A viral-fusion-peptide-like molecular switch drives membrane insertion of botulinum neurotoxin A1

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Botulinum neurotoxin (BoNT) delivers its protease domain across the vesicle membrane to enter the neuronal cytosol upon vesicle acidification. This process is mediated by its translocation domain (HN), but the molecular mechanism underlying membrane insertion of HN remains poorly understood. Here, we report two crystal structures of BoNT/A1 HN that reveal a novel molecular switch (termed BoNT-switch) in HN, where buried \( \alpha \)-helices transform into surface-exposed hydrophobic \( \beta \)-hairpins triggered by acidic pH. Locking the BoNT-switch by disulfide trapping inhibited the association of HN with anionic liposomes, blocked channel formation by HN, and reduced the neurotoxicity of BoNT/A1 by up to \( \sim180 \)-fold. Single particle counting studies showed that an acidic environment tends to promote BoNT/A1 self-association on liposomes, which is partly regulated by the BoNT-switch. These findings suggest that the BoNT-switch flips out upon exposure to the acidic endosomal pH, which enables membrane insertion of HN that subsequently leads to LC delivery.
Many bacterial toxins and viruses gain access to intracellular targets in their hosts by exploiting the endocytic pathway, such as anthrax toxin\(^1\), diphtheria toxin\(^2\), Ebola virus\(^3\), and vesicular stomatitis virus\(^4\). Some of the toxins and viruses detect acidification of the vesicle lumen and subsequently change from a water-soluble form to a membrane-embedded form to exert their functions. Botulinum neurotoxins (BoNTs), the most poisonous biological toxins for mammals, are speculated to exploit a similar mechanism to block neurotransmitter release at neuromuscular junctions that results in fatal botulism\(^5\). As some BoNTs, in particular type A1 (BoNT/A1), are powerful medicines as well as potential bio-weapons\(^6\)-\(^8\), a better understanding of the molecular mechanism underlying BoNT/A1 membrane translocation has great potential for improving its therapeutic efficacy and developing antitoxins.

There are seven established BoNT serotypes, designated as BoNT/A to G, which include more than 40 subtypes\(^9\). BoNTs are members of single-chain AB toxins that also include diphtheria toxin and tetanus neurotoxin\(^9\). A BoNT molecule consists of a light chain (LC) and a heavy chain (HC) that are linked by a disulfide bridge\(^10\)-\(^12\). LC is a zinc-dependent endopeptidase that is delivered into the cytosol where it degrades SNARE proteins to inhibit synaptic vesicle exocytosis. Membrane translocation of LC is mediated by HC, which can be subdivided into a translocation domain (HN) and a receptor-binding domain (HC) (Fig. 1a). The N-terminus of HN forms an extended “belt” that wraps around the LC and links HN to LC via a disulfide bond\(^13\). The BoNT HC binds a specific protein receptor and a ganglioside on the neuronal surface to enable toxin endocytosis\(^14\)-\(^39\).

The mechanisms underlying BoNT membrane insertion and LC translocation have been the subject of considerable debates. Earlier studies showed that BoNT/A1 translocated LC in planar lipid bilayer in the presence of a transmembrane pH gradient, an electrochemical potential, and a redox gradient\(^20\),\(^21\). It was proposed that acidic pH might induce a structural change of BoNT and promote its interaction with phospholipids\(^17\), which is similar to other pore forming toxins\(^2\),\(^22\),\(^23\). Although no conformational change was detected in BoNT at acidic pH in the available crystal structures\(^24\), it underwent an unknown structural rearrangement after binding to ganglioside-containing membranes\(^25\)-\(^29\). Furthermore, the HN core lacking the “belt” (termed tHN hereafter, Fig. 1a) was shown to form an ion channel independent of pH, and no clear structural change was detected by spectroscopic methods at acidic pH\(^30\)-\(^32\). Other regions of HN have also been proposed to be involved in membrane insertion\(^33\),\(^34\). However, the structural rearrangement of BoNT during LC delivery, particularly the trigger for membrane insertion of HN, has not been visualized.

To better understand the mechanism of BoNT membrane translocation, we determined the crystal structure of HN of BoNT/A1 (tHN, residues K547–K871) at pH 5.1, as well as a point mutation of tHN (tHN\(^{F668E}\)) at pH 8.5. To our surprise, these two structures revealed a previously unrecognized amphipathic region of HN (residues E620–F667, termed the BoNT-switch) that undergoes a profound pH-dependent structural rearrangement to form surface-exposed β-hairpins. Furthermore, the BoNT-switch shares a high sequence similarity with the internal fusion loop (IFL) of Ebola virus glycoprotein 2. A combination of structural studies, biochemical assays, single-molecule subunit counting, and functional assays confirmed that, upon endosome acidification, the BoNT-switch triggers the transformation of BoNT/A1 into a transmembrane state for LC delivery, which is essential for its extreme toxicity.

### Results

**Acidic pH triggers membrane insertion of tHN\(_A\).** The HN of BoNTs has evaded structural studies due to problematic protein folding during recombinant expression\(^31\),\(^32\). We and others found that HN\(_A\) primarily formed inclusion bodies in *Escherichia coli*, which could be refolded when stabilized by detergent. However, we found that the blltless HN (termed tHN\(_A\)) could be successfully expressed in soluble form and purified to high homogeneity (Supplementary Fig. 1a). Size-exclusion chromatography–coupled small-angle X-ray scattering (SEC-SAXS) showed that tHN\(_A\) was monomeric at neutral pH and displayed a conformation comparable to that observed in the crystal structure of full-length BoNT/A1\(^10\) (Supplementary Fig. 1b, c). Thus, deletion of the belt region improves protein folding of HN\(_A\) without altering its tertiary structure.

