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A Coincidence Detector Triggers Botulinum Neurotoxin Translocation

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Summary

Evaluation of Sun S, Suresh S, Liu H, Tepp WH, Johnson EA, Edwardson JM, Chapman ER: Receptor binding enables Botulinum neurotoxin B to sense low pH for translocation channel assembly. *Cell Host Microbe* 10, 237-247 (2011). Botulinum neurotoxins (BoNTs) are the deadliest poisons known to man that possess a particular duality, rapidly increasing clinical utility for a wide range of disorders and large concern as a possible weapon of bioterrorism. While great strides have been made in the structural and biochemical understanding of the mechanism of intoxication, the specific molecular details behind BoNT translocation out of endosomes remains elusive. In this study, it was conclusively demonstrated that light chain metalloprotease translocation can only occur in the presence of low pH, as is found in endosomes, and GT1b ganglioside co-receptor, who role was previously thought to only be in cell surface recognition by the toxin. As stated by the authors, the BoNT receptor-binding domain therefore serves as a “coincidence receptor” in that pH sensing and conformational change to a translocation competent form must be coupled in some way to receptor binding. Further study using atomic force microscopy also suggested the presence of oligomeric toxin channels that can be inhibited by the natural product toosendanin. This data revises the model of BoNT intoxication and shows the amazing temporal and spatial control possessed by this toxin, which ultimately manifests in its extreme potency.

Keywords

Botulinum neurotoxin; translocation; bioterrorism; atomic force microscopy; ganglioside

The Gram-positive bacterium *Clostridium botulinum* produces neurotoxic proteins, termed Botulinum neurotoxins (BoNTs), which target neuromuscular junctions on the corresponding motor neurons of the host organism [1]. By disrupting neurotransmitter signaling, these toxins cause flaccid paralysis, a hallmark of botulism, with exposure ultimately resulting in the death of the host due to respiratory failure. Their extreme potency (LD₅₀~1 ng/kg) places these toxins among the most potent naturally occurring compounds known. Consequently, the U.S. Centers for Disease Control and Prevention have classified BoNTs as one of the six highest-risk threat agents of bioterrorism [2].

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The Botulinum neurotoxin family consists of seven serotypes, BoNT/A-/G, which exhibit high amino acid sequence homology, with four of the seven serotypes (/A, /B, /E, and /F) causing human botulism. Each isoform is synthesized as a single 150-kDa polypeptide that is subsequently cleaved into an N-terminal 50-kDa light chain (LC) and a 100-kDa heavy chain (HC), which remain linked by a disulfide bond [1]. The LC is a Zn^{2+} -dependent metalloprotease that, depending on the serotype, cleaves a unique target protein in the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex, the critical protein machinery involved in vesicular fusion and exocytosis. Two of the serotypes, BoNT/A and /B, have been the most intensively studied and consequently, are also commonly used in a wide range of clinical applications despite their extreme toxicity [3].

The mechanism of action is virtually identical for all serotypes and can be subdivided into three stages: cellular binding and internalization, translocation, and proteolytic cleavage of the SNARE target (Figure 1A). Cell surface recognition and receptor binding represent the first step in the process of cellular intoxication. BoNT serotypes exhibit different cell tropisms and, hence, recognize and bind different receptors on the cellular surface. In addition, for productive interaction the toxin must bind two types of cell surface receptors, a protein receptor and a ganglioside (GD1b or GT1b) co-receptor. After binding, the toxin is subsequently internalized via receptor-mediated endocytosis. Once the lumen of the toxin-containing endosomes acidifies, the HC undergoes a conformational change that results in the formation of a HC protein pore in the endosomal membrane, enabling translocation of the LC to the cytosol where it can then cleave its cognate SNARE substrate. This cleavage event results in a total inhibition of acetylcholine neurotransmitter exocytosis, the ultimate stage in the pathogenesis of BoNT intoxication [3].

Whereas many questions regarding the elucidation of this rather complex process have been addressed in various studies clarifying aspects of BoNT pathogenesis, the mechanism of LC translocation into the cytosol remains poorly understood. In particular, recent years have seen a significant increase in crystallographic studies of both toxin-receptor interactions as well as the structural studies of the catalytic light chain metalloprotease [4–6]. One of the main reasons precluding scientists from effectively studying the processes of HC and LC conformational change, integration of HC into the membrane and final translocation of unfolded LC through the HC pore, lays in the difficulty of measuring and manipulating pH inside the small endosomal/synaptic vesicles.

An attractive approach to study the effects of low pH and receptor binding on BoNT translocation that elegantly circumvents the abovementioned difficulties has been reported in a recent issue of *Cell Host & Microbe* by Sun, *et al.* [7]. In this study, a method was developed to study the mechanism of BoNT/B translocation across the plasma membrane of cultured rat hippocampal neurons. The effect of pH on translocation was simply addressed by exposing cells to extracellular buffer of varying pH, and translocation of LC and its proteolytic activity in the cytosol of sensitive neurons was subsequently monitored by cleavage of its natural SNARE substrate synaptobrevin. Despite the simplicity of this experiment, striking results emerged. When acidic extracellular pH and GT1b cell surface co-receptor were present, BoNT/B could be translocated across the plasma membrane; however, neither low extracellular pH or GT1b ganglioside binding alone enabled the translocation (Figure 1B). In addition, only a combination of both factors, low pH and the presence of ganglioside, triggered the expected conformational change of the toxin, rendering it more hydrophobic and membrane bound, as measured by circular dichroism. These findings imply that: (i) the receptor-binding domain of the BoNT HC, if not bound to its cognate receptor on the cell surface, inhibits the conformational change of the HC, its insertion into the membrane and subsequent translocation of the LC, and inversely, (ii) when

