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DESCRIPTION

GOVERNMENT SUPPORT

[0001] This invention was made with government support under R01NS080833 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0002] *Clostridial Botulinum* neurotoxins (BoNTs) are among the most dangerous potential bioterrorism agents and are also used clinically to treat a growing list of medical conditions. There are seven serotypes of BoNTs (BoNT/A-G) known to date. In recent years, BoNTs have been widely used to treat a growing list of medical conditions: local injections of minute amount of toxins can attenuate neuronal activity in targeted regions, which can be beneficial in many medical conditions as well as for cosmetic purposes. As the application of BoNTs grows, limitations and adverse effects have been reported. The major limitation is the generation of neutralizing antibodies in patients, which renders future treatment ineffective. Termination of BoNT usage often leaves patients with no other effective ways to treat/relieve their disorders. Adverse effects associated with BoNT use range from transient non-serious events such as ptosis and diplopia to life-threatening events even death. The limitations and adverse effects of BoNTs are largely correlated with dose. There are considerable interests in developing novel BoNT types as therapeutic toxins. No new BoNT types have been recognized for the past 45 years.

SUMMARY

[0003] The present invention is defined by the appended set of claims. The present inventions provides an isolated *Clostridial Botulinum* neurotoxin (BoNT) polypeptide, comprising an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to SEQ ID NO: 3, optionally wherein the isolated BoNT polypeptide comprises the amino acid sequence of SEQ ID NO: 3, wherein the BoNT polypeptide does not cross react with an antibody against BoNT serotype A, B, C, D, E, F, or G

[0004] The present disclosure is based, at least in part, on the identification of a novel BoNT serotype, BoNT/X, from searching genomic database of *Clostridium Botulinum* strains. BoNT/X the lowest sequence identity with other BoNTs and it is not recognized by antisera raised against known BoNT types. BoNT/X cleaves SNARE proteins, like other BoNTs. However, BoNT/X also cleave several SNARE proteins that other BoNTs cannot cleave, e.g., VAMP4, VAMP5, and Ykt6. Compositions and methods for treating diseases using BoNT/X are provided. Also provided herein are methods of making BoNT/X.

[0005] Accordingly, some aspects of the present disclosure provide isolated *Clostridial Botulinum* neurotoxin (BoNT) polypeptides comprising the amino acid sequence of SEQ ID NO: 1.

[0006] Some aspects of the present disclosure provide isolated BoNT polypeptides comprising an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to SEQ ID NO: 1. In some embodiments, the isolated BoNT polypeptide consists of the amino acid sequence of SEQ ID NO: 1.

[0007] Some aspects of the present disclosure provide isolated BoNT polypeptides comprising the amino acid sequence of SEQ ID NO: 2. Some aspects of the present disclosure provide isolated BoNT polypeptides an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to SEQ ID NO: 2. In some embodiments, the isolated BoNT polypeptide consists of the amino acid sequence of SEQ ID NO: 2.

[0008] Some aspects of the present disclosure provide isolated BoNT polypeptides comprising the amino acid sequence of SEQ ID NO: 3. The present invention provides isolated BoNT polypeptides an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to SEQ ID NO: 3. In some embodiments, the isolated BoNT polypeptide consists of the amino acid sequence of SEQ ID NO: 3.

[0009] Some aspects of the present disclosure provide modified BoNT polypeptides comprising one or more substitution mutation(s) in a position corresponding to C461, C467, and C1240 of SEQ ID NO: 1. In some embodiments, the substitution mutation(s) corresponds to C461S, C461A, C467S, C467A, C1240S, C1240A, C461S/C1240S, C416S/C1240A, C461A/C1240S, C461A/C1240A, C467S/C1240S, C461S/C1240A, C467A/C1240S, or C467A/C1240A in SEQ ID NO: 1.

[0010] In some embodiments, the modified BoNT polypeptide comprises the amino acid sequence of any one of SEQ ID NO: 4-17. In some embodiments, the modified BoNT polypeptide comprises an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any of SEQ ID NOs: 4-17, wherein the polypeptide does not have the amino acid sequence of SEQ ID NO: 1. In some embodiments, the modified BoNT polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 4-17.

[0011] Some aspects of the present disclosure provide modified BoNT polypeptides comprising a single substitution mutation in a position corresponding to C461 or C467 of SEQ ID NO: 2.

[0012] In some embodiments, the substitution mutation corresponds to C461S, C461A, C467S, C467A, C1240S, C1240A, C461S/C1240S, C416S/C1240A, C461A/C1240S, C461A/C1240A, C467S/C1240S, C461S/C1240A, C467A/C1240S, or C467A/C1240A in SEQ ID NO: 2.

[0013] In some embodiments, the modified BoNT polypeptide comprises the amino acid sequence of any one of SEQ ID NOs: 18-21. In some embodiments, the modified BoNT polypeptide comprises an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any of SEQ ID NOs: 18-21, wherein the polypeptide does not have the amino acid sequence of SEQ ID NO: 2. In some embodiments, the modified BoNT polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 18-21.

[0014] Some aspects of the present disclosure provide chimeric BoNT polypeptides comprising the amino acid sequence of any one of SEQ ID NOs: 22-24. In some embodiments, the chimeric BoNT polypeptide comprises an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any one of SEQ ID NOs: 22-24, wherein the polypeptide does not have the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the chimeric BoNT polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 22-24.

[0015] In some embodiments, the chimeric BoNT polypeptide further comprises a single substitution mutation in a position corresponding to C461 or C467 of in SEQ ID NO: 2.

[0016] In some embodiments, the chimeric BoNT polypeptide comprises the amino acid sequence of any one of SEQ ID NOs: 25-30. In some embodiments, the chimeric BoNT polypeptide comprises an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any one of SEQ ID NOs: 25-30. In some embodiments, the chimeric BoNT polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 25-30.

[0017] In some embodiments, the BoNT polypeptide enters a cell. In some embodiments, the BoNT polypeptide cleaves a SNARE protein in the cell. In some embodiments, the SNARE protein is selected from the group consisting of: SNAP-25, VAMP1, VAMP2, VAMP3, VAMP4, VAMP5, Ykt6, and syntaxin 1.

[0018] In some embodiments, the SNARE protein is VAMP1. In some embodiments, the BoNT cleaves between amino acid residues corresponding to R66 and A67 of SEQ ID NO: 39.

[0019] In some embodiments, the SNARE protein is VAMP2. In some embodiments, the BoNT cleaves between amino acid residues corresponding to R66 and A67 of SEQ ID NO: 40.

[0020] In some embodiments, the SNARE protein is VAMP3. In some embodiments, the BoNT cleaves between amino acid residues corresponding to R66 and A67 of SEQ ID NO: 41.

[0021] In some embodiments, the SNARE protein is VAMP4. In some embodiments, the BoNT cleaves between amino acid residues corresponding to K87 and S88 of SEQ ID NO: 42.

[0022] In some embodiments, the SNARE protein is VAMP5. In some embodiments, the BoNT cleaves between amino acid residues corresponding to R40 and S41 of SEQ ID NO: 43.

[0023] In some embodiments, the SNARE protein is Ykt6. In some embodiments, the BoNT cleaves between amino acid residues corresponding to K173 and S174 of SEQ ID NO: 44.

[0024] In some embodiments, the BoNT polypeptide has increased stability compared to its corresponding wild type BoNT polypeptide.

[0025] In some embodiments, the cell is a secretory cell. In some embodiments, the cell is a neuronal cell. In some embodiments, the cell is an immune cell. In some embodiments, the BoNT polypeptide suppresses neuronal activity. In some embodiments, the BoNT polypeptide induces flaccid paralysis. In some embodiments, the cell is a cultured cell. In some embodiments, the cell is *in vivo.* In some embodiments, the cell is from a mammal. In some embodiments, the mammal is a human. In some embodiments, mammal is a rodent. In some embodiments, the rodent is a mice. In some embodiments, the rodent is a rat.

[0026] In the present invention, the BoNT polypeptide does not cross react with an antibody against BoNT serotype A, B, C, D, E, F, or G.

[0027] Other aspects of the present disclosure provide nucleic acid molecules comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5%, or 100% identity to the BoNT polypeptide described herein. Nucleic acid vectors comprising such nucleic acid molecules are provided. Cells comprising the nucleic acid molecules or the nucleic acid vectors described herein are provided. In some embodiments, such cells express the BoNT polypeptide described herein.

[0028] Methods of producing the BoNT polypeptide of the present disclosure are provided.

Such methods comprise the steps of culturing the cell expressing the BoNT polypeptides under conditions wherein said BoNT polypeptide is produced. In some embodiments, the methods further comprise recovering the BoNT polypeptide from the culture.