Some earlier studies suggested that low pH and anionic phospholipids may promote the membrane interaction of HN\(_A\)\(^30\),\(^32\).\(^33\).\(^35\). We conducted a liposome co-sedimentation assay to investigate the effect of pH and lipid composition on tHN\(_A\)-membrane association. We found that tHN\(_A\) was co-pelleted with asolectin liposomes at pH 4.4 or 5.0, but not at pH 7.5, indicating that acidic pH enhances the association of tHN\(_A\) with lipid (Fig. 1b). We then examined liposomes containing different ratios of anionic phosphatidyserine (PS) and neutral phosphatidylcholine (PC) lipids and found that tHN\(_A\) binding to liposomes at pH 4.4 was greatly enhanced by increasing concentrations of PS. This demonstrates that tHN\(_A\) shows selectivity for anionic lipids (Fig. 1c).

We further characterized the membrane insertion of tHN\(_A\) using two complementary assays. First, we monitored the ability of tHN\(_A\) to permeabilize calcein-entrapped liposomes. We found that tHN\(_A\) increased the rate of calcein release at acidic pH when liposomes contained the anionic phospholipid 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) or asolectin, but not when liposomes contained only the neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Fig. 1d).

**Acidic pH triggers a structural rearrangement of tHN\(_A\).** To better understand how acidic pH triggers membrane insertion of tHN\(_A\), we crystallized tHN\(_A\) at pH 5.1 and determined the structure at 2.7 Å resolution (Methods) (Table 1). Two molecules of tHN\(_A\) form a homodimer in the asymmetric unit (Fig. 2a). Each tHN\(_A\) is composed of two long coiled-coil helices that are wrapped around by several shorter helices at the two ends, and the overall structure of tHN\(_A\) is similar to that observed in full-length BoNT/A1 crystallized at a neutral pH\(^10\). However, we observed a major conformational change in a central helical bundle (residues E620–F667) of HN\(_A\). This region, which we term the “BoNT-switch”, is composed of disordered loops and three short helices (F662–F659) at neutral pH, but changes into five β strands (β1–β5) at acidic pH (Fig. 2b).

At neutral pH, α\(_A\) helix and the surrounding loops (residues I630–Y648, named β2/β3 loop thereafter, labeled green in Fig. 2b) are partially buried in a hydrophobic groove located between α\(_A\) (residues F652–S659) and the coiled-coil helices (Fig. 2b). The
conformation of this region is similar among BoNT/B, D, E, and TeNT. These contacts are stabilized by Y636 that interacts with F658, M732, and I784 (Fig. 2b). At acidic pH, the αA protrudes outward to form the center of the β2/β3 hairpin. This leaves the hydrophobic groove exposed to solvent, thus helices α9 and αC transform into the β4/β5 hairpin to partly shield the solvent-exposed surface. The low pH conformation is facilitated by several non-polar residues (V653, F658, and F667). These residues are solvent-exposed at neutral pH, but flip inward at acidic pH. Notably, the highly conserved Y636 that is important for stabilizing the local structure at neutral pH flips out to be solvent exposed at acidic pH. Interestingly, the original position of Y636 is taken over by F658. This permits F658 to participate in sensing low pH.

Fig. 1 Biochemical characterization of tH₃A. a The structure of BoNT/A1 (PDB code 3BTA). LC, the “belt”, tH₃, and H₃ are colored green, marine, orange, and cyan, respectively. The disulfide linkage between LC and H₃ is shown as salmon sphere and the BoNT-switch is highlighted in magenta. b, c Co-sedimentation of tH₃A with liposomes. tH₃A was incubated with asolectin liposomes at pH 7.5, 5.0 or 4.4 (b); or incubated with liposomes containing 60/40 mol% PC/cholesterol, 30/30/40 mol% PC/PS/cholesterol, or 60/40 mol% PS/cholesterol at pH 4.4 (c). After liposomes were pelleted, the proteins in input, supernatant, and pellet fractions were analyzed by SDS-PAGE. These experiments were performed in triplicate and quantified by protein band intensities are shown in Supplementary Fig. 11. Uncropped images of gels are shown in Supplementary Fig. 13. d Membrane depolarization assay. Liposomes composed of 70/20/10 mol% DOPC/DOPS/cholesterol were polarized at a positive internal voltage by adding valinomycin in the presence of a transmembrane KCl gradient. Membrane potential was measured using the voltage-sensitive fluorescence dye ANS. After 3 min, tH₃A was added at the indicated buffer pH. The data are presented as mean ± S.D., n = 3

Interestingly, the Propka algorithm predicted that three acidic residues (E620, D629, and E666) located within the BoNT-switch have increased pKa values in the context of the neutral pH conformation. Two of these residues, D629 and E666, are locked in hydrogen bonds to S794 and Y250, respectively, while these interactions are released at acidic pH (Supplementary Fig. 2). Therefore, we suspect that these titratable charged residues could be involved in sensing low pH.

As this is the only crystal structure for the isolated tH₃A and all the other available structures are for the full-length BoNT/A or LC/A-H₃A, it would be informative for structural analysis if we could obtain the structure of tH₃A, without the influence of other BoNT/A domains, at a neutral pH. Interestingly, diffraction-quality crystals of tH₃A could only be obtained at an acidic pH despite thorough crystallization screens at various pH. We suspected that the isolated tH₃A domain may be prone
Interestingly, these hydrophobic patches in β²/β³, which are energetically not favored in an aqueous environment, are shielded by forming a homodimer in the crystal lattice, which buries a surface area of ~2,625 Å² per molecule (PDBePISA). Specifically, the tHNA homodimer forms a two-side flattened β-barrel that is stabilized by swapping the β²/β³ hairpin between the two protomers, which forms an antiparallel β-sheet with β₁, β₄, and β₅ (Fig. 2a). Dimer formation is stabilized by hydrogen bonds primarily involving the main-chain atoms of β₂ and β₅ (Supplementary Fig. 5a). As a result, most of the exposed hydrophobic side-chains of the β-hairpins are buried within the tHNA homodimer in the crystal structure (Supplementary Fig. 5b, c).

