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Architecture of the botulinum neurotoxin complex: a molecular machine for protection and delivery

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Botulinum neurotoxins (BoNTs) are extremely poisonous protein toxins that cause the fatal paralytic disease botulism. They are naturally produced in bacteria with several nontoxic neurotoxin-associated proteins (NAPs) and together they form a progenitor toxin complex (PTC), the largest bacterial toxin complex known. In foodborne botulism, the PTC functions as a molecular machine that helps BoNT breach the host defense in the gut. Here, we discuss the substantial recent advance in elucidating the atomic structures and assembly of the 14-subunit PTC, including structures of BoNT and four NAPs. These structural studies shed light on the molecular mechanisms by which BoNT is protected against the acidic environment and proteolytic destruction in the gastrointestinal tract, and how it is delivered across the intestinal epithelial barrier.

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Introduction

Botulinum neurotoxins (BoNTs) are secreted by the bacterium *Clostridium botulinum* and less frequently, by *Clostridium butyricum* and *Clostridium baratii*. There are seven serotypes of BoNTs, designated type A through G (BoNT/A–G), which include at least 40 different subtypes (for a recent review, see [1]). An eighth serotype, BoNT/H, has been reported recently, but is pending further validation [2,3]. BoNT/A, B, E and F are known to cause botulism in both human and other animals, while BoNT/C and D mainly affect cattle, poultry, and wild birds (for a recent review, see [4]). All BoNTs carry out their damage as potent blockers of neurotransmission in the peripheral cholinergic nerve terminals [5].

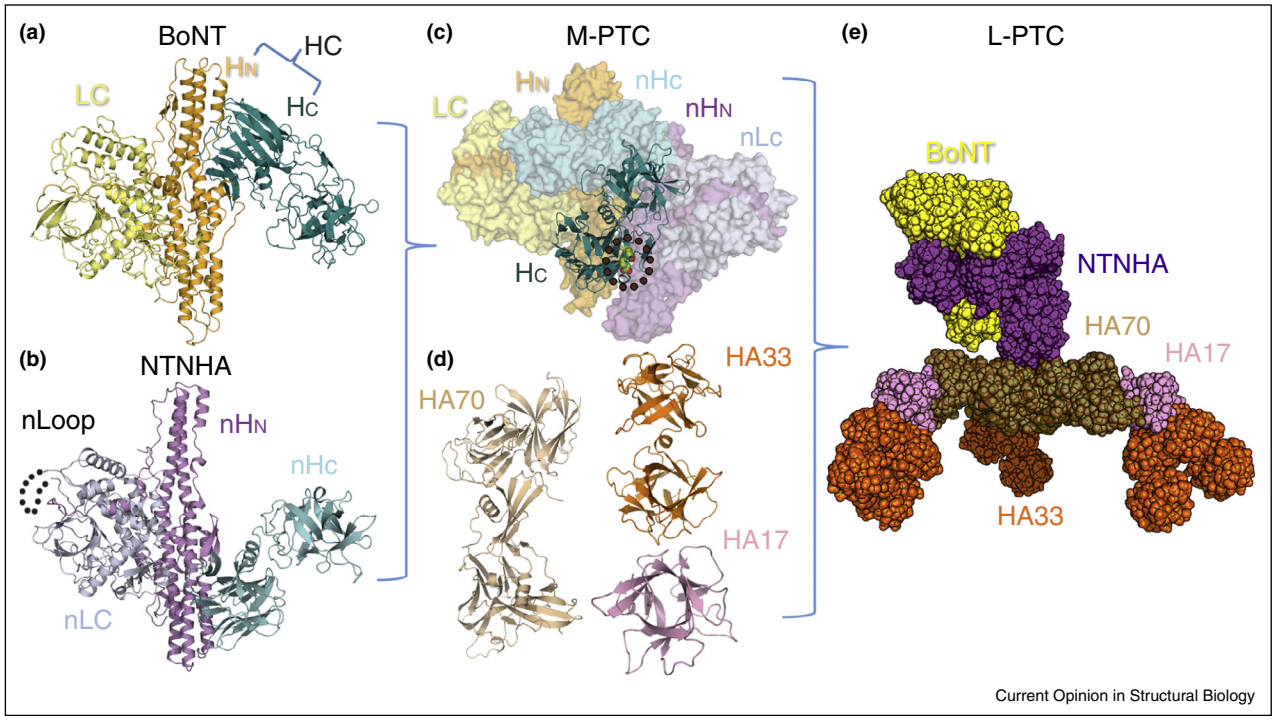
BoNTs are tripartite proteins consisting of a ~50 kDa light chain (LC) and a ~100 kDa heavy chain (HC). HC can be further divided into an N-terminal translocation domain (H_N) and a C-terminal receptor-binding domain (H_C or RBD) (Figure 1a). Upon arriving at neuromuscular junctions, H_C helps BoNT attach to the neuronal membrane by binding to gangliosides and specific synaptic vesicle proteins (e.g., synaptotagmin or synaptic vesicle glycoprotein 2) [6–8]. The toxin is then endocytosed with its receptors, followed by H_N-mediated translocation of LC across the vesicle membrane to the cytosol. LC is a Zn^{2+}-endopeptidase that cleaves SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) components and arrests the synaptic recycling. The resulting blockade of cholinergic neurons subsequently leads to fatal muscle paralysis [9].