[0029] Other aspects of the present disclosure provide modified BoNT polypeptides comprising: (a) a protease domain; (b) a modified linker region; and (c) a translocation domain; wherein (a), (b), and (c) are from BoNT serotype X, and wherein the modified linker region comprises one single substitution mutation in a position corresponding to C461 or C467 of SEQ ID NO: 1.

[0030] In some embodiments, the modified BoNT polypeptide further comprises: (d) a receptor binding domain.

[0031] In some embodiments, modified linker region comprises a substitution mutation corresponding to C461S or C461A in SEQ ID NO: 1. In some embodiments, the modified linker region comprises a substitution mutation corresponding to C467S or C467A in SEQ ID NO: 1.

[0032] In some embodiments, the receptor binding domain is from BoNT/X. In some embodiments, the receptor binding domain is modified. In some embodiments, the receptor binding domain comprises a substitution mutation corresponding to C1240S or C1240A in SEQ ID NO: 1.

[0033] In some embodiments, the receptor binding domain is from a serotype selected from the group consisting of A, B, C, D, E, F, and G.

[0034] In some embodiments, the modified BoNT polypeptide enters a cell. In some embodiments, the modified BoNT polypeptide cleaves SNARE proteins in the cell. In some embodiments, the SNARE protein is selected from the group consisting of: SNAP-25, VAMP1, VAMP2, VAMP3, VAMP4, VAMP5, Ykt6, and syntaxin 1.

[0035] In some embodiments, the SNARE protein is VAMP1. In some embodiments, the BoNT cleaves between amino acid residues corresponding to R66 and A67 of SEQ ID NO: 39.

[0036] In some embodiments, the SNARE protein is VAMP2. In some embodiments, the BoNT cleaves between amino acid residues corresponding to R66 and A67 of SEQ ID NO: 40.

[0037] In some embodiments, the SNARE protein is VAMP3. In some embodiments, the BoNT cleaves between amino acid residues corresponding to R66 and A67 of SEQ ID NO: 41.

[0038] In some embodiments, the SNARE protein is VAMP4. In some embodiments, the BoNT cleaves between amino acid residues corresponding to K87 and S88 of SEQ ID NO: 42.

[0039] In some embodiments, the SNARE protein is VAMP5. In some embodiments, the BoNT cleaves between amino acid residues corresponding to R40 and S41 of SEQ ID NO: 43.

[0040] In some embodiments, the SNARE protein is Ykt6. In some embodiments, the BoNT cleaves between amino acid residues corresponding to K173 and S174 of SEQ ID NO: 44.

[0041] In some embodiments, the BoNT polypeptide has increased stability compared to its corresponding wild type BoNT polypeptide.

[0042] In some embodiments, the cell is a secretory cell. In some embodiments, the cell is a neuronal cell. In some embodiments, the cell is an immune cell. In some embodiments, the BoNT polypeptide suppresses neuronal activity. In some embodiments, the BoNT polypeptide induces flaccid paralysis. In some embodiments, the cell is a cultured cell. In some embodiments, the cell is *in vivo.* In some embodiments, the cell is from a mammal. In some embodiments, the mammal is a human. In some embodiments, mammal is a rodent. In some embodiments, the rodent is a mice. In some embodiments, the rodent is a rat.

[0043] In the present invention, the BoNT polypeptide does not cross react with an antibody against BoNT serotype A, B, C, D, E, F, or G.

[0044] In some embodiments, the modified linker region comprises an artificial linker. In some embodiments, the artificial linker contains a cleavage site of a protease. In some embodiments, the protease is selected from the group consisting of Thrombin, TEV, PreScission (3C protease), Factor Xa, MMP-12, MMP-13, MMP-17, MMP-20, Granzyme-B, and Enterokinase. In some embodiments, the linker comprises the amino acid sequence of any of SEQ ID NOs: 50-60).

[0045] Other aspects of the present disclosure provide nucleic acid molecules comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5%, or 100% identity to the BoNT polypeptide described herein. Nucleic acid vectors comprising such nucleic acid molecules are provided. Cells comprising the nucleic acid molecules or the nucleic acid vectors described herein are provided. In some embodiments, such cells expresse the BoNT polypeptide described herein.

[0046] Methods of producing the BoNT polypeptide of the present disclosure are provided. Such methods comprise the steps of culturing the cell expressing the BoNT polypeptides under conditions wherein said BoNT polypeptide is produced. In some embodiments, the methods further comprise recovering the BoNT polypeptide from the culture.

[0047] Other aspects of the present disclosure provide modified BoNT polypeptides comprising one or more substitution mutation(s) in positions corresponding to R360, Y363, H227, E228, or H231 in SEQ ID NO: 1. In some embodiments, the one or more substitution mutation corresponds to R360A/Y363F, H227Y, E228Q, or H231Y in SEQ ID NO: 1.

[0048] In some embodiments, the modified BoNT polypeptide comprises the amino acid sequence of any one of SEQ ID NOs: 31-38. In some embodiments, the modified BoNT polypeptide comprises an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any of SEQ ID NOs: 31-38, wherein the polypeptide does not have the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2. In some embodiments, the modified BoNT polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 31-38.

[0049] Other aspects of the present disclosure provide modified BoNT/X polypeptide comprising: a) an inactive protease domain; b) a linker region; and c) a translocation domain. In some embodiments, the modified BoNT/X further comprises a receptor binding domain.

[0050] In some embodiments, the inactive protease domain comprises one or more substitution mutations in positions corresponding to R360, Y363, H227, E228, or H231 of SEQ ID NO: 1. In some embodiments, the one or more substitution mutations correspond to R360A/Y363F, H227Y, E228Q, or H231Y of SEQ ID NO: 1.

[0051] In some embodiments, the modified BoNT polypeptide enters a cell. In some embodiments, the modified BoNT polypeptide does not cleave a SNARE protein.

[0052] In some embodiments, the modified BoNT/X polypeptide further comprises a modification in the linker region of (b). In some embodiments, the modification in the linker region comprises one single substitution mutation in a position corresponding to C461 or C467 of SEQ ID NO: 1. In some embodiments, the single substitution mutation corresponds to C461A, C461S, C467A, or C467S in SEQ ID NO: 1. In some embodiments, the modified BoNT/X polypeptide further comprises a modification in the receptor binding domain of (d).

[0053] In some embodiments, the modification in the receptor binding domain comprises a substitution mutation in a position corresponding to C1240 of SEQ ID NO: 1.

[0054] Other aspects of the present disclosure provide nucleic acid molecules comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5%, or 100% identity to the BoNT polypeptide described herein. Nucleic acid vectors comprising such nucleic acid molecules are provided. Cells comprising the nucleic acid molecules or the nucleic acid vectors described herein are provided. In some embodiments, such cells expresse the BoNT polypeptide described herein.

[0055] Methods of producing the BoNT polypeptide of the present disclosure are provided. Such methods comprise the steps of culturing the cell expressing the BoNT polypeptides under conditions wherein said BoNT polypeptide is produced. In some embodiments, the methods further comprise recovering the BoNT polypeptide from the culture.

[0056] Further provided herein are use of the modified BoNT polypeptide described herein as a delivery vehicle to deliver therapeutics into neurons.

[0057] Some aspects of the present disclosure provide chimeric molecules comprising a first portion linked to a second portion, wherein the first portion is a modified BoNT polypeptide described herein.

[0058] In some embodiments, the first portion and the second portion are linked covalently. In some embodiments, the first portion and the second portion are linked non-covalently.

[0059] In some embodiments, wherein the second portion is selected from the group consisting of a small molecule, a nucleic acid, a short polypeptide and a protein. In some embodiments, the second portion is a bioactive molecule. In some embodiments, the second portion is a non-polypeptide drug. In some embodiments, the second portion is a therapeutic polypeptide.

[0060] Other aspects of the present disclosure provide nucleic acid molecules comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5%, or 100% identity to the chimeric BoNT polypeptide described herein. Nucleic acid vectors comprising such nucleic acid molecules are provided. Cells comprising the nucleic acid molecules or the nucleic acid vectors described herein are provided. In some embodiments, such cells expresse the chimeric BoNT polypeptide described herein.

[0061] Methods of producing the chimeric BoNT polypeptide of the present disclosure are provided. Such methods comprise the steps of culturing the cell expressing the chimeric BoNT polypeptides under conditions wherein said chimeric BoNT polypeptide is produced. In some embodiments, the methods further comprise recovering the chimeric BoNT polypeptide from the culture.

[0062] Other aspects of the present disclosure provide pharmaceutical compositions comprising the BoNT polypeptides described herein.

[0063] In some embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable excipient.

[0064] Kit comprising such pharmaceutical compositions and directions for therapeutic administration of the pharmaceutical composition are also provided.

