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

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### Abstract

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.

### 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<sub>N</sub>) and a C-terminal receptor-binding domain (H<sub>C</sub> or RBD) (Figure 1A). Upon arriving at neuromuscular junctions, H<sub>C</sub> 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<sub>N</sub>-

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mediated translocation of LC across the vesicle membrane to the cytosol. LC is a  $\text{Zn}^{2+}$ -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 ( $\text{pH} < 3$ ) 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  $\sim 360$ – $16,000$ -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 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<sub>50</sub>) 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<sub>N</sub>, and nH<sub>C</sub>, because they resemble LC, H<sub>N</sub>, and H<sub>C</sub> of BoNT/A, respectively. However, BoNT/A residues that are important for its Zn<sup>2+</sup>-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<sub>C</sub> fragment of BoNT/A, leaving LC largely exposed (Figure 1C), which is consistent with the biochemical finding that H<sub>C</sub> is more susceptible to proteolytic cleavage than LC and H<sub>N</sub> [21,22]. Mechanistically, H<sub>C</sub>-mediated receptor binding is the earliest step during neuron invasion and likely one of the most crucial, because damage to H<sub>C</sub> would otherwise jeopardize the enrichment of BoNT/A on the neuron membrane [23]. Therefore, the apparently biased molecular safeguard for H<sub>C</sub>, as opposed to the other toxin domains, is likely the most efficient strategy to protect such a large protein [19••].

Interestingly, BoNT is released from the PTC upon transition from acidic to neutral pH, as occurs during absorption from the intestine into the general circulation [24]. This is achieved by the presence of pH-dependent interactions between BoNT and NTNHA [19••]. Recent small-angle X-ray scattering studies indicate that NTNHA-A is able to sense the change of environmental pH, and that acidic conditions induce NTNHA-A to adopt a specific conformation that initiates a mutual induced fit between NTNHA-A and BoNT/A [25•,26]. At the same time, pH-sensing residues on BoNT/A (e.g., Glu982 and Asp1037) and NTNHA-A are protonated to allow favorable local electrostatic interactions between them to strengthen the binding (Figure 1C) [19••]. The inherent pH sensing feature of the M-PTC is crucial to ensure stable binding to protect BoNT in the GI tract and release it in systemic circulation.

Currently, a high-resolution structure of an M-PTC is only available for BoNT/A. The structures of the M-PTC of BoNT/B and BoNT/E revealed by negative stained EM and 3D reconstruction closely resemble that of BoNT/A [17••]. The crystal structure of the free-form of NTNHA-D is highly similar to NTNHA-A [26]. Therefore, the BoNT–NTNHA binding module is likely conserved across different BoNTs serotypes. It is worth noting that a unique peptide fragment in nLC of NTNHA, termed the nLoop, is conserved in HA-containing BoNT serotypes, and likely mediates the interaction between the M-PTC and the HA complex [19••,27]. But the molecular details of this interaction have yet to be determined.

## Structure and function of the HA complex

### Architecture of the HA complex

Atomic models of the fully assembled HA complexes of BoNT/A (HA/A) and BoNT/B (HA/B) have been determined recently [16••,17••,28•]. In addition, the subcomponent structures of HAs are available for BoNT/C (HA33 and HA70) [29-32] and BoNT/D (HA17–HA33 complex) [18]. The HA complex features three prominent triangular blades (Figure 2). The center of the complex is the trimeric HA70 hub. Each HA70 contains three domains (named D1–3): D1 and D2 participate in homo-trimerization and D3 sits at the periphery of the trimer and interacts with HA17. HA17 contains a compact  $\beta$ -trefoil fold and simultaneously binds two HA33 molecules. Each HA33 is composed of two  $\beta$ -trefoil domains linked by an  $\alpha$  helix (Figure 2). Although the N-terminal domain of HA33 is restrained by docking to HA17, its C-terminal domain is exposed and exhibits significant conformational plasticity [16••,28•]. The overall structure of the HA complex is likely to be flexible and its three arms may adopt different conformations [17••].