By contrast to the well-studied BoNT–neuron interaction, it is not known how BoNTs in foodborne botulism manage to achieve efficient absorption through the gastrointestinal (GI) tract, which is possibly the most challenging route of entry into the systemic circulation. After ingestion with toxin-contaminated food, BoNTs have to tolerate the extremely acidic (pH < 3) and protease-rich environment of the stomach, and the tightly regulated intestinal barrier. We now know that BoNTs overcome the host defense in the form of a large multi-protein complex, the progenitor toxin complex (PTC). The PTC of some BoNT serotypes exhibits ~360–16,000-fold greater oral toxicity than the free BoNT [10–13]. In this review, we summarize recent progress in understanding the structure and assembly of the PTC, emphasizing the structural determinants that guard the toxin when circumventing the primary host defense in the gut.

The progenitor toxin complex, BoNT's landing gear

BoNTs are naturally produced as PTCs, which are composed of BoNT and several auxiliary components termed nontoxic neurotoxin-associated proteins (NAPs). The NAPs are encoded together with the *bont* gene in one of two different gene clusters, the HA cluster or the orfX cluster [14]. Both clusters encode the non-toxic non-hemagglutinin (NTNHA) protein (Figure 1b), which assembles with BoNT to form the minimally functional PTC (M-PTC, also termed the 12S toxin) (Figure 1c). The HA gene cluster, as observed in BoNT/A–D and G, encodes three hemagglutinins (HA70, HA17, and HA33; also called HA3, HA2, and HA1, respectively) (Figure 1d), which together with BoNT and NTNHA constitute the

Figure 1



Macromolecular assembly of the L-PTC. **(a)** BoNT is composed of the N-terminal LC (pale yellow), H_N (bright orange), and the C-terminal H_C (deep teal) (PDB: 3BTA). **(b)** NTNHA displays a similar domain organization as BoNT, which contains nLC (blue white), nH_C (cyan), and nH_N (violet). The nLoop likely mediates interaction with HAs (dotted line) (PDB: 3V0A). **(c)** The assembly of the interlocked M-PTC is regulated by environmental pH, and two pH sensing residues on BoNT/A have been identified (green sphere and circled). **(d–e)** The M-PTC (BoNT/yellow–NTNHA/purple) further assembles with three HA70 (sand) (PDB: 4LO4), three HA17 (pink), and six HA33 (orange) (PDB: 4LO0) to form the bimodular L-PTC.

large PTC (L-PTC or the 16S toxin) (Figure 1e) [15]. By contrast, some BoNTs, such as BoNT/A2–4, E, and F, are encoded in the orfX gene cluster, which contains several *orfX* genes but not the *HA* genes. The function of the corresponding orfX proteins remains elusive.

Atomic models of the L-PTC of BoNT/A (L-PTC/A) and BoNT/B (L-PTC/B) have been recently elucidated using a combination of X-crystallography and electron microscopy (EM) [16••,17••]. They display a similar structural organization, which is composed of 14 protein subunits including BoNT, NTNHA, HA70, HA17 and HA33 in a 1:1:3:3:6 stoichiometry. The overall architecture of the L-PTC consists of two structurally and functionally independent entities, an ovoid-shaped M-PTC and a triskelion-shaped HA complex (Figure 1e). The M-PTC protects BoNT from destruction in the GI tract and the HA complex allows BoNT to dock onto the receptors located on the lumen of the small intestine. Based on an earlier EM study, the L-PTC of BoNT/D (L-PTC/D) likely adopts a similar structure [18], suggesting that the L-PTC structure may be conserved across HA-containing BoNT serotypes.

Structure and function of the M-PTC

In the absence of M-PTC formation, free BoNT/A is easily inactivated by digestive proteases or by incubation under an acidic environment. Its oral median lethal dose (LD_{50}) is reduced 10–20-fold when it forms the M-PTC with NTNHA. The crystal structure of the M-PTC of BoNT/A offers the first molecular insight into the protection mechanism (Figure 1c) [19••,20]. NTNHA-A has a strikingly similar tripartite architecture as BoNT/A, despite their low amino acid sequence identity. The three domains of NTNHA-A are therefore named as nLC, nH_N, and nH_C, because they resemble LC, H_N, and H_C of BoNT/A, respectively. However, BoNT/A residues that are important for its Zn^{2+}-dependent endopeptidase activity and receptor binding are lost in NTNHA-A, which therefore lacks the neurotoxicity. BoNT/A and NTNHA-A form an inter-locked complex that buries a large solvent-accessible area of ~3200 Å per subunit. Interestingly, all three domains of NTNHA-A bind to the H_C fragment of BoNT/A, leaving LC largely exposed (Figure 1c), which is consistent with the biochemical finding that H_C is more susceptible to proteolytic cleavage than LC and H_N [21,22]. Mechanistically, H_C-mediated