Remarkably, we found that the β²/β³ loop contains a stretch of hydrophobic residues rich in aliphatic side-chains, which shares a high sequence similarity to a lipid-binding peptide of the glycoprotein 2 (GP2) IFL of Ebola virus, a member of the Filoviridae family of enveloped viruses. IFL is known to control viral-host membrane fusion at low endosomal pH by directly engaging the host membrane 46 (Fig. 2b). The loops in both BoNT/A1 and GP2 contain an “aromatic-hydrophobic-glycine” tripeptide motif flanked by two proline residues, which would favorably interact with the interface region of lipid bilayers 46. Amino acid sequence alignments among the 44 known BoNT subtypes, including the recently identified BoNT/HA 47, BoNT/X 48, and eBoNT/J (aka BoNT/En) 49,50, as well as TeNT, showed that the sequence of the β²/β³ loop is highly conserved, suggesting a functional role for this loop in BoNTs (Fig. 2c).

We noted that the second proline (P639) is conserved among BoNT/A, C, D, G, X, and TeNT, but this residue is replaced by a leucine in BoNT/B, E, F, and HA or an asparagine in eBoNT/J (Supplementary Fig. 6). Such variation may indicate that the structure of the BoNT-switch when inserted into membrane could be different from that of the viral-fusion-loop of GP2.

The BoNT-switch is similar to the Ebola GP2 fusion loop. The structural rearrangement of the BoNT-switch causes a ~21% increase in the solvent-accessible hydrophobic surface in tHNA 45, which is largely ascribed to the formation of the β²/β³ loop (Fig. 2b, c). The structural and sequence analyses of the BoNT-switch indicate that the solvent-exposed β²/β³ loop is favorable for interacting with lipids, as supported by the hydropathy analysis of the BoNT/A sequence (Supplementary Fig. 4).

Interestingly, these hydrophobic patches in β²/β³, which are to adopting the acidic pH conformation needed for LC translocation, and its neutral pH conformation could be stabilized by LC/A and/or HCA. Taking advantage of our new crystal structure, we set out to look for point mutations on tHNA that shift the structural equilibrium of tHNA more towards the neutral pH conformation as opposed to the acidic pH conformation. After extensive search and screening, we found that tHNA carrying a single point mutation of F658E could be crystallized at pH 8.5 and we determined its structure at 3.02 Å (Table 1 and Supplementary Fig. 3a). This result is consistent with our structural analysis that suggested F658E may destabilize the structural change induced by acidic pH. Therefore, the engineered disulfide tHNAF658E is highly similar to the corresponding region in full-length BoNT/A that was crystallized at a neutral pH (root-mean-square deviation of ~0.96 Å). Notably, the BoNT-switch adopts an almost identical structure in tHNAF658E and the full-length toxin (Supplementary Fig. 3b). Taken together, the structures of tHNA at pH 5.1 and tHNAF658E at pH 8.5 allow us to directly visualize how the environmental pH regulates the conformation of the BoNT-switch.

The BoNT-switch is important to the membrane insertion of H₃Nₐ. To further characterize the role of the BoNT-switch in membrane insertion of tHNA, we engineered a covalent tethering between loopₐ and a₉ by replacing S622 and V653 with cysteines (named tHNA₁₂₃₅) (Fig. 2b and Supplementary Fig. 7). The crystal structures showed that these two solvent-exposed residues are 5.3 Å apart (Cβ–Cβ distance) when BoNT/A1 is at a neutral pH, but they move away from each other (15.5 Å) upon the conformational change induced by acidic pH. Therefore, the disulfide bridge between S622C and V653C will inhibit the helix-to-strand transition of a₉ when oxidized, and the inhibition will be released when reduced. To monitor exposure of hydrophobic surface in tHNA, we used 8-anilinonaphthalene-1-sulfonic acid (ANS) as an indicator that displays increased fluorescence that was released when reduced (Fig. 3a).

The BoNT-switch contains a stretch of hydrophobic residues rich in aliphatic side-chains, which shares a high sequence similarity to a lipid-binding peptide of the glycoprotein 2 (GP2) IFL of Ebola virus, a member of the Filoviridae family of enveloped viruses. IFL is known to control viral-host membrane fusion at low endosomal pH by directly engaging the host membrane 46 (Fig. 2b). The loops in both BoNT/A and GP2 contain an “aromatic-hydrophobic-glycine” tripeptide motif flanked by two proline residues, which would favorably interact with the interface region of lipid bilayers 46. Amino acid sequence alignments among the 44 known BoNT subtypes, including the recently identified BoNT/HA 47, BoNT/X 48, and eBoNT/J (aka BoNT/En) 49,50, as well as TeNT, showed that the sequence of the β²/β³ loop is highly conserved, suggesting a functional role for this loop in BoNTs (Fig. 2c).

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Table 1 Data collection and refinement statistics

|                 | tHNA | tHNAF658E |
|-----------------|------|-----------|
| Data collection |      |           |
| Space group     | H 3 2| P 6 2 2   |
| Cell dimensions |      |           |
| a, b, c (Å)     | 167.66, 167.66, | 158.27, 158.27, |
|                 | 222.83 | 123.97    |
| α, β, γ (°)     | 90, 90, 120 | 90, 90, 120 |
| Resolution (Å)  | 52.01-2.70 | 42.87-3.02 |
| Rmerge (%)      | (2.83-2.70)² | (3.20-3.02)² |
| CC1/2           | 0.999 (0.711) | 0.999 (0.886) |
| l/e(Å)          | 12.8 (2.0) | 15 (3.6)  |
| Completeness (%)| 99.3 (99.1) | 99.9 (100) |
| Redundancy      | 4.6 (4.1) | 9.1 (9.3) |
| Refinement      |      |           |
| Resolution (Å)  | 52.01-2.70 | 42.87-3.02 |
| No. reflections | 32906 | 18514     |
| Reflections used for | 1662 | 946       |
| Rmerge/Rmerge   | 0.209 / 0.223 | 0.182 / 0.237 |
| No. atoms       | 5176  | 2647      |
| Protein         | 5144  | 2602      |
| Ligand/ion      | 5     | 45        |
| Water           | 27    |           |
| B-factors       | 85.70 | 83.40     |
| Protein         | 85.80 | 83.00     |
| Ligand/ion      | 117.60| 107.10    |
| Water           | 65.50 |           |
| R.m.s. deviations| 0.009 | 0.010     |
| Bond lengths (Å)| 1.167 | 1.116     |

