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How a Neurotoxin Survives

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Botulinum neurotoxins are secreted by the spore forming Gram-positive bacteria *Clostridium botulinum* and some other *Clostridium* strains under suitable anaerobic conditions (1). Ingestion or inhalation of botulinum neurotoxin leads to a severe neuromuscular disease, termed botulism, which is characterized by muscle paralysis and autonomic dysfunction. Both conditions stem from inhibition of acetylcholine release, which results from botulinum neurotoxin-mediated proteolysis of one of three SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins involved in the release of this neurotransmitter from nerve terminals (2). On page 977 of this issue, Gu et al. (3) provide a key insight into how botulinum neurotoxin can survive passage through the gastrointestinal tract, allowing the intact toxin to reach the bloodstream.

Botulinum neurotoxin is synthesized as a single 150 kD protein, which is modified posttranslationally to the active two-chain form (2). The smaller light chain (LC) catalyzes SNARE protein cleavage by a Zn^{2+} -dependent mechanism. The larger heavy chain contains a binding domain (HC) for selective attachment to cholinergic nerve terminals and a translocation domain (HN) for intracellular delivery of the LC to the nerve-terminal cytosol, where it can cleave the appropriate SNARE protein. Owing to its extreme potency and history of weaponization, botulinum neurotoxin has been designated by the U.S. Centers for Disease Control and Prevention as a category A bioterrorism agent (4). Yet, its exquisite selectivity and long duration of action has also enabled dilute formulations to be used for clinical indications such as focal dystonias, spasticity, wound healing, and aesthetics (5).

Botulinum neurotoxin can intoxicate the host by inhalation or ingestion. Both routes involve transcytosis of the neurotoxin across epithelial barriers to reach the circulation, and from there, the nerve terminal targets (6). During passage through the gastrointestinal tract the toxin must endure extremes in pH and potential destruction by proteolysis to emerge as active toxin in the bloodstream. Gu et al. demonstrate how botulinum neurotoxin achieves this feat, the subject of conjecture for more than half a century. The key to this mystery lies in the accessory proteins that are cosecreted by botulinum neurotoxin-producing bacteria. Thus, botulinum neurotoxin exists in nature not as the pure toxin but rather as a complex bound noncovalently with neurotoxin-associated proteins.

Unlike botulinum neurotoxin/A, which is the most lethal substance known, neurotoxin-associated proteins are completely devoid of toxicity (2). Three of these (HA17, HA33, and HA70) have hemagglutinin activity; the fourth and largest lacks this activity and is called nontoxic non-hemagglutinin (NTNHA). When associated with these proteins, botulinum neurotoxin is referred to as the toxin complex or progenitor toxin, with molecular weights of 300 to 900 kD depending on the degree of aggregation (6).

Gu et al. focus on the role of NTNHA, which together with botulinum neurotoxin constitutes the minimally functional progenitor toxin complex (M-PTC) (3). The authors coexpressed a

catalytically inactive mutant of botulinum neurotoxin/A (BoNT/Ai) with an NTNHA identical to that associated with wild-type botulinum neurotoxin/A (NTNHA-A) in *Escherichia coli*. The proteins form a tight 1:1 complex at acidic pH similar to that found in native M-PTC. The crystal structure of this complex at high resolution (2.7 Å) shows the detailed interactions between the toxin and the associated protein. Unexpectedly, NTNHA-A exhibits a very similar domain architecture to BoNT/Ai (see the figure, panel A), although the two proteins share only 20% sequence identity. Despite this structural similarity, NTNHA-A lacks the specialized motifs required for binding to nerve terminals or cleaving SNARE proteins.

Gu et al. subjected NTNHA-A, BoNT/Ai and M-PTC to conditions resembling those relevant to the absorption of botulinum neurotoxin in the gastrointestinal tract (7). Only M-PTC was stable in pepsin at pH 2.6 and in trypsin at pH 6. Neutral or alkaline pH promoted dissociation of the complex, resulting in loss of protection against trypsin inactivation. The essential residues involved in the interaction between BoNT/Ai and NTNHA-A were identified by site-directed mutagenesis. The results suggest that small molecule inhibitors could be developed to weaken this interaction and inactivate the neurotoxin early in intoxication, when intervention would be most effective. However, because there are no signs of botulism until SNARE protein cleavage is in progress (2), the inhibitors would need to be taken before exposure, requiring prior knowledge of an attack.

Examination of the crystal structure of the M-PTC complex helps to understand how NTNHA-A protects BoNT/Ai. The domain of botulinum neurotoxin most susceptible to proteolysis is the H_C (8), and all three domains of NTNHA-A interact extensively with this domain, thereby protecting it against proteolysis (see the figure, panel B). In contrast, the catalytically active LC domain of botulinum neurotoxin does not appear to interact with NTNHA-A, suggesting that it is inherently resistant to proteolytic degradation in the M-PTC complex. These results suggest that it may be possible to orally deliver protein-based therapeutics—both for botulism and for other conditions where a protein- or peptide-based drug is indicated—by coupling them to modified M-PTCs to protect the cargo from degradation.

The seminal findings of Gu et al. raise important questions for future studies. Is the domain homology of NTNHA unique to serotype A, or is it the general pattern for all serotypes? What are the roles of the other neurotoxin-associated proteins, especially HA33, which resists proteolysis and enhances transcytosis of neurotoxin (9)? Finally, given that the 900 kD complex provides the best protection against degradation of botulinum neurotoxin (5), how is this complex assembled, and how does each component contribute to keeping the toxin structurally intact and able to invade and inactivate cholinergic nerve cells in the host organism?

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Figure. Interlocked complex

Gu et al. report the crystal structure of a complex between the botulinum neurotoxin BoNT/Ai and the neurotoxin-associated protein NTNHA-A. The two proteins have surprisingly similar domain structures (**A**). The complex formed by the two proteins (**B**) can survive the conditions of the gastrointestinal tract, whereas neither protein can survive these conditions in isolation. nLC, nH_N, and nH_C denote the regions in NTNHA-A that correspond to the LC, H_N, and H_C of the neurotoxin, respectively. Data from Gu et al.'s (3) supporting online material: (A) modified from figure S6; (B) modified from figure S3.