[0065] Some aspects of the present disclosure provide a therapeutically effective amount of the BoNT polypeptide, the chimeric molecule, or the pharmaceutical composition described herein for use in methods of treating a condition, comprising administering a therapeutically effective amount of the BoNT polypeptide, the chimeric molecule, or the pharmaceutical composition described herein to a subject to treat the condition.

[0066] In some embodiments, the condition is associated with overactive neurons or glands. In some embodiments, the condition is selected from the group consisting of, spasmodic dysphonia, spasmodic torticollis, laryngeal dystonia, oromandibular dysphonia, lingual dystonia, cervical dystonia, focal hand dystonia, blepharospasm, strabismus, hemifacial spasm, eyelid disorder, cerebral palsy, focal spasticity and other voice disorders, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia and other muscle tone disorders and other disorders characterized by involuntary movements of muscle groups, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions, secretory disorders, pain from muscle spasms, headache pain, dermatological and obesity/reduced appetite.

[0067] In some aspects, there is provided a non-therapeutic method of treating a cosmetic or aesthetic condition using the BoNT polypeptide described herein, optionally wherein the condition is brow furrows or skin wrinkles.

[0068] In some embodiments, the condition is not associated with unwanted neuronal activity. In some embodiments, the condition is selected from the group consisting of: psoriasis, allergy, haemophagocytic lymphohistiocytosis, and alcoholic pancreatic diseases.

[0069] In some embodiments, the administering is via injection to where unwanted neuronal activity is present.

[0070] Yet other aspects of the present disclosure provide methods of producing a *Clostridial Botulinum* neurotoxin (BoNT) polypeptide, the method comprising:

1. (i) obtaining a first BoNT fragment comprising a light chain (LC) and a N-terminal domain of a heavy chain (H_N), wherein the first BoNT fragment comprises a C-terminal LPXTGG (SEQ ID NO: 60) motif;
2. (ii) obtaining a second BoNT fragment comprising a C-terminal domain of the heavy chain (H_C); wherein the second BoNT fragment comprise a specific protease cleavage site at its N-terminus;
3. (iii) cleaving the second BoNT fragment with a specific protease, wherein the cleavage results in a free Glycine residue at the N-terminus; and
4. (iv) contacting the first BoNT fragment and the second BoNT fragment in the presence of a transpeptidase, thereby ligating the first BoNT fragment and the second BoNT fragment to form a ligated BoNT.

[0071] In some embodiments, the first BoNT fragment further comprises an affinity tag. In some embodiments, the affinity tag is fused to the first BoNT fragment at the N-terminus. In some embodiments, the affinity tag is fused to the first BoNT fragment at the C-terminus. In some embodiments, the affinity tag is selected from the group consisting of: His6, GST, Avi, Strep, S, MBP, Sumo, FLAG, HA, Myc, SBP, E, Calmodulin, Softag 1, Softag 3, TC, V5, VSV, Xpress, Halo, and Fc.

[0072] In some embodiments, the second BoNT fragment further comprises an affinity tag. In some embodiments, the affinity tag is fused to the first BoNT fragment at the N-terminus. In some embodiments, the affinity tag is fused to the second BoNT fragment at the C-terminus. In some embodiments, the affinity tag is selected from the group consisting of: His6, GST, Avi, Strep, S, MBP, Sumo, FLAG, HA, Myc, SBP, E, Calmodulin, Softag 1, Softag 3, TC, V5, VSV, Xpress, Halo, and Fc.

[0073] In some embodiments, the protease is selected from the group consisting of: thrombin, TEV, PreScission, MMP-12, MMP-13, MMP-17, MMP-20, Granzyme-B, Enterokinase, and SUMO protease. In some embodiments, the cognate protease is thrombin.

[0074] In some embodiments, the first BoNT fragment is from BoNT serotype A, B, C, D, E, F, G, or X. In some embodiments, the first BoNT fragment is from BoNT/X. In some embodiments, the second BoNT fragment is from BoNT serotype A, B, C, D, E, F, G, or X. In some embodiments, the second BoNT fragment is from BoNT/A. In some embodiments, the second BoNT fragment is from BoNT/B. In some embodiments, the second BoNT fragment is from BoNT/C. In some embodiments, the second BoNT fragment is from BoNT/X.

[0075] In some embodiments, the transpeptidase is a sortase. In some embodiments, the sortase is from Staphylococcus aureus (SrtA).

[0076] These and other aspects of the disclosure, as well as various advantages and utilities will be apparent with reference to the Detailed Description of the Invention. Each aspect of the disclosure can encompass various embodiments as will be understood.

BRIEF DESCRIPTION OF THE DRAWINGS

[0077] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein. The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0078] FIGs. 1A-1E show the identification of BoNT/X as a new BoNT. FIG. 1A shows a phylogenic tree of the protein sequence alignment for BoNT/A-G, BoNT/F5, TeNT, and BoNT/X, analyzed by ClustalW method. The percentages of sequence identity between each toxin and BoNT/X are denoted after each toxin. The percentages of sequence identity between

BoNT/E and BoNT/F, and between BoNT/B and BoNT/G were also noted. FIG. 1B, upper panel, shows a schematic drawing of the three domains of BoNT/X, with conserved protease motif in the LC and the ganglioside binding motif in the H_C noted. FIG. 1B, lower panel, shows a sliding sequence comparison window demonstrating that BoNT/X has a low similarity evenly distributed along its sequence to all other seven BoNTs and TeNT. FIG. 1C is a schematic drawing of the orf gene cluster that hosts BoNT/X gene (upper panel), which has two unique features compared to two known variants of orfX cluster (middle and lower panels): (1) there is an additional orfX2 protein (designated as orfX2b) located next to the BoNT/X gene; (2) the reading frame of orfX genes has the same direction with BoNT/X gene. FIG. ID is a schematic illustrating the unique gene directionality and additional OrfX2 gene found in BoNT/X. FIG. 1E shows a preliminary structure of the BoNT/X light chain. The dark dot represents the active site zinc. The structure is shown at a 1.9Å resolution.

[0079] FIGs. 2A-2J show the LC of BoNT/X (X-LC) cleaves VAMPs at a unique site. FIG. 2A shows X-LC, with or without pre-treated with EDTA, incubated with rat brain detergent extracts (BDE). Immunoblot analysis was carried out to detect syntaxin 1, SNAP-25, and VAMP2. Synaptophysin (Syp) was also detected as a loading control. The LC of BoNT/A (A-LC) and BoNT/B (B-LC) were analyzed in parallel. Cleavage of VAMP2 by B-LC results in loss of immunoblot signals, while cleavage of SNAP-25 by A-LC generates a smaller fragment of SNAP-25 that can still be detected on immunoblot (marked by an asterisk). Incubation with X-LC resulted in loss of VAMP2 immunoblot signals, suggesting that X-LC cleaved VAMP2. EDTA blocked the activity of X-, A-, and B-LCs. FIG. 2B shows VAMP2 (residues 1-96) purified as a His6-tagged recombinant protein and incubated with X-LC. Samples were analyzed by SDS-PAGE and Coomassie Blue staining. X-LC converted VAMP2 (1-96) into two smaller fragments, indicating that X-LC cleaved VAMP2. FIGs. 2C-2E show VAMP2 (1-96) incubated with X-LC. Whole protein samples were then analyzed by mass spectrometry (LC-MS/MS) to determine the precise molecular weight of cleaved fragments. Eluted peptide peaks from the HPLC column were plotted in FIG. 2C over running time (RT, X-axis). The mass spectrometry data for the two cleavage products are shown in FIGs. 2D and 2E, respectively, with mass-to-charge ratio (m/z) noted for each signal. The molecular weight is deducted by multiplying m with z, followed by subtracting z. The protein sequences for the two cleavage products correspond to SEQ ID NO: 61 and 62 from top to bottom and are shown in FIG. 2C. FIG. 2F shows a sequence alignment between VAMP 1, 2, 3, 4, 5, 7, 8, Sec22b and Ykt6, with cleavage sites for BoNT/B, D, F, G, and X underlined, and two SNARE motifs boxed. The sequences correspond to SEQ ID NOs: 63-71 from top to bottom. FIG. 2G shows HA-tagged VAMP1, 3, 7, and 8, and myc-tagged Sec22b and Ykt6 expressed in HEK293 cells via transient transfection. Cell lysates were incubated with X-LC and subjected to immunoblot analysis detecting the HA or Myc tag. Actin served as a loading control. X-LC cleaved VAMP1, 3 and Ykt6, but not VAMP7, 8 and Sec22. FIG. 2H shows GST-tagged Ykt6 incubated with X-LC (100 nM) for the indicated times. Samples were analyzed by SDS-PAGE and Coomassie Blue staining. X-LC cleaved Ykt6. FIG. 2Ishows GST-tagged cytoplasmic domains of VAMP2 (33-86), VAMP4 (1-115), and VAMP5 (1-70) incubated with X-LC for the indicated times. Samples were analyzed by SDS-PAGE and Coomassie Blue staining. X-LC cleaved both VAMP4 and VAMP5. A longer incubation time (360 min) is required to cleave majority of VAMP5. Note that VAMP5 protein contains an additional band that is either degradation product or bacterial protein contaminant, which runs close (but not identical) to the cleavage product on SDS-PAGE. FIG. 2J shows experiments carried out as described in FIG. 2A, except that VAMP4 and Sec22b were detected. Synaptotagmin I (Syt I) was detected as a loading control. X-LC cleaved native VAMP4 in BDE.