One crystal was used for each structure.
*Statistics for the highest resolution shell are shown in parentheses.
structure and stability of tHNA<sup>DS</sup> (Supplementary Fig. 8). At neutral pH, the melting temperature (Tm) of the oxidized tHNA<sup>DS</sup>, but not the reduced tHNA<sup>DS</sup>, increased by ~15% relative to WT tHNA, indicating that the engineered disulfide bond stabilized tHNA. The Tm could not be measured at pH 4.6 for either WT tHNA or the reduced tHNA<sup>DS</sup> due to high fluorescence intensity, because large hydrophobic surfaces in tHNA were exposed at acidic pH. However, the Tm of the oxidized tHNA<sup>DS</sup> could be measured at pH 4.6, which only decreased by ~6% in comparison to the Tm at neutral pH, suggesting that the oxidized tHNA<sup>DS</sup> is trapped in the neutral-pH-like conformation and insensitive to pH changes. These findings demonstrate that the BoNT-switch regulates the exposure of hydrophobic surface at acidic pH.

Using tHNA<sup>DS</sup> as a unique molecular probe, we further examined lipid association of tHNA using a liposome co-sedimentation assay (Fig. 3b). The WT tHNA and the reduced tHNA<sup>DS</sup> co-sedimented with anionic liposomes containing 20% DOPS at pH 4.6 in a lipid concentration-dependent fashion. In contrast, association of the oxidized tHNA<sup>DS</sup> with liposomes was significantly reduced at all lipid concentrations tested. Furthermore, the oxidized tHNA<sup>DS</sup> exhibited a marked decrease in both the rate of calcein dye release and the ability to dissipate the membrane potential of a polarized membrane in liposomes, while the reduced tHNA<sup>DS</sup> behaved like the WT tHNA (Fig. 3c, d). These findings prove that the acidic pH induced structural change of the BoNT-switch is necessary for tHNA to transform from a water-soluble state to a transmembrane state.
The BoNT-switch regulates self-association of BoNT/A1. Since the tHNA homodimer observed in the crystal lattice could be affected by crystal packing, we used analytical size-exclusion chromatography to examine the effect of pH on self-association of tHNA in solution. We found that tHNA shifted to a higher oligomeric state at acidic pH (pH 4.6) in a concentration-dependent manner (Supplementary Fig. 9c). Therefore, tHNA self-association seems to be needed to protect the unique conformation of the BoNT-switch at acidic pH, whereas such protection could be provided by other BoNT domains in the context of the full-length toxin or by the vesicle membrane in vivo. Furthermore, the micromolar tHNA concentration used in this assay is much higher than the physiological concentration of BoNT/A1 at the neuromuscular junction. Therefore, tHNA dimerization may not be directly relevant for BoNT/A1 function in vivo. In support of this, we found that the full-length BoNT/A1i (a genetically inactivated BoNT/A1 carrying R363A/Y366F mutations) does not exhibit pH-dependent self-association in solution (Supplementary Fig. 9d).

To investigate whether membrane binding may trigger BoNT/A1 self-association, we used single-molecule photobleaching to count the number of BoNT/A1 molecules recruited to single liposomes containing gangliosides. Photobleach step counting has been widely used to study protein oligomerization on the membrane. Since BoNT/A1 contains several functionally important cysteine residues, we could not chemically-label BoNT/A1. To circumvent this, we fluorescently labeled a BoNT/A1-specific camelid heavy-chain-only antibody (ciA-D12) that binds to LC with high affinity (Kd ~0.2 nM) and does not affect BoNT/A1 function. Importantly, the ciA-D12-BoNT/A1i interaction is not affected by acidic pH (Supplementary Fig. 10). An engineered cysteine residue (S142C) in ciA-D12 was labeled to high affinity (Kd ~0.2 nM) and does not affect BoNT/A1i-D12* complex assembly and purification by size-exclusion chromatography.

The BoNT/A1i-D12* complex was reconstituted on 100 nm anionic liposomes containing 10% polysialoganglioside GT1b. The liposomes also contained 0.5 mol% Rhodamine-labeled lipids to allow liposomes to be attached to a streptavidin-coated microscope slide while unbound proteins were washed away by rinsing. To allow co-localization of protein and liposomes, we included 0.5 mol% Rhodamine-labeled lipids and imaged the samples with two-color Total Internal Reflection Fluorescence (TIRF) microscopy. Liposomes were deposited under dilute conditions to achieve optical resolution of individual liposomes, which appeared as diffraction-limited spots (Fig. 4a). Under our incubation conditions (10 nM protein with 0.5 mg/mL lipid), we estimated that ~10% of liposomes were occupied by protein molecules. The low surface density and low liposome occupancy both help to minimize the probability of false co-localization of proteins.