### The HA complex binds to cell-surface carbohydrates

The intestinal microvilli are covered by a stratified layer of mucus. The HA complex is believed to anchor the L-PTC on the intestinal surface through its multiple carbohydrate-binding sites. HA70 binds one Neu5Ac-containing carbohydrate [16••]. HA33, on the other hand, binds one galactose-containing carbohydrate through its C-terminal  $\beta$ -trefoil domain in serotypes A and B [16••,33•]. HA33 serotype C, however, displays a lower affinity for galactose, but carries an extra Neu5Ac-binding site near the Gal-binding pocket [30,34]. These serotype-specific HA–glycan interactions may partially contribute to the different oral toxicity and host susceptibility among different BoNT serotypes [35-38]. Altogether, each HA complex likely displays multivalent carbohydrate binding, involving up to 9 carbohydrates in L-PTC/A and B and up to 15 carbohydrates in L-PTC/C (Figure 2) [16••, 30,34]. Moreover, the carbohydrate-binding domain of HA33 is located at the very tip of the HA complex and displays significant conformational flexibility, which may allow the complex to adjust itself on the intestinal surface to achieve optimal multivalent glycan binding [35,39]. The physiological importance of the HA–carbohydrate interactions has been further validated by *in vivo* studies using a mouse oral toxicity assay. Carbohydrate receptor mimics (for instance, IPTG) extended survival of mice following lethal BoNT/A oral intoxication [16••], and a mutated L-PTC/A that is unable to bind carbohydrate displayed drastically reduced oral toxicity [40••].

### The HA complex disrupts the E-cadherin-mediated cell-cell junctions

The intestinal epithelial cells are tightly regulated by adhesion proteins that physically separate the lumen from the lamina propria. Therefore, it is fascinating that the HAs can hijack the adhesion protein E-cadherin to disrupt the intestinal epithelial barrier [41,42]. E-cadherin contains five tandem extracellular cadherin (EC) domains (EC1–EC5), which share a common seven-stranded  $\beta$ -barrel fold. They are typically located below tight junctions and mediate cell-cell recognition and adhesion via trans-dimerization between their N-terminal EC1 domains, which extend from the apposed cells, and cis-dimerization between molecules on the same cell [43,44]. In the trans-dimer, the EC1 domains from apposed cells form a

“strand-swapped” conformation in which residue Trp2 acts as an anchor by docking into a complementary Trp-binding pocket in the partner E-cadherin molecule (Figure 2, left panel). This occurs via a two step-binding process that involves a fast-binding intermediate conformation named the “X-dimer” [43,45]. The dimeric E-cadherin is energetically more favorable than a monomeric conformation that places a conformational strain on its N-terminal 10 amino acids (termed the A\*/A strand) [46].

A major advance was provided by the crystal structure of an HA/A subcomplex bound with EC1–EC2 of E-cadherin [40••]. This structure showed that the HA complex stabilizes the A\*/A strand of E-cadherin in its monomeric conformation with Trp2 binding intramolecularly into its own Trp-binding pocket, therefore relieving the driving force for trans-dimerization (Figure 2, left panel). Furthermore, the HA complex occupies the E-cadherin dimer interface that is required to form the trans-dimer and the X-dimer. Consistent with this finding, HA/A binds the monomeric EC1–EC2 domains with an affinity that is much stronger than the affinity of E-cadherin homo-dimerization [40••,44,47]. The model that disruption of the adherens junctions of epithelial cells by the HA complex opens up a paracellular route to facilitate BoNT absorption has been supported by extensive in vitro and ex vivo studies [33•,40••,42] (Figure 3), and was further confirmed by an in vivo study showing that an E-cadherin binding deficient L-PTC/A has markedly decreased oral toxicity in mouse [40••].