[0080] FIGs. 3A-3E show activation of BoNT/X by proteolytic cleavage of the linker region between LC and H_N. FIG. 3A shows a sequence alignment of the linker regions between LC and H_N of the seven BoNTs and BoNT/X. The sequences correspond to SEQ ID NOs: 72-79 from top to bottom. BoNT/X has the longest linker region among all BoNTs, which contains an extra cysteine in addition to the two conserved cysteines in the LC and in the H_N. The Lys-C cutting site under limited proteolysis was identified by mass spectrometry approach. FIG. 3B shows cultured rat cortical neurons exposed to indicated concentrations of X-LC-H_N in media for 12 hours. Cell lysates were harvested and immunoblot analysis was carried out to examine syntaxin 1, SNAP-25, and VAMP2 in neurons. Actin served as a loading control. Trypsin-activated LC-H_N of BoNT/A (A-LC-H_N) and BoNT/B (B-LC-H_N) were analyzed in parallel as controls. X-LC-H_N entered neurons and cleaved VAMP2, as evidenced by loss of VAMP2 immunoblot signals. X-LC-H_N activated by Lys-C showed a drastically increased potency than non-activated X-LC-H_N. X-LC-H_N is more potent than trypsin-activated B-LC-H_N and A-LC-H_N, which did not show any detectable cleavage of their SNARE substrates in neurons under the same assay concentrations. FIG. 3C shows X-LC-H_N mutants with indicated cysteine mutated, as well as WT X-LC-H_N, activated by limited proteolysis and analyzed by SDS-PAGE and Coomassie Blue staining, with or without DTT. C423S mutation resulted in two 50 kDa fragments, with or without DTT. Mutants harboring C461S or C467S showed a single band at 100 kDa in the absence of DTT, and it separated into two ~50 kDa bands in the presence of DTT, demonstrating that both C461 and C467 on the H_N can form the inter-chain disulfide bond with C423 on the LC. A portion of WT X-LC-H_N formed aggregates at the top of the SDS-PAGE gel. These aggregates are due to formation of inter-molecular disulfide bond, as they disappeared in the presence of DTT. Mutating any one of three cysteines abolished aggregates, indicating that formation of inter-molecular disulfide bond is due to existence of an extra cysteine in the linker region. The majority of activated WT X-LC-H_N also separated to two ~50 kDa bands on SDS-PAGE gel without DTT. This is due to disulfide bond shuffling described in FIG. 3D. FIG. 3D shows WT X-LC-H_N activated by limited proteolysis, followed by pre-incubation with indicated concentrations of NEM to block disulfide bond shuffling. The samples were then analyzed by SDS-PAGE and Coomassie Blue staining, with or without the presence of DTT. Majority of WT X-LC-H_N exist as a single band at 100 kDa without DTT after NEM treatment, indicating that WT X-LC-H_N mainly contains inter-chain disulfide bond. FIG. 3E shows experiments carried out as described in FIG. 3B, except that neurons were exposed to either WT or indicated X-LC-H_N mutants. Mutating the cysteine on the LC (C423) abolished the activity of X-LC-H_N, while mutating one of the two cysteines on the H_N (C461 or C467) did not affect the activity of X-LC-H_N on neurons. These results confirmed that formation of the inter-chain disulfide bond is essential for the activity of X-LC-H_N.

[0081] FIGs. 4A-4F show full-length BoNT/X is active on cultured neurons and *in vivo* in mice. The sequences are as follows: LVPR-GS (SEQ ID NO: 80), LPETGG-His6 (SEQ ID NO: 81), GG-His6 (SEQ ID NO: 82) and LPETGS (SEQ ID NO: 59). FIG. 4A shows a schematic drawing illustrates synthesis of full-length BoNT/X using sortase ligation method. FIG. 4B shows that sortase ligation reaction mixture and indicated control components were analyzed by SDS-PAGE and Coomassie Blue staining. The asterisk marks aggregates of proteins due to inter-molecular disulfide bond, as these aggregates disappeared in the presence of DTT. The molecular weight marker is in lane 1 (starting from the left side). Full-length BoNT/X (X-FL) only appeared in the sortase ligation mixture (lane 7 and lane 14). FIG. 4C shows that neurons exposed to the same amount (5 µl) of sortase ligation mixture or indicated control components for 12 hours in media. Cell lysates were analyzed by immunoblot. X-LC-HN alone cleaved some VAMP2 due to its high concentration in the reaction mixture. The control mixture containing both X-LC-HN and X-HC but not sortase, slightly enhanced cleavage of VAMP2 as compared to X-LC-HN alone, likely because X-HC associates with X-LC-HN via non-covalent interactions. Ligating X-LC-HN and X-HC by sortase enhanced cleavage of VAMP2 over the mixture of X-LC-HN and X-HC without sortase, demonstrating that ligated X-FL is functional in neurons. FIG. 4D shows that sortase reaction mixture as prepared as described in panel b (lane 7) is active in vivo analyzed using DAS assay in mice. The injected limb developed flaccid paralysis and the toes failed to spread within 12 hours. The left limb was not injected with toxins, serving as a control. FIG. 4E shows that BoNT/A-G, a mosaic toxin BoNT/DC, and BoNT/X were subjected to dot blot analysis (0.2 µg per toxin, spotted on nitrocellulose membranes), using four horse antisera (trivalent anti-BoNT/A, B, and E, anti-BoNT/C, anti-BoNT/DC, and anti-BoNT/F), as well as two goat antisera (anti-BoNT/G and anti-BoNT/D). BoNT/X is composed of purified X-LC-HN and X-HC at 1:1 ratio. These antisera recognized their corresponding target toxins, yet none of them recognized BoNT/X. FIG. 4F shows that full-length inactive form of BoNT/X (BoNT/XRY) was purified as a His6-tagged recombinant protein in E.coli and analyzed by SDS-PAGE and Coomassie Blue staining, with or without DTT.

[0082] FIG. 5 is a phylogenetic tree showing the distribution and relationship of Clostridial neurotoxins. The tree represents the relationships of different BoNTs and TeNT sequences from the Jackhmmer search. BoNT/X is circled.

[0083] FIG. 6 shows a mass spectrometry analysis of intact VAMP2 (1-96). His6-tagged VAMP2 (1-96) was analyzed by LC-MS/MS mass spectrometry. The HPLC profile is listed in the left panel, together with the protein sequence. The mass spectrometry data for full-length VAMP2 (1-96) was shown in the right panel and corresponds to SEQ ID NO: 83, with m/z value marked for each signal.

[0084] FIGs. 7A-7F shows the identification of the cleavage site on GST-VAMP2 (33-86) by X-LC. FIG. 7A shows a GST-tagged VAMP2 (33-86) incubated with or without X-LC. Samples were analyzed by SDS-PAGE and Coomassie Blue staining. FIGs. 7B-7C show intact GST-tagged VAMP2 (33-86) analyzed by LC-MS/MS mass spectrometry. The HPLC profile was shown in FIG. 7B. The mass spectrometry data was shown in FIG. 7C, with protein sequence

(SEQ ID NO: 84) noted in FIG. 7C. VAMP2 (33-66) and VAMP2 (67-86) are marked. FIGs. 7D-7E show GST-tagged VAMP2 (33-86) incubated with X-LC. Samples were then analyzed by LC-MS/MS mass spectrometry. The HPLC profile is shown in FIG. 7D. The mass spectrometry data for the C-terminal fragment (SEQ ID NO: 85) generated by X-LC is shown in FIG. 7E. The mass spectrometry data for the N-terminal fragment (SEQ ID NO: 86) was shown in FIG. 7F. The protein sequences of the C- and N- terminal fragments were indicated in FIGs. 7E-7F, and correspond to SEQ ID NOs: 85 and 86 respectively.