The number of BoNT/A1i-D12* complexes on a liposome was determined by counting Alexa Fluor 647 photobleaching steps for diffraction-limited spots that also contained Rhodamine emission (Fig. 4b). At pH 4.6, we found that a major fraction of BoNT/A1i-D12* fluorescent spots displayed single-step bleaching with a smaller fraction showing multi-step bleaching up to five steps (Fig. 4c). We observed 66.2 ± 1.7%, 23.2 ± 2.4%, 8.2 ± 1.5%, 1.9 ± 1.2%, and 0.5 ± 0.5% for one to five-step events, respectively.
At neutral pH, we found a decrease in protein co-localization events. Specifically, two- and three-step events were reduced and higher order events disappeared entirely while the one-step bleaching events increased by 16.8 ± 3.8%. This finding suggests that, while BoNT/A1i binds to membranes predominantly as monomer at neutral or acidic pH on liposomes we tested here, an acidic environment increased the degree of BoNT/A self-association. Interestingly, low-pH-dependent self-association of BoNT was previously observed for BoNT/B on artificial membranes27.

To test whether locking the BoNT-switch could affect self-association of BoNT/A1 on membranes, we incorporated S622C and V653C into the full-length BoNT/A1i (termed BoNT/AiDS). BoNT/AiDS was oxidized and then assembled with the labeled antibody D12*. We found that the oxidized BoNT/AiDS showed a similar liposome occupancy as the WT toxin, suggesting that locking the BoNT-switch did not significantly affect membrane association of BoNT/A1. We then examined the oligomerization state of BoNT/AiDS by analyzing the photobleaching time traces as described above. Notably, locking the BoNT-switch showed significant effect on oligomerization of the full-length toxin (Fig. 4d). The increase in protein oligomerization at pH 4.6 was completely eliminated in the oxidized BoNT/AiDS, even though BoNT/AiDS showed the same level of monomers at pH 7.4 (82.8 ± 1.5%). Taken together, we found that acidic pH tends to promote BoNT/A1 oligomerization on liposomes, which is partly regulated by the BoNT-switch. The physiological relevance of BoNT/A1 self-association on the vesicle membrane in vivo warrants further investigation.

**The BoNT-switch is crucial to the extreme potency of BoNT/A1.** To explore the functional relevance of the BoNT-switch to BoNT toxicity, we incorporated the S622C and V653C mutations...
into active BoNT/A1 (termed BoNT/A1\(^{DS}\)). Since an endogenous disulfide linkage between LC and HC is crucial to LC translocation\(^ {35,58}\), we could not directly measure the toxicity of BoNT/A1\(^{DS}\) under reducing conditions. Instead, we generated two single cysteine mutations (BoNT/A1\(^{S622C}\) and BoNT/A1\(^{V653C}\)) as controls. We then examined the neurotoxicity of BoNT/A1 mutants at motor nerve terminals using ex vivo mouse phrenic nerve hemidiaphragm (MPN) assay\(^ {57}\) (Fig. 5a). Remarkably, the naturally oxidized BoNT/A1\(^{DS}\) displayed a ~100-fold decreased toxicity, while BoNT/A1\(^{V653C}\) and BoNT/A1\(^{S622C}\) behaved like the WT toxin. When BoNT/A1\(^{DS}\) was further oxidized with copper phenanthroline, the decrease in neurotoxicity was ~180-fold. BoNT/A1\(^{F658E}\) has a ~5-fold decreased toxicity, which is likely due to the energetic costs of their protonated forms\(^ {59}\) are positioned at the base of the β2/β3 loop, which would favorably interact with a negatively charged membrane at acidic pH.

Our model of membrane binding would require an acidic pH-triggered structural re-orientation of BoNT/A1 to position H\(_N\)A on the membrane surface. Such a structural rearrangement is possible because the flexible H\(_N\)-H\(_C\) linker allows a 140° rotation between H\(_N\) and H\(_C\) when BoNT/A1 is bound to the non-toxic nonhemagglutinin (NTNHA) protein at pH < 6.5 in the medium progenitor toxin complex (M-PTC)\(^ {60}\). The driving force for the structural change likely involves acidic pH and the electrostatic attraction between the electropositive surface of BoNT/A1 with the negatively charged membrane\(^ {27}\). Interestingly, the H\(_N\)-H\(_C\) linker of TeNT is also modulated by pH, which may play a role in reorienting TeNT for the membrane insertion\(^ {51}\). Furthermore, we speculate that the belt of BoNT/A1 may modulate the conformational change of H\(_N\)A by interacting with and shielding the hydrophobic surface of the BoNT-switch. The belt is likely unfolded upon membrane binding and vesicle acidification that may help to release the BoNT-switch.

It is well known that some viral-fusion proteins, such as influenza virus hemagglutinin, vesicular stomatitis virus GP, and Ebola virus GP, contain a hydrophobic loop that is buried at neutral pH, but released at acidic pH to insert the fusion protein into the lipid bilayer\(^ {3,4,6}\). Our complementary structural and functional studies reveal a surprising similarity between the β2/β3 loop of the BoNT-switch, which is conserved in almost all known BoNTs, and the IFL of Ebola virus GP2. Moreover, H\(_N\)A carries a pair of long α-helices that resemble the coiled-coil viral-fusion proteins (e.g., influenza hemagglutinin and HIV-1 gp41)\(^ {10}\). These viral proteins undergo dramatic structural reorganization of these helices after the fusion loop penetrates the membrane\(^ {3,62,63}\). We speculate that the formation of protein-conducting channel of BoNT may similarly involve a large reorganization of the long helices through H\(_N\)A.

Interestingly, the β2/β3 loop of BoNT is situated at the middle of the long helices that likely break down into shorter helical segments following membrane insertion. In this scenario, our model may represent the first step of membrane insertion, in which the β2/β3 loop brings the long helices of H\(_N\)A closer to the vesicle membrane and promotes its reorganization to form the translocation channel. Another possibility is that the β2/β3 loop may constitute part of the transmembrane hairpin and H\(_N\)A may form β-hairpin channels reminiscent of anthrax toxin\(^ {22}\). Formation of a β-hairpin channel requires oligomerization of BoNT, which is also consistent with our observation that acidic pH tends to promote BoNT/A1 oligomerization on liposomes, which is partly regulated by the BoNT-switch. It is worth noting that our data argues that the mechanism of BoNT membrane insertion is different from some other bacterial toxins, such as diphtheria toxin and colicin, which also have an α-helical translocation domain similar to BoNT. Those toxins do not have a viral-fusion-peptide-like switch, and they insert into membranes through exposure of a helical hairpin without a significant change in secondary structures\(^ {54}\).

