinguishes them from conventional proteases is that they require an extended substrate segment for optimal catalytic activity, and a major role is played by a nine residue-long motif present within the SNARE proteins characterized by three carboxylate residues alternated with hydrophobic and hydrophilic residues.[70–75] A X-ray structure of a binary complex between LC/A and SNAP25 (146–204) defined additional regions of interaction external to the cleavage site and to exosites.[75] More recently, the fitting of an extended region of the substrate (residues 189–203) within the long active site cleft was defined following extensive mutagenesis of LC/A and SNAP-25[76,77] and of LC/B or LC of TeNT and VAMP2.[78] A comparative analysis of the mode of interaction of VAMP-specific CNTs revealed that BoNT/F and TeNT required a more extended interface interaction than BoNT/B and /D and that the interactions sites are located upstream the respective scissile bonds.[79] Therefore exosites diversity seems to dictate the serotype substrate-specific binding, whereas sequence variations around the toxin active sites would determine the scissile bond specificity.[75,80]

8. Therapeutic uses of Botulinum Neurotoxins

Botulinum neurotoxins are the most poisonous poisons but the demonstration that their inhibition of the nerve-muscle impulse is followed by a functional recovery of the NMJ provided the scientific basis of their use in the therapy of a variety of human diseases caused by hyperfunction of cholinergic terminals.[16,81,82] Injections of minute amounts of BoNT into the muscle(s) to be paralyzed lead to a depression of the symptoms lasting months. Owing to the long lasting duration of its effect, BoNT/A has almost invariably been used. Also BoNT/B is commercially available but it has a rather short duration of action, and longer paralysis can only be achieved with very high doses, thus increasing the possibility of an immune response.[83–87] Studies performed in humans (reviewed in[88]) and in mice,[89] show that BoNT/C has a general profile of action similar to that of BoNT/A and could be a valid alternative in human therapy. In addition to dystonias or spasticity, other pathological conditions such as hyperhydrosis, which are due to excessive Ach release from autonomic nerve terminals which innervate glands, benefit from BoNT/A treatment.[90,91] Moreover, the use of BoNT/A has been increasingly reported in many conditions of pathological pain, including migraine and other headache disorders,[92,93] musculoskeletal pain, such as myofascial pain, low back pain and other chronic pain syndromes.[94–96]

BoNT therapy has a remarkable record of safety and this depends partly on the toxin ability to remain relatively localized at the site of injection.[17] However there is substantial evidence that injection of BoNT/A into some facial sites or behind the eye can produce a retroaxonal transport of the toxin to CNS sites. These findings have been usually ascribed to plastic rearrangements subsequent to the peripheral blockade. The finding of a retrograde transport of catalytically active BoNT/A suggests that BoNT/A may also have direct central effects, especially if high doses are injected.[97,98]

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Povzetek

Tetanus in botulinum toksini, ki jih proizvajajo anaerobne bakterije iz rodu *Clostridium*, so najbolj toksične znane beljakovine in edini dejavniki, odgovorni za patogenezo tetanusa in botulizma. Pri svojem delovanju vstopijo v periferne živčne končiče in cepijo določene beljakovine, udeležene v procesu eksocitoze živčne celice, kar privede do dolgotrajne, vendar reverzibilne, inhibicije sproščanja nevrotransmiterja. Botulinski nevrotoksini se uporabljajo pri zdravljenju številnih človeških sindromov, pogojenih s hiperaktivnostjo holinergičnih živčnih končičev. V predstavljenem članku smo se osredotočili na zadnje pomembne dosežke, ki so prispevali k boljšemu razumevanju molekulskega mehanizma delovanja klostridijskih nevrotoksinov, in na njihovo uporabo pri zdravljenju ljudi.