[0085] FIGs. 8A-8B show that XA chimeric toxin is active on neurons. FIG. 8A shows a XA chimeric toxin generated by ligating X-LC-H_N with A-H_C by sortase, similar to generating X-FL as described in FIG. 4A. The sortase ligation mixture and indicated control components were analyzed by SDS-PAGE and Coomassie Blue staining. The ligation is efficient as majority of X-LC-H_N was ligated into XA chimeric toxin. FIG. 8B shows rat cortical neurons exposed to the indicated control components or sortase ligated XA mixture (5 µl) for 12 hours in media. Cell lysates were analyzed by immunoblot. X-LC-H_N alone cleaved some VAMP2 due to its high concentration in the reaction mixture. Ligated XA cleaved VAMP2 in neurons.

[0086] FIG. 9 shows that mutating the extra cysteine in the H_N and the cysteine in the H_C does not affect activity of BoNT/X. X-H_C (C1240S) was ligated with WT X-LC-H_N, X-LC-H_N (C461S), or X-LC-H_N (C467S) by sortase ligation. Neurons were exposed to sortase ligation mixture or control components (5 µl) for 12 hours in media. Cell lysates were analyzed by immunoblot. Mutating C1240 and one of the cysteine on H_N (C461 or C467) did not affect the activity of BoNT/X, as ligated mutant toxins are capable of entering neurons and cleaved VAMP2.

[0087] FIG. 10 shows antisera raised against the seven serotypes of BoNTs neutralizing their target BoNTs on neurons. Cultured rat cortical neurons were exposed to indicated BoNTs, with or without pre-incubation with indicated antisera. Cell lysates were harvested 12 hours later and subjected to immunoblot analysis. All antisera specifically neutralized their target BoNTs, without affecting the activity of a different serotype of BoNTs, thus validating the specificity and potency of these antisera. The concentrations for BoNTs were: BoNT/A (50 pM), BoNT/B (2 nM), BoNT/C (1.5 nM), BoNT/D (100 pM), BoNT/E (0.5 nM), BoNT/F (0.5 nM), BoNT/G (5 nM). The antiserum against BoNT/A/B/E was used at 20 µl per well. All the other antisera were used at 10 µl per well. BoNTs were pre-incubated with indicated antisera for 30 mins at 37 °C prior to adding into culture media.

[0088] FIGs. 11A-11C show that BoNT/X_{RY} is not active on neurons. FIG. 11A shows cultured rat cortical neurons exposed to BoNT/X_{RY} at indicated concentrations. Cell lysates were analyzed by immunoblot. VAMP2 was not cleaved, indicating that BoNT/X_{RY} is not active on neurons. FIG. 11B shows the SDS-PAGE analysis of cell lysate and supernatant (S/N) expression of BoNT/X_{RY} (4-12% BisTris, MOPS buffer). A band at 150kDa corresponding to BoNT/X is clearly visible in both lysate and soluble fraction. FIG. 11C shows the SDS-PAGE analysis of a final sample of highly purified BoNT/X_{RY} (4-12% BisTris, MOPS buffer). A single band at 150 kDa corresponding to BoNT/X is clearly visible and shows ~90% purity.

[0089] FIGs. 12A-12F show that BoNT/X binds to all four brain gangliosides. FIGs. 12A-12D show BoNT/X (squares), and A-Hc (circles) binding to GD1a (FIG. 12A), GT1b (FIG. 12B), GD1b (FIG. 12C), and GM1 (FIG. 12D), respectively. Curves correspond to an average of triplicate ELISA assays and were fitted with Prism7 (GraphPad software). FIG 12E shows a summary of BoNT/X binding to all four gangliosides compared with the overall binding of BoNT/A in FIG 12F.

[0090] FIGs. 13A-13D show the identification of the cleavage sites of X-LC on Ykt6 by mass spectrometry analysis. FIGs. 13A-13D show 10 µg GST-tagged Ykt6 (1-192), with or without pre-incubation with X-LC, were separated on SDS-PAGE (FIG. 13A). The protein bands were excised as indicated and digested by chymotrypsin. Digested peptides were desalted and analyzed by reversed phase HPLC via C18 column coupled with ESI-MS. The HPLC profiles of GST-Ykt6 without pre-treatment with X-LC was shown in FIG. 13B, and the sample pretreated with X-LC was shown in FIG. 13C. One peptide was identified to be ~100-fold higher intensity in the samples pre-treated with X-LC than in the samples that was not exposed to X-LC (denoted with an asterisk). This peptide was eluted at 37 min RT, with m/z = 611 (FIG. 13D), which can only fit the peptide sequence ESLLERGEKLDDLVSK (SEQ ID NO: 87) in Ykt6, indicating that this is the peptide located at the N-terminal side of the cleavage site for X-LC. Therefore the cleavage site is K173-S174 in Ykt6.

[0091] FIGs. 14A-14E show the identification of the cleavage sites of X-LC on VAMP4 and VAMP5 by mass spectrometry analysis. FIGs. 14A-14E show experiments carried out as described in FIG. 13, except that VAMP4 (FIGs. 14B, 14C) and VAMP5 (FIGs. 14D, 14E) were analyzed. FIG. 14B is the peptide that marks the N-terminal site of the cleavage site in VAMP4. The sequence of the peptide DELQDK corresponds to SEQ ID NO: 88. FIG. 14C is the peptide that marks the C-terminal site of the cleavage site in VAMP4. The sequence of the peptide SESLSDNATAF corresponds to SEQ ID NO: 89. FIG. 14D is the peptide that marks the N-terminal site of the cleavage site in VAMP5. The sequence of the peptide AELQQR corresponds to SEQ ID NO: 90. FIG. 14E is the peptide that marks the C-terminal site of the cleavage site in VAMP5. The sequence of the peptide SDQLLDMSSTF corresponds to SEQ ID NO: 91. Thus, the cleavage sites were determined to be K87-S88 in VAMP4 and R40-S41 in VAMP5.

DETAILED DESCRIPTION OF SOME EMBODIMENTS

[0092] *Clostridium Botulinum* neurotoxins (BoNTs) are a family of bacterial toxins produced by clostridium bacteria, with seven well-established serotypes (BoNT/A-G) [1-3]. They are one of the most dangerous potential bio-terrorism agents, classified as a "Category A" select agent by Center for Disease Control (CDC) of United States [4]. These toxins are produced as a single polypeptide and can be separated by bacterial or host proteases into a light chain (LC, ~ 50 kDa) and a heavy chain (H_C, ~ 100 kDa). The two chains remain connected via an inter-chain disulfide bond. The H_C contains two sub-domains: the N-terminal H_N domain that mediates translocation of the LC across endosomal membranes, and the C-terminal H_C domain that mediates binding to receptors on neurons. The inter-chain disulfide bond is reduced once the LC translocates into the cytosol [5,6]. Released LC acts as a protease to specifically cleave a set of neuronal proteins: BoNT/A, C, and E cleave at distinct sites on a protein known as SNAP-25; BoNT/B, D, F, and G cleave at different sites on a vesicle protein VAMP; and BoNT/C also cleaves a transmembrane protein syntaxin 1 [1-3]. These three proteins form a complex, known as SNARE complex, which is essential for release of neurotransmitters [7,8]. Cleavage of any one of these three SNARE proteins blocks neurotransmitters release from neurons, thus paralyzing muscles.

[0093] BoNTs are the most potent toxins known and cause the human and animal disease known as botulism [3]. The major form of botulism is caused by ingesting food contaminated with BoNTs (food botulism). Other forms also exist such as infant botulism, which is due to colonization of the intestine by toxin-producing bacteria in infants. BoNTs are always produced together with another 150 kDa protein known as NTNHA (non-toxic non-hemagglutinin protein), which forms a pH-dependent complex with BoNTs and protects BoNTs from proteases in the gastrointestinal tract [9]. Genes encoding BoNT and NTNHA are found in two types of gene clusters: (1) HA cluster, containing genes for three conserved proteins HA17, HA33 and HA70, which form a complex with BoNT/NTNHA and facilitate absorption of toxins across the intestinal epithelial barrier [10-12]. (2) OrfX cluster, which encodes conserved OrfX1, OrfX2, OrfX3 and P47 proteins with unknown functions [13].

[0094] Because local injections of minute amounts of toxins can attenuate neuronal activity in targeted regions, BoNTs have been used to treat a growing list of medical conditions [14-16], including muscle spasms, chronic pain, overactive bladder problems, as well as for cosmetic applications. The market for BoNTs has already surpassed $1.5 billion in 2011 and is projected to reach 2.9 billion by 2018.