In summary, our studies demonstrate that BoNT/A1 contains a molecular switch that regulates channel formation and LC delivery, so it only happens in the right cellular location at the right time. The importance of the BoNT-switch to the neurotoxicity of BoNT/A1 suggests that blocking the conformational transition with an antibody or small molecule inhibitors holds promise as an alternative strategy to neutralize these extremely lethal toxins. This is also supported by a report that antibodies from the sera of some cervical dystonia patients with immune-resistance to BoNT/A1 bound to a region of BoNT/A1 (S659–G677) that is within the BoNT-switch\(^ {63}\). These unique human antibodies are worthy of further characterization. Because
of its functional importance and high sequence conservation among all known BoNTs, the BoNT-switch could be an ideal target for the development of the long sought-after cross-serotype therapeutic intervention for botulism.

Methods

Statistical method. No statistical method was used to predetermine sample size. The experiments were not randomized and were not performed with blinding to the conditions of the experiments.

Construct design and cloning. The sequences corresponding to full-length BoNT/A1 carrying two substitutions at the active site (R363A/Y366F) (BoNT/A1i), and ciA-D12 (residues K547–K871) from BoNT/A1-producing C. botulinum strain 62A were cloned into expression vectors pGEX-6p-1 and pCDFDuet-1, respectively. ciA-D12 was cloned into pGEX-6p-1. Sequences of PCR primers are provided in Supplementary Table 1. To facilitate protein purification, a His-tag followed by a PreScission cleavage site was introduced to the N-terminus of hHsA, BoNT/A1i and ciA-D12 were produced with an N-terminal GST-tag followed by a PreScission cleavage site. All hHsA, BoNT/A1i, BoNT/A1i and ciA-D12 mutations were generated by QuikChange site-directed mutagenesis (Agilent).

Protein expression and purification. All recombinant proteins were expressed in the E. coli BL21 Bhi (DE3) (Novagen). Bacteria expressing recombinant full-length activated BoNT/A1 and ciA-D12 were grown at 37 °C in LB medium in the presence of the appropriate selecting antibiotics. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when OD600 reached 0.6. The temperature was then decreased to 30 °C and expression was continued for 16 h. For the expression of hHsA, bacteria were grown at 37 °C in TB medium and expression was induced with 1 mM IPTG when OD600 reached 0.9. The temperature was then decreased to 20 °C and expression was continued for 16 h. The cells were harvested by centrifugation and stored at −20 °C until use.

For protein purification, bacteria were resuspended in Tris buffer (50 mM Tris, pH 8.0, 400 mM NaCl) with 0.4 mM PMSE and lysed by sonication. His6-tagged proteins (hHsA wild type and mutants) were purified using a Ni-NTA (nitrilotriacetic acid, Qiagen) affinity column in a buffer containing 50 mM Tris (pH 8.0), 400 mM NaCl, and 10 mM imidazole and subsequently eluted in the same buffer containing 150 mM imidazole. The eluted fractions of each protein were pooled and dialyzed overnight at 4 °C against a buffer composed of 20 mM Hepes (pH 8.0) and 150 mM NaCl followed by a Superdex-200 size-exclusion chromatography using Superdex-200.

The protein was further purified by MonoQ ion-exchange chromatography (GE Healthcare) in a buffer containing 20 mM Hepes (pH 8.0) and eluted with a NaCl gradient, followed by Superdex-75 size-exclusion chromatography (SEC, GE Healthcare, Germany) in 10 mM Hepes (pH 7.0) and 150 mM NaCl. GST-tagged BoNT/A1i and the ciA-D12 were purified using Glutathione Sepharose 4B resin (GE Healthcare) in 50 mM Tris (pH 8.0), 400 mM NaCl, and eluted from the resin after on-column cleavage using PreScission protease. The protein was further purified by Superdex-200 increase or Superdex-75 SEC (GE Healthcare, Munich, Germany) in 10 mM Hepes (pH 7.0) and 150 mM NaCl, respectively. Each protein was concentrated to ~5 mg/mL using Amicon Ultra centrifugal filters (Millipore) and stored at −80 °C until use for further characterization or crystallization.

Wild type and mutant recombinant full-length activated BoNT/A1i were produced under low-sodium 2% divalent cation (project number GA A 3806543/123) respectively in E. coli B strain in Dr. Rummels lab66. BoNT/A1i variants carrying C-terminal His-tag were purified on Co2＋-Talon matrix (Takara Bio Europe S.A.S., France) and eluted with 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 250 mM imidazole. For proteolytic activation, BoNT/A1i was incubated for 16 h at room temperature with 0.01 U bovine thrombin (Sigma-Aldrich Chemie GmbH, Germany) per µg of BoNT/A1i. Subsequent gel filtration (Superdex-200 SEC, GE Healthcare, Germany) was performed in phosphate buffered saline (pH 7.4).

The purified ciA-D12 (S124C) was labeled with Alexa Fluor C2 647 fluorescent dye (Molecular Probes) according to the manufacturer’s instructions. The labeled ciA-D12 was further purified by MonoQ ion-exchange chromatography in 10 mM Hepes (pH 8.0) and eluted with a NaCl gradient. The calculated dye to protein ratio was ~1 molecule of dye per molecule of ciA-D12. The purified ciA-D12 was further purified by MonoQ ion-exchange chromatography in 10 mM Hepes (pH 8.0) and eluted with a NaCl gradient. The calculated dye to protein ratio was ~1 molecule of dye per molecule of ciA-D12. The purified ciA-D12 was complexed with ciA-D12 complex prepared by mixing BoNT/A1i and ciA-D12 in 1:1:5 molar ratio and the complex was purified by size-exclusion chromatography using Superdex-200.

