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Point of VIEW

Peculiar Binding of Botulinum Neurotoxins

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ABSTRACT Botulinum neurotoxin (BoNT) is a bacterial toxin that causes paralysis. Recent models have suggested that BoNT recognizes and enters nerve endings by interacting with protein receptors and gangliosides, which are glycosphingolipid components of the cell membrane that modulate cell signaling. Recent structures provide insight into how BoNT interacts with these cell surface components and open the door for the development of inhibitors against this neurotoxin.

One of the emerging drugs used in human therapy and in cosmetics is botulinum neurotoxin (BoNT) (*1, 2*). This is not the traditional small-molecule or peptide drug. BoNTs are a family of seven (BoNT/A–G) bacterial proteins of 150 kDa that are made of a heavy chain (H, 100 kDa) and a light chain (LC, a metalloprotease of 50 kDa) (*2*). BoNTs are the toxins responsible for the flaccid paralysis of botulism, a disease with relatively minor impact on human health; nevertheless, BoNTs are a considerable bioterrorism scourge. BoNT is a perfect example of Janus, a molecule better known as a therapeutic agent massively produced by the millions every year in two different types: BoNT/A, under the trade names Botox, Dysport, and Xeomin, and BoNT/B, commercially known as Myobloc and Neurobloc (*3*). These neurotoxins are specific biochemical scalpels that bind and enter peripheral cholinergic nerve terminals (both skeletal and autonomic terminals), where they inhibit the Ca^{2+}-evoked release of acetylcholine for months. The result of this inhibition is that the innervated muscle or gland is paralyzed (*2, 3*). Thus, any syndrome caused by the hyperfunctionality of cholinergic nerve terminals can be treated with injection of minute amounts of BoNT.

BoNTs are endowed with characteristics that make them wonder drugs. Upon injection, they stay *in loco* and affect only the cholinergic terminals present within the very restricted area reached by the carrier solvent. Furthermore, BoNTs are the most poisonous substances known to humans, with lethal doses on the order of a few nanograms per kilogram. Despite this latter characteristic, these neurotoxins have one of the safest usage records.

These characteristics are linked in some way to the binding properties of these molecules. In fact, their very high specificity for presynaptic nerve terminals leads to the impairment of essential physiological functions, such as vision and respiration, causing overall high toxicity. BoNTs also exert their paralyzing activity around the injection site, with very limited spreading. These facts have puzzled scientists for decades. A review of the available literature led to the proposal of a double-receptor model for the binding of BoNTs to neuronal membranes 20 yr ago (*4*). The model suggested that BoNT first interacts with the oligosaccharide portion of polysialogangliosides, which are highly enriched at nerve terminals, causing the BoNT molecule to adhere to the surface of the membrane. The toxin–ganglioside complex was suggested to move laterally to find and bind to a second, less abundant, protein receptor. This double binding ensures high trapping efficiency, because of the abundance of polysialogangliosides, and high specificity, conferred by the protein receptor. Furthermore, this dual-binding mode would provide the high-binding affinity necessary to account for the high toxicity displayed by BoNTs at subpicomolar concentrations in the tissue fluids (*4*). Subsequently, the protein receptor has been suggested to be the lumenal domain of a synaptic vesicle protein to account for the binding of BoNT/B to synaptotagmin (Syt) (*5*) and for the subsequent entry of BoNT into the nerve terminal *via* endocytosis (*6*). This suggestion added peculiarity to

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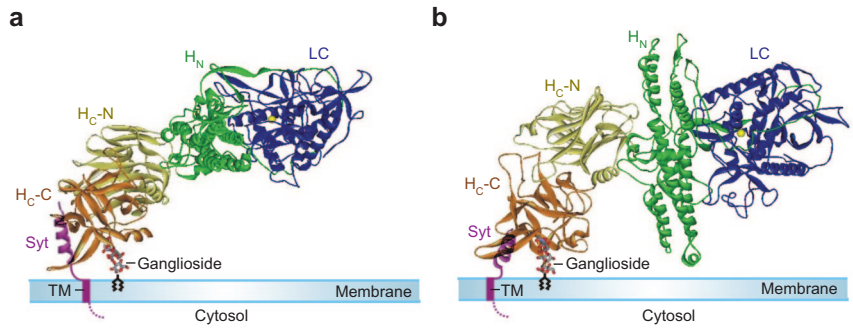

Figure 1. Binding of BoNT/B to the presynaptic neuronal membrane. Two different models have been proposed (*11, 12*). The C-terminal part of the binding domain (H_C-C in orange) binds both polysialogangliosides and the luminal domain of Syt-II (magenta). a) The helices of the translocation domain (H_N) may be oriented in a parallel fashion (*12*) or b) sit orthogonal to the plane of the membrane surface (*11*). Proteins deposited in the Protein Data Bank under accession codes 1F31 (*9*), 2NM1 (*11*), and 2NP0 (*12*) were used to model the complexes. The LC, the N-terminal part of the heavy chain (H_N), and the two C-terminal subdomains of the heavy chain (H_C) are shown in blue, green, yellow, and orange, respectively. The yellow sphere represents the atom of zinc at the active site of the LC metalloprotease. TM = transmembrane domain.

the model, because the majority of the proposed BoNT protein receptors would be located inside synaptic vesicles and, therefore, would not be accessible to ligands at the neuron surface. These protein receptors only become exposed to the external medium for a short time, between the times when the vesicle fuses with the presynaptic membrane (this event is accompanied by the release of neurotransmitters) and the times when the fission event allows vesicle endocytosis and recycling (*7*). In this model, the synaptic vesicle cycling is the cellular event that brings the toxin inside the nerve terminal; synaptic vesicles, hence, act as neuronal Trojan horses for the BoNTs.