[0095] BoNTs were traditionally typed by neutralization assays in mice, by injecting culture supernatant of clostridium bacteria into mice, with or without antisera against known BoNTs. The first distinguished serotypes, BoNT/A and BoNT/B, were established in 1919 by Georgina Burke [18]. The last of the seven type, BoNT/G, was recognized in 1969 from soil samples in Argentina [19]. No new serotype of BoNTs has been recognized since 1970. This classification held true after protein sequences for each BoNT was determined in 1990's. The sequence identity between any two pairs among the seven BoNTs ranges from 32% to 65.3%. All seven BoNTs have been identified and characterized before the era of their medical use. Therefore, there is no patent on any of these toxins. Any company is free to produce and market any one of these seven BoNTs. Among the seven types, BoNT/A and BoNT/B are the two toxins that are currently FDA-approved for use in humans [14-16]. BoNT/A is the dominant type used for both medical and cosmetic applications, marketed as *Botox* from Allergan Inc., *Dysport* from IPSEN Inc., and *Xeomin* from Merz Inc.. BoNT/B is marketed as *Myobloc* by USWorld Med. There are considerable interests in developing other BoNT types as therapeutic toxins, for two major reasons:

1. (1) A major limitation in treatment is generation of neutralizing antibody against BoNT/A or BoNT/B in patients, which renders future treatment with the same toxin ineffective [20]. In this case, patients will need to be treated with a different type of BoNTs. This is why BoNT/B is often utilized to treat patients who have generated neutralizing antibodies against BoNT/A during treatment, but there is a need for alternative toxins for patients who have generated antibodies against both BoNT/A and BoNT/B.
2. (2) Although all BoNTs share the same structure and function, there are also considerable differences between them. For instance, BoNT/A cleaves SNAP-25 and uses a protein SV2 as its receptor, whereas BoNT/B cleaves VAMP and uses a protein synaptotagmin (Syt) as its receptor [21-27]. These functional variations may translate to potential differences in therapeutic efficacy targeting distinct types of neurons. In addition, the stability and therapeutic duration can be also different among seven types of toxins. Therefore, a different toxin type may have its advantage over BoNT/A and BoNT/B.

[0096] Rapid progress on genomic sequencing in recent years has revealed a remarkable diversity of BoNTs [28,29]. First, there are multiple subtypes, which can be recognized by the same antiserum, but contain significant levels of variations on protein sequences (2.6%~31.6% differences) [28,30]. For instance, BoNT/A contains 8 known subtypes, designated as BoNT/A1-A8 [13]. Furthermore, multiple mosaic toxins exist, likely derived from recombination of toxin genes. For instance, a "type H" was reported in 2013, but it was later recognized as a chimeric toxin because its LC shares ~ 80% identity with the LC of a BoNT/F subtype, BoNT/F5, and its H_C shares ~ 84% identity to the H_C of BoNT/A1 [31-34]. Consistently, this toxin can be recognized and neutralized by available antisera against BoNT/A [33].

[0097] The gene cluster encoding BoNTs can be on plasmids, bacterial phage, or chromosomes, indicating that the toxin genes are mobile and subject to horizontal gene transfer [13]. There are also cases that a clostridium bacteria strain contains two or even three different toxin genes [32,35,36]. In these cases, one toxin is usually expressed at higher levels (designed with a capital letter) than the other toxin (designated with a lower case letter). For instance, strains that express high levels of BoNT/B and low levels of BoNT/F are known as BoNT/Bf strains. There are also cases that one toxin is expressed, but the other toxin is not expressed, which is known as silent toxin (usually marked with ()). For instance, a survey for infant botulism cases in California showed that 8% strains were BoNT/A(B), which means these strains contain genes for both BoNT/A and BoNT/B, but only express detectable levels of

BoNT/A [37-39].

[0098] As illustrated in the drawings and examples of the present disclosure, published clostridium bacteria genomic sequence databases were searched, and a novel BoNT gene (hereafter designated "BoNT/X") encoded on the chromosome of *Clostridium botulinum* strain 111 was identified. Strain 111 was first isolated from an infant botulism patient in Japan in 1996 [40]. It has been shown that toxicity from strain 111 in mice can be neutralized by BoNT/B antisera [40]. It was later confirmed that this strain expresses a subtype of BoNT/B, BoNT/B2, encoded on a plasmid [41,42]. The sequence of BoNT/X was deposited into PubMed database in February of 2015, as part of genomic sequence of Strain 111. BoNT/X has not been characterized before. It remains unknown whether it is expressed in the strain 111 and whether it is a functional toxin.

[0099] Also provided herein are the characterization of BoNT/X at functional levels. Its LC was found to cleave VAMP at a site distinct from known target sites of all other BoNTs. The full-length toxin, produced by covalently linking non-toxic fragments via sortase, was found to enter cultured neurons and cleave VAMP in neurons, inducing flaccid paralysis in mice. Finally, it was found that the toxin is not recognized by antisera raised against all seven known BoNTs, establishing BoNT/X as a novel BoNT serotype. Its identification poses an urgent challenge for developing effective countermeasures. It also has the potential to be developed into a new therapeutic toxin and can be used to generate chimeric toxins with potentially distinct pharmacological properties.

[0100] As used herein, the term "Clostridial Botulinum neurotoxin (BoNT) polypeptide" encompasses any polypeptide or fragment from a Botulinum neurotoxin described herein. In some embodiments, the term BoNT refers to a full-length BoNT. In some embodiments, the term BoNT refers to a fragment of the BoNT that can execute the overall cellular mechanism whereby a BoNT enters a neuron and inhibits neurotransmitter release. In some embodiments, the term BoNT simply refers to a fragment of the BoNT, without requiring the fragment to have any specific function or activity. For example, a BoNT polypeptide may refer to the light chain (LC) of a BoNT, e.g., BoNT/X. Other terms that may be used throughout the present disclosure for "Clostridial Botulinum neurotoxins" may be BoNTs, Botulinum toxins, or C. Botulium toxins. It is to be understood that these terms are used interchangeably. "BoNT/X" refers to the novel BoNT serotype described and characterized in the present disclosure. The BoNT/X protein sequence (GenBank No. BAQ12790.1; four cysteines are underlined and bolded) is also provided:

MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPERYNFTNNTNDLNIPSEPIME
ADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKPEGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNI
VSNLQANLVIYGPGPDIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFVKRE
FAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEELLTFGGIDSKAISSLIIKKIIET
AKNNYTTLISERLNTVTVENDLLKYIKNKIPVQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVR
KHYLKERPIDPIYVNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKI**C**PRNGLLYNAI
YRNSKNYLNNIDLEDKKTTSKTNVSYP**C**SLLNG**C**IEVENKDLFLISNKDSLNDINLSEEKIKPETTVFFKD
KLPPQDITLSNYDFTEANSIPSISQQNILERNEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIR

VELTDSVDEALSNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSSDTLAIVP
YIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIGGELAREQVEAIVNNALDKRDQKWA
EVYNITKAQWWGTIHLQINTRLAHTYKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLN
KSVEQAMKNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLSSSLRRKVSIR
LNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGKIKDLSGTTSDINIGSDIELADGRENKAIKIK
GSENSTIKIAMNKYLRFSATDNFSISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDH
NNSIKIVTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRLKNNRDTQAFTLLD
QFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYNKKYYLQTQDKPGKGLIREYWSSFGYDYVILS
DSKTITFPNNIRYGALYNGSKVLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTH

DLTTDFEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSAWYFQNYENLNLRKHT
KTNWYFIPKDEGWDED (SEQ ID NO: 1)

[0101] A "modified Clostridial Botulinum neurotoxin (BoNT)" encompasses a BoNT comprising any modifications in the amino acid sequence, e.g., truncation, addition, amino acid substitution, and any combination thereof. For example, a BoNT/X comprising amino acid substitution mutations in C461 or C467 is a modified BoNT. In another example, a fragment or a domain of the full-length BoNT *(e.g.,* the protease domain, or LC) is considered a modified BoNT. In some embodiments, a domain of the BoNT may also comprise amino acid substitution mutations, e.g., a protease domain comprising substitution mutations at positions C461 or C467 of BoNT/X.

[0102] The term "enters a cell" when used to describe the action of a BoNT of the present disclosure, encompasses the binding of a BoNT to a low or high affinity receptor complex, binding of a BoNT to ganglioside, the internalization of the toxin, the translocation of the toxin light chain into the cytoplasm and the enzymatic modification of a BoNT substrate.

[0103] As used herein, the term "Clostridial Botulinum neurotoxin (BoNT) protease domain" is synonymous to "light-chain (LC)." The BoNT protease domain is located in the light chain of the BoNT, and thus is also referred to as the LC. The term means a BoNT domain that can execute the enzymatic target modification step of the intoxication process. If the LC from a specific BoNT serotype is referred to, the term "serotype-LC" is used. For example, "X-LC" means the LC polypeptide from BoNT/X. A BoNT protease domain specifically targets a C. Botulinum toxin substrate and encompasses the proteolytic cleavage of a C. Botulinum toxin substrate, such as, *e.g.,* SNARE proteins such as a SNAP-25 substrate, a VAMP substrate and a Syntaxin substrate. In BoNT (e.g., BoNT/X, BoNT/A, BoNT/B, BoNT/C, etc.). The protease domain or the LC is considered to correspond to about amino acid 1-439 of BoNT/X. The domain boundary may vary by about 25 amino acids. For example, the protease domain may correspond to amino acids 1-414 or 1-464 of BoNT/X. In some embodiments, the protease domain may correspond to amino acids 1-438, 1-437, 1-436, 1-435, 1-434, 1-433, 1-432, 1-431, 1-430, 1-429, 1-439, 1-440, 1-441, 1-442, 1-443, 1-444, 1-445, 1-446, 1-447, 1-448, or 1-449 of BoNT/X.