Interestingly, the complete triskelion-shape of the HA complex is crucial for its function, because a sub-complex representing one arm of the HA complex failed to disrupt cell-cell junctions [40••]. The fully assembled HA complex might simultaneously bind three E-cadherins, which would greatly strengthen binding through avidity effects and is likely necessary to achieve potent binding in vivo. Furthermore, the bulky triskelion-shaped HA complex (~260 Å wide and ~100 Å height) might disrupt the condensed arrays of E-cadherin dimers that normally stabilize adherens junctions. Additionally, sequestration of E-cadherin by the HA complex might destabilize adherens junctions by affecting the turnover of E-cadherin at adherens junctions [48].

Most of the E-cadherin-binding residues are conserved between the HAs of BoNT/A and BoNT/B, suggesting that the HA–E-cadherin binding mode is likely conserved between these human toxins. By contrast, the HAs of BoNT/C and D, which predominantly cause botulism in birds and cattle, do not bind E-cadherin [33•,40••]. Future studies should aim to understand whether or how HA/C and HA/D disrupt the intestinal epithelial barrier and the functional role of HAs in determining host tropism of various BoNTs [49,50].

## Current models for trans-epithelial delivery of the L-PTC

Many structural and functional studies suggest that BoNTs have two different routes of passing through the intestinal epithelial cells (Figure 3). In one scenario, BoNTs in the forms of L-PTC, M-PTC or the free form may cross the epithelial cells by transcytosis without interfering with the epithelial barrier. Once the HA complexes gain access to the basolateral surface, they disrupt E-cadherin-mediated cell-cell adhesion, thereby opening up the paracellular route for BoNT absorption. However, many fundamental questions remain

unanswered. For example, the mechanism of BoNT transcytosis is largely unknown. Some data suggest that BoNT might directly recognize specific receptors on intestinal cell surface that mediate transcytosis [51,52]. Alternatively, it is possible that there are transcytosis hot spots on intestinal epithelia, for instance microfold cells (M cells) and neuroendocrine crypt cells, which could mediate BoNT transcytosis [42,53]. Notably, E-cadherin is lumenally accessible around mucus-expelling goblet cells, around extruding enterocytes at the tip and lateral sides of villi, and in villus epithelial folds [54]. Hence, the HA complex might access E-cadherin in the intestinal lumen to mediate transcytosis and/or paracellular crossing.

## Conclusion and future perspectives

Structures of the 760 kDa L-PTC have revealed a sophisticated macromolecular machine of bacterial toxins that evades host defense systems. Besides stabilizing BoNT in the harsh environment of the GI tract, the L-PTC efficiently delivers BoNT into the general circulation through up to 15 binding sites for cell surface carbohydrates and 3 binding sites for the crucial host adhesion protein E-cadherin. It is worth noting that BoNTs use a dual-receptor mechanism to recognize nerve terminals by interacting with both a protein receptor and gangliosides to mediate cell entry at neuromuscular junctions [23]. It is remarkable that BoNTs use the “same” strategy twice, targeting different host receptors at different times and locations, to ensure its extreme toxicity.

The advances in understanding the structure and function of the L-PTC will promote the development of novel chemical inhibitors or antibody/peptide inhibitors that block the L-PTC from recognizing intestinal glycan and protein receptors, thereby preventing toxin invasion. The L-PTC could also be exploited for alternative applications. For example, coupling of protein-based therapeutics to a modified non-toxic L-PTC or the HA complex might facilitate drug delivery by enhancing permeability of the intestinal epithelium. Thus, the improved structural understanding of these fascinating macromolecular assemblies informs efforts to treat a deadly toxin and opens opportunities to develop novel therapeutics.