Crystallization. Initial crystallization screens were performed using a Phoenix crystallization robot (Art Robbins Instruments) and high-throughput crystallization screen kits (Hampton Research), followed by extensive manual optimization. The best single crystals of hHsA were chosen at 18 °C by the hanging drop vapor diffusion method. The protein at a concentration of 5 mg/mL was mixed in a 2:1 (v/v) ratio with a reservoir solution containing 1.4 M sodium potassium phosphate (pH 5.1) and 0.1 M potassium sodium tartrate tetrahydrate. Crystals of hHsA were grown in a protein concentration at 3 mg/mL at 18 °C with a reservoir solution containing 0.1 M sodium phosphate, 0.1 M Tris (pH 8.5), and 0.05% n-dodecyl β-D-maltoside. The crystals of hHsA were cryoprotected in the original mother liquor supplemented with 30% (v/v) ethylene glycol. The crystals were dehydrated by air for 5 minutes and then frozen in liquid nitrogen. The crystals of hHsA were cryoprotected with 2.5 M lithium sulfate.

Data collection and structure determination. After screening numerous crystals at the Stanford Synchrotron Radiation Lightsource (SSRL) or Advanced Photon Source (APS), the best X-ray diffraction datasets for hHsA and hHsA were collected at 100 K at beam line BL9-2, SSRl. The data were processed with HKL2000. The data collection statistics are summarized in Table 1. The structure of hHsA and hHsA was determined by molecular replacement using the Phaser software with hHsA of BoNT/A1 as the search model (PDB code 3V0A)60. Manual model building and refinement was performed in COOT69, PHENIX70, and CCP4 suite71 in an iterative manner. The refinement progress was monitored with the free R value using a 5% randomly selected test set72. The structures were validated with the MolProbity73 and showed excellent stereochemistry. Structural refinement statistics are listed in Table 1. The residues 858–871 of chain A and residue 871 of chain B of hHsA and residues 647–649 of hHsA are invisible in the electron density map that likely indicates local flexibility.

One amino-phosphonate molecules were identified in the crystal structures of hHsA and hHsA, respectively, that could be attributed to the high sodium concentration in the corresponding crystallization buffer. All structure figures were prepared with PyMol (http://www.pymol.org).

8-anilinophenanthroline-1-sulfonic acid binding assay. The wild type or mutant hHsA was incubated at 0.1 mg/mL (~2.68 μM) with 100 μM ANS for ten minutes in either 50 mM sodium acetate (pH 4.0–5.6), 50 mM sodium cacodylate (pH 6.2), or 50 mM sodium phosphate buffer (pH 7.0). All buffers contained 100 mM NaCl. Fluorescence spectra were recorded at 25 °C in a Molecular Devices SpectraMax M2e spectrophotometer with excitation at 370 nm. The emission spectrum was collected from 420 to 640 nm. The fluorescence intensity was corrected by subtraction of background fluorescence from ANS in buffer lacking protein. Fluorescence emission was calculated as the area under the spectra. Error bars indicate SD of three replicate measurements.

Thermal denaturation assay. The thermal stability of wild type or mutant hHsA was measured using a fluorescence-based thermal shift assay on a StepOne real-time PCR machine (Life Technologies). Immediately before the experiment, the protein (2.7 μM) was mixed with the fluorescent dye SYPRO Orange (Sigma-Aldrich) at pH 7.0 or pH 4.6. The samples were heated from 25 °C to 90 °C in ~45 min. The midpoint of the protein-melting curve (Tm) was determined using the analysis software provided by the instrument manufacturer. The data obtained from three independent experiments were averaged to generate the bar graph. The Tm of hHsA wild type and reduced hHsA at pH 4.6 is not determined due to high fluorescence signal at starting temperature.

Analytical size-exclusion chromatography. hHsA at protein concentrations ranging from 0.1 to 1 mg/mL (~2.7–27 μM) were incubated at 4 °C for 30 minutes in various buffers that contained 0.1 M NaCl and either 50 mM sodium phosphate (pH 4.4–5.6) or 50 mM MOPS (pH 7.4). BoNT/A1i at 4 mg/mL (27 μM) was incubated in buffers at pH 4.4 or pH 7.4. Reduced hHsA was prepared by pre-incubation with 5 mM TCEP. Protein oligomerization was resolved using Superdex-200 SEC.

Liposome co-sedimentation assays. Liposomes were prepared by extrusion method using Avanti Mini Extruder according to manufacturer’s protocol. Briefly, lipids (Avanti Polar Lipid) were dissolved in chloroform while G1Tβ trisodium salt (Santa Cruz Biotechnology) was dissolved in methanol. The lipids at the indicated molar ratios were mixed and then dried under nitrogen gas and placed under vacuum for at least 2 h. The dried lipids were rehydrated and were subjected to five cycles of freezing and thawing cycles. Unilamellar vesicles were prepared by extrusion through a 100 nm pore membrane using an Avanti Mini Exxtruder according to the manufacturer’s instructions.

To study the pH-dependence of hHsA–lipid association, 0.2 μM protein was incubated with 0.13 mg/mL asolectin liposomes (Sigma-Aldrich) in 150 mM NaCl with 10 mM sodium acetate (pH 4.4–5.6) or 50 mM MOPS (pH 7.4). BoNT/A1i at 4 mg/mL (27 μM) was incubated in buffers at pH 4.4 or pH 7.4. Reduced hHsA was prepared by pre-incubation with 5 mM TCEP. Protein oligomerization was resolved using Superdex-200 SEC.
containing 1-oleoyl-2-(9,10-dibromostearoyl)phosphatidylcholine (OBPC)74. OBPC has density of 1.2 g/cm³ and sediments easily in relatively low centrifugal field52. A volume of 0.2 μM H₄A or mutants were incubated with liposomes containing 80% OBPC and 20% DOPS in 0.1 M NaCl, 50 mM sodium acetate (pH 4.6), 1 mM KCl, and 1 mM EDTA at room temperature for 2 h. The samples were spun progressively at 4000×, 9000×, and 16,000× g for 30 min each. Supernatant and pellet were separated and analyzed by SDS-PAGE.