[0104] As used herein, the term "Clostridial Botulinum neurotoxin (BoNT) translocation domain" is synonymous with " H_N domain" and means a BoNT domain that can execute the translocation step of the intoxication process that mediates BoNT light chain translocation. Thus, an H_N facilitates the movement of a BoNT light chain across a membrane into the cytoplasm of a cell. Non-limiting examples of a H_N include a BoNT/A H_N, a BoNT/B H_N, a BoNT/Cl H_N, a BoNT/D H_N, a BoNT/E H_N, a BoNT/F H_N, a BoNT/G H_N, and a BoNT/X H_N. The translocation domain is located in the N-terminus of the heavy chain (H_C), and thus is also referred as H_N. It is to be understood that these terms are used interchangeably herein.

[0105] As used herein, the term "linker region" refers to the amino acid sequence between the BoNT protease domain and the translocation domain. The linker comprises two cysteines at position 461 and 467, one of which forms an inter-molecular disulfide bond with a cysteine in the protease domain, C423 (C461-C423 disulfide bond, or C467-C423 disulfide bond). The formation of this disulfide bond is essential for the activity of BoNT/X.

[0106] As used herein, the term "LC-H_N" refers to a BoNT polypeptide encompassing the protease domain, the linker region, and the translocation domain. If the LC-H_N from a specific BoNT serotype is referred to, the term "serotype-LC-H_N" is used. For example, "X-LC-H_N" means the LC-H_N polypeptide from BoNT/X. The LC-H_N polypeptide is considered to correspond to about amino acid 1-892 of BoNT/X. The domain boundary may vary by about 25 amino acids. For example, LC-H_N polypeptide may correspond to about amino acid 1-917 or 1-867 of BoNT/X. In some embodiments, the LC-H_N polypeptide may correspond to amino acids 1-893, 1-894, 1-895, 1-896, 1-897, 1-898, 1-899, 1-900, 1-901, 1-902, 1-892, 1-891, 1-890, 1-889, 1-888, 1-887, 1-886, 1-885, 1-884, or 1-883 of BoNT/X.

[0107] As used herein, the term "Clostridial Botulinum neurotoxin (BoNT) receptor-binding domain" is synonymous with "H_C domain" and means any naturally occurring BoNT receptor binding domain that can execute the cell binding step of the intoxication process, including, *e.g.,* the binding of the BoNT to a BoNT-specific receptor system located on the plasma membrane surface of a target cell. Some aspects of present disclosure relate to modified BoNT receptor binding domains from serotype X (BoNT/X). In some embodiments, a "modified BoNT/X receptor binding domain" comprises amino acid substitutions in a position corresponding to C1240 in BoNT/X (SEQ ID NO: 1). The receptor binding domain, or the H_C, is considered to correspond to about amino acid 893-1306 of BoNT/X. The domain boundary may vary by about 25 amino acids. For example, the receptor binding domain or H_C may correspond to amino acids 868-1306 or 918- 1306. In some embodiments, the receptor binding domain or H_C may correspond to amino acids 893-1306, 894-1306, 895-1306, 896-1306, 897-1306, 898-1306, 899-1306, 900-1306, 901-1306, 902-1306, 892-1306, 891-1306, 890-1306, 889-1306, 888-1306, 887-1306, 886-1306, 885-1306, 884-1306, or 883-1306 of BoNT/X.

[0108] By "isolated" is meant a material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings, e.g., from a cell or from flanking DNA or from the natural source of the DNA. The term "purified" is used to refer to a substance such as a polypeptide that is "substantially pure", with respect to other components of a preparation *(e.g.,* other polypeptides). It can refer to a polypeptide that is at least about 50%, 60%>, 70%>, or 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to other components. The terms "substantially pure" or "essentially purified", with regard to a polypeptide, refers to a preparation that contains fewer than about 20%, more preferably fewer than about 15%, 10%, 8%, 7%, most preferably fewer than about 5%, 4%, 3%, 2%, 1%, or less than 1%, of one or more other components (e.g., other polypeptides or cellular components).

[0109] The term "substitution mutation" without the reference to a specific amino acid, may include any amino acid other than the wild type residue normally found at that position. Such substitutions may be replacement with non-polar (hydrophobic) amino acids, such as glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, and proline. Substitutions may be replacement with polar (hydrophilic) amino acids such as serine, threonine, cysteine, tyrosine, asparagine, and glutamine. Substitutions may be replacement with electrically charged amino acids, e.g., negatively electrically charged amino acids such as aspartic acid and glutamic acid and positively electrically charged amino acids such as lysine, arginine, and histidine.

[0110] The substitution mutations described herein will typically be replacement with a different naturally occurring amino acid residue, but in some cases non-naturally occurring amino acid residues may also be substituted. Non-natural amino acids, as the term is used herein, are non-proteinogenic (*i.e.*, non-protein coding) amino acids that either occur naturally or are chemically synthesized. Examples include β-amino acids (β3 and β2), homo-amino acids, proline and pyruvic acid derivatives, 3 -substituted alanine derivatives, glycine derivatives, ring-substituted phenylalanine and tyrosine derivatives, linear core amino acids, diamino acids, D-amino acids, and N-methyl amino acids. In some embodiments, the amino acid can be substituted or unsubstituted. The substituted amino acid or substituent can be a halogenated aromatic or aliphatic amino acid, a halogenated aliphatic or aromatic modification on the hydrophobic side chain, or an aliphatic or aromatic modification.

[0111] The "percent identity" of two amino acid sequences is determined using the algorithm of Karlin and Altschul Proc. Natl. Acad. Sci. USA 87:2264-68, 1990, modified as in Karlin and Altschul Proc. Natl. Acad. Sci. USA 90:5873-77, 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. J. Mol. Biol. 215:403-10, 1990. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the protein molecules of interest. Where gaps exist between two sequences, Gapped BLAST can be utilized as described in Altschul et al., Nucleic Acids Res. 25(17):3389-3402, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

[0112] The present invention provides isolated BoNT polypeptides, wherein the isolated Clostridial Botulinum neurotoxin (BoNT) polypeptide comprises an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to SEQ ID NO: 3, optionally wherein the isolated BoNT polypeptide comprises the amino acid sequence of SEQ ID NO: 3, and wherein the BoNT polypeptide does not cross react with an antibody against BoNT serotype A, B, C, D, E, F, or G.

[0113] In some embodiments, the isolated BoNT polypeptide is a full-length BoNT/X polypeptide. In some embodiments, the isolated BoNT polypeptide comprise the a amino acid sequence of SEQ ID NO: 1. In some embodiments, the isolated BoNT/X polypeptide comprises an amino acid sequence that has at least 85% identity to SEQ ID NO: 1. For example, the isolated BoNT polypeptide may comprise an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to SEQ ID NO: 1. In some embodiments, the isolated BoNT polypeptide comprises an amino acid sequence that has 85%, 86%, 87%, 88%, 89% ,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 1. In some embodiments, the isolated BoNT polypeptide consists of the amino acid sequence of SEQ ID NO: 1.

[0114] In some embodiments, the isolated BoNT polypeptide is an X-LC-H_N polypeptide. In some embodiments, the isolated BoNT polypeptide comprise the a amino acid sequence of SEQ ID NO: 2. In some embodiments, the isolated BoNT polypeptide comprises an amino acid sequence that has at least 85% identity to SEQ ID NO: 2. For example, the isolated BoNT polypeptide may comprise an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to SEQ ID NO: 2. In some embodiments, the isolated BoNT polypeptide comprises an amino acid sequence that has 85%, 86%, 87%, 88%, 89% ,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 2. In some embodiments, the isolated BoNT polypeptide consists of the amino acid sequence of SEQ ID NO: 2.

[0115] In some embodiments, the isolated BoNT polypeptide is an X-LC polypeptide. In some embodiments, the isolated BoNT polypeptide comprise the a amino acid sequence of SEQ ID NO: 3. In the present invention, the isolated BoNT polypeptide comprises an amino acid sequence that has at least 85% identity to SEQ ID NO: 3. For example, the isolated BoNT polypeptide may comprise an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to SEQ ID NO: 3. In the present invention, the isolated BoNT polypeptide comprises an amino acid sequence that has 85%, 86%, 87%, 88%, 89% ,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 3. In some embodiments, the isolated BoNT polypeptide consists of the amino acid sequence of SEQ ID NO: 3.