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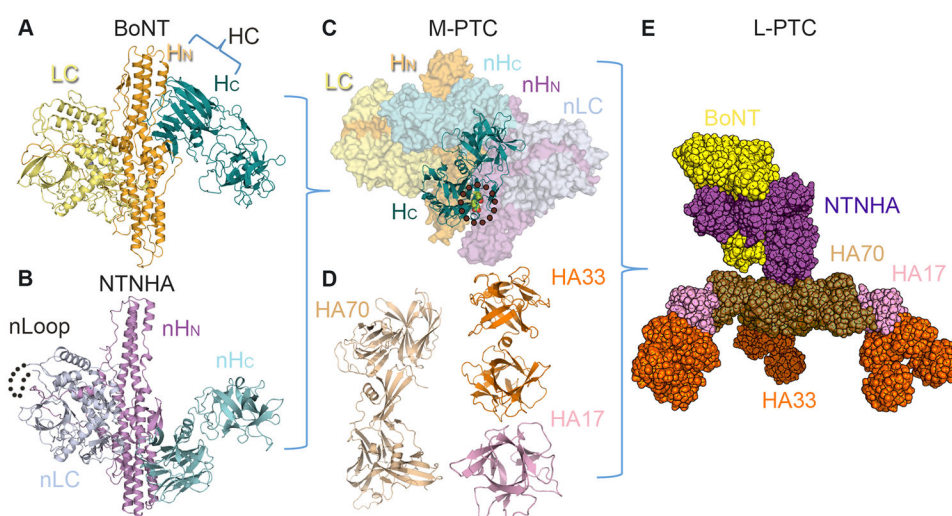
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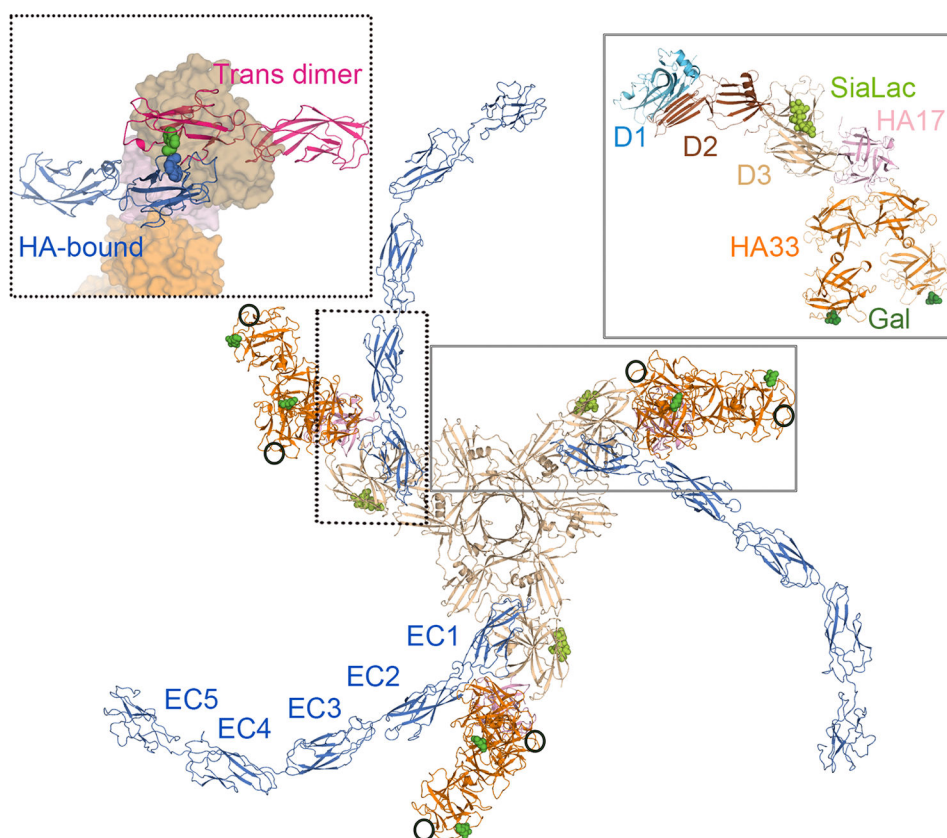
### Highlights

- Botulinum neurotoxins are highly potent oral toxins.
- The large progenitor complex of BoNT is a bimodular 14-subunit complex.
- NTNHA protects BoNT in the acidic and protease-rich gastrointestinal tract.
- The HA complex displays multivalent binding with the host glycans in the intestine.
- The HA complex hijacks E-cadherin to cross epithelial cell junctions.