These remarkable features remained known only to a small scientific community even after the publication of a major result supporting this model. In fact, the solution of the crystallographic structure of BoNT/A (*8*) and BoNT/B (*9*) showed a three-domain structure (LC, H_N, and H_C). The binding domain, H_C, was composed of two parts: H_C-N and H_C-C (Figure 1). The N-terminal subdomain H_C-N adopts the same folds as the carbohydrate-binding lectin family of proteins. The amino acid sequence of the determinants of the secondary structure of this H_C-N is conserved among BoNTs and TeNT; suggesting a similar fold with some nonconserved loops. However, the sequence of the C-terminal part of H_C, H_C-C, is poorly conserved, and it folds similarly to proteins of the trypsin inhibitor family (*8, 9*). This structure suggested that H_C-N is involved in the binding of polysialogangliosides, and that the nonconserved protein–protein interacting subdomain H_C-C was involved in binding to the protein receptor localized on the synaptic vesicle membrane.

Recent papers provided a major twist to this binding saga showing that both the polygangliosides- and the protein receptor-binding sites are located in the H_C-C subdomain (*10, 11, 12*). In fact, the crystal structures of the complex between BoNT/B and the luminal domain of the synaptic vesicle protein synaptotagmin II (*11, 12*) show that Syt binds H_C-C *via* its segment 47–60 at a site partially overlapping the polysialoganglioside binding site. This luminal domain of Syt is largely unstructured in solution. However, upon binding to the H chain of BoNT/B, it folds into a helix that fits into a cleft generated by a group of residues that are highly similar between BoNT/B and BoNT/G, a toxin type that binds Syt-I and -II as well (*13*). Extensive mutagenesis of the polysialoganglioside binding site and of the Syt binding cleft provided functional evidence of the role of the two H_C-C sites and indicated that Syt binding is perhaps more important than polysialoganglioside binding (*10–12*).

This finding fits very well with the original dual-receptor model that predicted that the toxin–protein receptor interaction would transform a reversible toxin membrane interaction into a productive one, such as one followed by intoxication (*4, 14*). However, this finding contradicts the expectations of the role of the two subdomains of H_C. In fact, both the glycolipid and the protein receptor binding are mediated by H_C-C, and this opens the question of the role of H_C-N. Is it a mere rigid spacer that physically projects H_C-C in a way that makes it easier to bind to the membrane? Or does it help BoNT bind to the presynaptic membrane *via* the interaction with additional receptors (*15*)? Alternatively, does H_C-N play a role in the transmembrane chaperoning of the translocation of LC? The answers to these questions will also help scientists understand the mode of membrane translocation of the catalytic LC. The way BoNT orientates itself with respect to the membrane plane after binding remains unknown. Radically different binding modes for BoNTs may be obtained by using molecular modeling with the same crystallographic data (Figure 1) (*11, 12*). The two 110-Å long helices that characterize the H_N domain may be oriented either perpendicularly (*11*) or in a parallel fashion (*12*) to the membrane surface. Interestingly, H_N at low pH forms transmembrane ion channels (*16*) and may assist with a chaperone-like activity, the translocation of the LC from the lumen of the synaptic vesicle to the cytoplasm (*17*).

These findings are very important because they provide the structural basis for designing specific inhibitors of toxin bind-

ing. Although their possible use as therapeutic agents is not evident at the moment, it is likely that bivalent drugs capable of binding to both the polysialoganglioside and the Syt binding sites would strongly inhibit BoNT binding and the ensuing intoxication of neurons.

As recently discussed, these developments, as important as they are, do not elucidate the sequence of events leading to membrane binding of BoNT (*7*). One possibility is that the toxin binds to both the glycolipid and the protein receptors simultaneously, after the vesicle interior opens during neurotransmitter release. Although the existence of polysialoganglioside on the vesicle lumen is yet to be demonstrated (*7*), it seems very unlikely that BoNT can manage to display the exceptional binding properties mentioned above with receptors that are present on the cell surface only a restricted length of time. A second possibility is that BoNT binds to one of the many polysialoganglioside molecules present on the presynaptic membrane. Then, after a lateral search, it might interact with Syt exposed on the surface by a readily fused vesicle or to the few molecules of Syt present on the nerve terminal surface as a consequence of imperfect synaptic vesicle membrane retrieval (*18*).

These studies should be actively pursued not only because they will offer scientists a better understanding of the scientific basis of the therapeutic applications of BoNTs, but also because they could shed light on the binding to cell membranes of other biological ligands.

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