the toxin is exposed only to low pH but not bound to its cognate surface receptor, for example, in the host gastrointestinal tract where epithelial cells do not express the requisite receptors on their cell membranes, it does not penetrate the membrane, and the LC cannot translocate and cleave its substrate, representing an impressive mechanism for the distinctive neuronal specificity of BoNTs. This data is in agreement with previous studies that showed that deletion of the receptor-binding domain of BoNT/A leads to a toxin that spontaneously forms large conductance channels, independent of pH [8]. Effectively, this argues that the heavy chain of the toxin is, as termed by the authors, a “coincidence detector”: two distinct events must occur in temporal proximity in order to achieve the requisite conformational change for productive translocation.

Although the described method represents an attractive and simplified approach to clarify the roles of low pH and GT1b binding on BoNT conformational change and LC translocation across the membrane, it is not able to uncouple the two physiologically and functionally distinguished processes of internalization via receptor-mediated endocytosis and translocation across the endosomal membrane. Indeed, the data indicates that these two processes are more tightly intertwined than previously thought. It is important to note that the translocation across the endosomal membrane may take less than ~3 minutes (the time BoNT/A channel requires to exhibit maximal conductance; ref. [8]), which is less time the cultured rat hippocampal neurons were exposed to low extracellular pH [7], thereby raising a possibility that the toxin may “leak” from acidic endosomes in cells previously exposed to low extracellular pH in the absence of the vacuolar H⁺-ATPase inhibitor bafilomycin. This mechanistic disparity does not compromise the conclusions of this study, but instead highlights a point for further study, that is, the relative roles of endocytosed toxin translocating out of endosomes versus toxin directly translocating across the plasma membrane when exposed to low pH and GT1b.

The paper also presents an intriguing model on the molecular mechanism behind how the BoNT/B HC forms channels in membranes and the specific architecture of the assembled pore. By using atomic force microscopy, oligomerization of the BoNT/B holotoxin and formation of donut-shaped structures on artificial lipid membranes was observed when exposed to low pH and in the presence of GT1b; interestingly, both processes could be prevented by the addition of toosendanin, a characterized inhibitor of BoNT/A LC translocation [3, 9]. Notably, this data is in contrast to existing evidence that single BoNT molecules are sufficient to form channels in cell membranes and translocate the LC metalloprotease [10, 11]. At this time, while this new data does support existing hypotheses that oligomerization plays a role in BoNT function, it is unable to explain the advantage to or reason for the oligomerization; indeed, the authors measured pore size and conductance of the oligomeric donut-shaped pores and found them to be similar to those of a monomeric channel.

Future Perspective

Taken together, the article by Sun et al. [7] addresses important questions about the roles of low pH and ganglioside co-receptor binding on BoNT/B channel formation and translocation. Their data clearly shows that through the receptor binding domain of the HC, BoNT/B is able to sense low pH and cell type specificity by binding to GT1b ganglioside receptor, thereby ensuring spatial and temporal accuracy for internalization only by the target neuronal cells. Given that translocation has been the most poorly understood step in the intoxication process, this study provides a significant advance to the field and as all intriguing findings do, provides new avenues for further study. Future study into the fate of the HC channel after completion of translocation as well as direct imaging of the LC during

the translocation event are surely warranted to validate the biological significance of these findings.

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Executive Summary

Objective

- To understand the role of GT1b ganglioside co-receptors on BoNT/B translocation across membranes.
- To explain the process of the BoNT/B HC translocation channel assembly.
- To clarify the mechanism of BoNT/B LC translocation across membranes.

Methods

- Novel model system for monitoring direct translocation of BoNT/B across the plasma membrane.
- Biophysical characterization (i.e., circular dichroism, hydrophobicity assays, patch clamp recording) of BoNT/B translocation domain in the presence and absence of low pH and GT1b.
- Atomic force microscopy studies of the oligomerization of BoNT/B in the presence of low pH and GT1b.

Result

- Conformational change of BoNT/B HC and translocation of LC only occurs in the presence of both low pH and GT1b ganglioside.
- BoNT/B HC transforms into a hydrophobic membrane-associated protein in the presence of low pH and GT1b.
- Toosendanin, a drug with therapeutic effects against botulism, alters properties of the BoNT/B channel and hinders translocation of BoNT/B LC.

Conclusion

- Novel model system gives unprecedented insights into the mechanisms of BoNT channel formation and LC translocation.
- The BoNT/B HC receptor-binding domain is an inhibitor of the conformational change required for translocation.

Figure 1.

A) Mechanism of BoNT/B intoxication showing the steps of receptor binding and endocytosis, endosome acidification, heavy chain (HC) conformational change and membrane insertion, and light chain (LC) unfolding and subsequent translocation through the HC channel to the cytosol. After LC translocation and refolding, the ultimate fate of the acidified endosome containing the BoNT/B HC channel is currently unknown. Red – BoNT/B HC; Blue – BoNT/B LC; Green – SytII protein receptor; Black – GT1b ganglioside co-receptor. B) Model system of BoNT/B translocation directly across the plasma membrane utilized by Sun, *et al.* [7].