**Mouse phrenic nerve hemidiaphragm assay**

Three times independently. monitored for 3 minutes with excitation at 380 nm. The experiments were repeated containing 1-oleoyl-2-(9,10-dibromostearoyl)phosphatidylcholine (OBPC)74. OBPC has density of 1.2 g/cm³ and sediments easily in relatively low centrifugal field52. A volume of 0.2 μM H₄A or mutants were incubated with liposomes containing 80% OBPC and 20% DOPS in 0.1 M NaCl, 50 mM sodium acetate (pH 4.6), 1 mM KCl, and 1 mM EDTA at room temperature for 2 h. The samples were spun progressively at 4000×, 9000×, and 16,000× g for 30 min each. Supernatant and pellet were separated and analyzed by SDS-PAGE.

**Calcine dye release assay**

Dried lipid (20 mg/ml) was resuspended in 150 mM NaCl, 20 mM Hepes (pH 7.0), 1 mM EDTA, 50 mM calcine. Free calcine dye was separated from calcine-entrapped liposomes by desalting (Zeba). Fluorescence was measured on a Spectramax M2e cuvette module with excitation at 349 nm and emission at 525 nm. In the assay, liposomes were diluted either in 150 mM NaCl, 20 mM sodium acetate (pH 4.6), 1 mM EDTA, or in 150 mM NaCl, 20 mM Hepes (pH 7.0), 1 mM EDTA to give a final concentration of 0.2 μM and incubated until the fluorescence signal was stable. Wild type or mutant H₄A was added at 0.2 μM and the fluorescence intensity was recorded for 10 minutes. The reaction was stopped by adding 0.1% Trion X100. The percentage of fluorescence change was calculated as the ((F – F₀)/F₀) × 100%

**Membrane depolarization assay**

Depolarization was measured as previously described with some modifications56. Briefly, liposomes composed of 70% DOPC, 20% DOPS, 10% cholesterol were prepared in 200 mM NaCl, 1 mM KCl, 10 mM Hepes (pH 7.0). To create a trans–positive membrane potential (Δθ → +150 mV), liposomes were diluted in 200 mM KCl, 1 mM NaCl with either 10 mM sodium acetate (pH 4.6) or 10 mM Hepes (pH 7.0). Membrane potential was monitored for 0.5–1 h. Membrane ANS is sensitive to membrane potential and has been used as a measure of the magnitude of the diffusion potential37. ANS is strongly fluorescent when bound to lipids and gives an additional increase in fluorescence intensity when a trans–positive potential is present. Valinomycin was added at time 0–second to give a final concentration of 30 nM. At 180-second, wild type, or mutant H₄A were added at 0.2 μM and the fluorescence intensity at 490 nm was monitored for 3 minutes with excitation at 380 nm. The experiments were repeated three times independently.

**Mouse phrenic nerve hemidiaphragm assay**

The MPN assay was performed employing 20–30 g NMRI mice (Janvier SA, France) as described previously53,57. According to §4 Abs. 3 (killing of animals for scientific purposes, German animal protection law, §28, SGB II, SchG), animals were sacrificed by trained personnel before dissection of organs and its number reported yearly to the animal welfare officer of the Central Animal Laboratory and to the local authority, Veterinärämter Hannover. First, mice were euthanized by CO₂ anesthesia and subsequent exsanguination via an incision of the ventral aspect of the throat. The chest of the cadaver was opened, the phrenic nerve hemidiaphragm tissue was excised and placed into an iced bath. The phrenic nerve was continuously stimulated at 5–25 mA with a frequency of 1 Hz and with a 0.1 ms pulse duration. Isometric contractions were transformed using a force transducer and recorded with VitroDat Online software (FMI GmbH, Germany). The time required to decrease the amplitude to 50% of the starting value (paralytic half-time) was determined. To allow comparison of the altered neurotoxicity of mutants with BoNT/A1 wild type, a power function (y(BoNTA1; x) = a + bx) was fitted to a dataset of 40 independent ab initio models was generated by the program DAMAVER80. The script fplcplots, implemented in the program AUTORG77, was used for data analysis.

**SAXS data collection and analysis**

Purified H₄A was exchanged into a buffer containing 200 mM NaCl, 5 mM DTT, 0.1 mM EDTA, 55 mM Hepes (pH 7.0), and the protein samples were concentrated to 10 mg/ml. SEC-SAXS experiment was performed using Bio-SAXS beam line BL4-2 at Stanford Synchrotron Radiation Lightsource (SSRL) in a similar manner to our previous report58. Experimental setup and data analysis are summarized in Supplementary Table 2. Briefly, the program SasTool (http://srl.slac.stanford.edu/~sas/analysis/sasstool.html) was employed for data reduction to generate background-subtracted scattering profiles.

**Data availability**

Atomic coordinates and structure factors for H₄A and H₄A-F658E have been deposited in the Protein Data Bank under accession codes 6DKK [https://doi.org/10.2210/pdb6DKK/pdb] and 6MHJ [https://doi.org/10.2210/pdb6MHJ/pdb], respectively. All relevant data are available within the paper and its Supplementary Information files.

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**Author contributions**

K.-h.L. and R.J. conceived the project. A.R., N.K. and J.W. generated the active full-length BoNT/A1 variants and performed the MPN assay. G.Z. and M.E.B performed the single particle counting. T.M. carried out the SAXS measurements. K.P. collected the X-ray diffraction data. K.L., R.J., M.E.B. and A.R. wrote the manuscript with inputs from all other authors.

**Additional information**

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