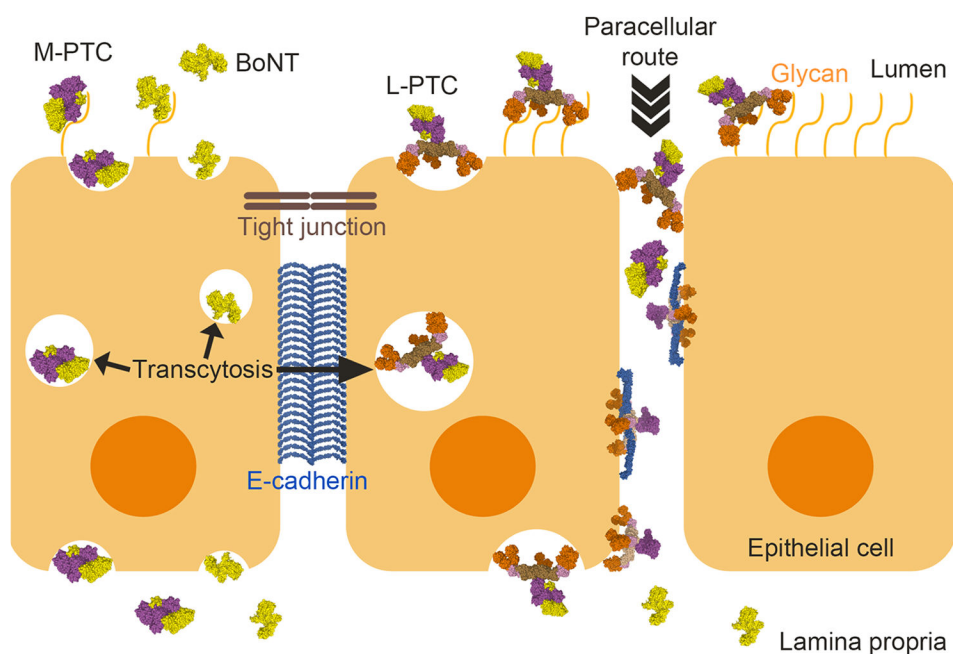
**Figure 1.**

Macromolecular assembly of the L-PTC. **(A)** BoNT is composed of the N-terminal LC (pale yellow), H<sub>N</sub> (bright orange), and the C-terminal H<sub>C</sub> (deep teal) (PDB: 3BTA). **(B)** NTNHA displays a similar domain organization as BoNT, which contains nLC (blue white), nH<sub>C</sub> (cyan), and nH<sub>N</sub> (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.



**Figure 2.**

The triskelion-shaped HA complex in complex with carbohydrates and E-cadherin. As highlighted in the right box, each arm of the HA complex contains one HA70 (sand), one HA17 (pink), and two HA33 (orange). HA70 D1 (light blue) and D2 (brown) domains are crucial for the trimer formation of HA70. HA/A contains nine glycan-binding sites with three Neu5Ac-binding sites on HA70 (lime) and six galactose-binding sites on HA33 (forest) (PDB: 4LO1 and 4LO5). HA/C may contain one additional Neu5Ac-binding site on each HA33 (circled). E-cadherin has five extracellular domains (EC1–EC5). To form a trans dimer, residue Trp2 (green sphere) of an E-cadherin (blue molecule) needs to dock into the complementary Trp-binding pocket on the partner E-cadherin (pink molecule). As shown in the left box, the HA complex (surface representation) sequesters E-cadherin (blue molecule) in the monomeric state with the Trp2 (blue sphere) resting in its own Trp-binding pocket, and also blocks the access of its potential dimer partner (PDB: 4QD2).



**Figure 3.**

Models for the intestinal absorption of the BoNT complexes. L-PTC, M-PTC or BoNT could be adsorbed by transcytosis that is mediated by carbohydrates and/or unknown protein receptors on the cell surface. When the L-PTC gains access to E-cadherin, the HA complex could disrupt the epithelial barrier, leading to efficient absorption of BoNTs and PTCs through the paracellular route. After crossing the epithelial barrier, BoNT dissociates from the complex and enters into the systemic circulation.