[0116] The X-LC polypeptide may be introduced alone into cells where the cleavage of a BoNT substrate (e.g., a SNARE protein) is desired for research or therapeutic purpose, by any known techniques of expression an exogenous protein in the art, e.g., transfection of LC coding sequence directly into cells, via lentiviral vectors, via AAV vectors, or fusing X-LC with cell penetrating peptides).

[0117] In some embodiments, the BoNT polypeptides of the present disclosure is a full-length BoNT/X comprising a protease domain (LC), a linker region, a translocation domain (H_N), and a receptor binding domain (H_C), wherein the linker region is located between the protease domain and the translocation domain. Like other BoNTs, BoNT/X is initially produced as a single polypeptide and is activated via the cleavage of the linker region between LC and H_N either bacterial or host proteases. This process is known as "activation" and is essential for the activity of BoNT/XAfter the cleavage, the LC and H_N remain connected via an inter-chain disulfide bond prior to translocation of LC into the cytosol of cells, where the disulfide bond is reduced in order to release the LC into the cytosol. BoNT/X contains two cysteines that are conserved compared to other BoNTs, C423 and C467. Interestingly, BoNT/X also contains an additional cysteine (C461), which is unique to BoNT/X. The formation of the inter-chain disulfide bond (C423-C461, or C423-C467) is required for BoNT/X activity.

[0118] In addition to the cysteines in the linker region, the receptor binding domain of BoNT contains another cysteine, C1240, which can also form inter-molecular disulfide bonds with other cysteines in BoNT/X. These intermolecular disulfide bonds causes BoNT/X to aggregate and destabilizes the protein (FIG. 4B). Replacing the cysteines that are not required for BoNT/X activity may produces BoNT/X polypeptides with increased stability.

[0119] Accordingly, some aspects of the present disclosure provide modified BoNT/X polypeptide comprising one or more substitution mutation(s) in C461, C467, or C1240, which are more stable than the wild-type BoNT/X and have comparable activities. The cysteines may be substituted with any amino acids that abolish the formation of disulfide bonds. In some embodiments, the cysteines are substituted with serine (S) or alanine (A). Possible combinations of substitution mutations that may be present in the modified BoNTs of the present disclosure are, without limitation: C461S, C461A, C467S, C467A, C1240S, C1240A, C461S/C1240S, C461A/C1240S, C461S/C1240A, C467A/C1240A, C467S/C1240S, C467A/C1240S, C467S/C1240A, and C467A/C1240A. "/" indicates double mutations. In some embodiments, the modified BoNT/X polypeptide of the present disclosure comprises an amino acid sequence of any one of SEQ ID NOs: 4-17. In some embodiments, the modified BoNT/X polypeptide comprises an amino acid sequence that has at least 85% identity to any one of SEQ ID NO: 4-17, and does not have the amino acid sequence of SEQ ID NO: 1. For example, the modified BoNT/X polypeptide may comprise an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any one of SEQ ID NOs: 4-17, and does not have the amino acid sequence of SEQ ID NO: 1. In some embodiments, the modified BoNT/X polypeptide comprises an amino acid sequence that has 85%, 86%, 87%, 88%, 89% ,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to any one of SEQ ID NOs: 4-17, and does not have the amino acid sequence of SEQ ID NO: 1. In some embodiments, the modified BoNT/X polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 4-17.

[0120] In some embodiments, the modified BoNT polypeptide of the present disclosure is a modified BoNT/X-LC-H_N polypeptide comprising the substitution mutations described herein. In some embodiments, the modified BoNT/X-LC-H_N comprises one single substitution mutation in a position corresponding to C461 or C467 in SEQ ID NO: 2. In some embodiments, the modified BoNT/X-LC-H_N comprises one single substitution mutation corresponding to C461A, C461S, C467A, or C467S in SEQ ID NO: 2. In some embodiments, the modified BoNT/X polypeptide of the present disclosure comprises an amino acid sequence of any one of SEQ ID NOs: 18-21. In some embodiments, the modified BoNT/X-LC-H_N polypeptide comprises an amino acid sequence that has at least 85% identity to any one of SEQ ID NO: 18-21, and does not have the amino acid sequence of SEQ ID NO: 2. For example, the modified BoNT/X-LC-H_N polypeptide may comprise an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any one of SEQ ID NOs: 18-21, and does not have the amino acid sequence of SEQ ID NO: 2. In some embodiments, the modified BoNT/X-LC-H_N polypeptide comprises an amino acid sequence that has 85%, 86%, 87%, 88%, 89% ,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to any one of SEQ ID NOs: 18-21, and does not have the amino acid sequence of SEQ ID NO: 2. In some embodiments, the modified BoNT/X-LC-H_N polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 18-21.

[0121] The modified BoNT polypeptide comprising one or more substitution mutation(s) (e.g., in C461, C467, or C1240) described herein does not form inter-molecular disulfide bonds that cause aggregation of the protein, and are therefore more stable than their corresponding wild type proteins. The activity of the BoNT polypeptides are not affected by the substitution mutations in the cysteines. Thus, the modified BoNT/X may be more suitable for therapeutic use than the wild type BoNT/X due to its increased stability.

[0122] Other aspects of the present disclosure provide chimeric BoNTs comprising BoNT/X-LC-H_N described herein and the receptor binding domain (H_C) from a different BoNT. For example, the receptor binding domain may be from any one of BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/E, BoNT/F, and BoNT/G. Thus, the chimeric BoNTs contemplated herein include BoNT/X-LC-H_N-A-H_C, BoNT/X-LC-H_N-B-H_C, BoNT/X-LC-H_N-C-H_C, BoNT/X-LC-H_N-D-H_C, BoNT/X-LC-H_N-E-H_C, BoNT/X-LC-H_N-F-H_C, and BoNT/X-LC-H_N-G-H_C. It is to be understood that the H_C domain of any subtypes of the seven known serotypes *(e.g.,* A, B, C, D, E, F, or G) are suitable for the chimeric toxin. When BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F, or BoNT/G is referred to, it encompasses all the subtypes. For example, BoNT/A has 8 subtypes, BoNT/A1, BoNT/A2, BoNT/A3, BoNT/A4, BoNT/A5, BoNT/A6, BoNT/A7, or BoNT/A8, and the H_C of any one of these BoNT/A subtypes are suitable for use in the chimeric BoNT of the present disclosure. Similarly, the H_C of any one of the 8 subtypes of BoNT/B, *i.e.,* BoNT/B1, BoNT/B2, BoNT/B3, BoNT/B4, BoNT/B5, BoNT/B6, BoNT/B7, or BoNT/B8, are suitable for use in the chimeric BoNT of the present disclosure.

[0123] In some embodiments, BoNT/X-LC-H_N-A1-H_C (SEQ ID NO: 22), BoNT/X-LC-H_N-B1-H_C (SEQ ID NO: 23), and BoNT/X-LC-H_N-C1-H_C (SEQ ID NO: 24) are provided. In some embodiments, the chimeric BoNT polypeptide comprises an amino acid sequence that has at least 85% identity to any one of SEQ ID NO: 22-24. For example, the chimeric BoNT polypeptide may comprise an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any one of SEQ ID NOs: 22-24. In some embodiments, the chimeric BoNT polypeptide comprises an amino acid sequence that has 85%, 86%, 87%, 88%, 89% ,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to any one of SEQ ID NOs: 22-24. In some embodiments, the chimeric BoNT polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 22-24.

[0124] In some embodiments, the chimeric BoNT of the present disclosure comprises a modified BoNT/X-LC-H_N comprising a substitution mutation in the linker region, *e.g.,* in a position corresponding to C461 or C467 of SEQ ID NO: 2. For example, the BoNT/X-LC-H_N in the chimeric BoNT may comprise a substitution mutation corresponding to C461A, C467A, C461S, or C467S of SEQ ID NO: 2. For example, the chimeric BoNT polypeptide of the present disclosure may comprise an amino acid sequence of any one of SEQ ID NOs: 25-30. In some embodiments, the chimeric BoNT polypeptide comprises an amino acid sequence that has at least 85% identity to any one of SEQ ID NO: 25-30. For example, the chimeric BoNT polypeptide may comprise an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any one of SEQ ID NOs: 25-30. In some embodiments, the chimeric BoNT polypeptide comprises an amino acid sequence that has 85%, 86%, 87%, 88%, 89% ,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to any one of SEQ ID NOs: 25-30. In some embodiments, the chimeric BoNT polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 25-30.

[0125] To generate the chimeric toxins, e.g., the BoNT/X-LC-H_N-A1-H_C toxin, the X-LC-H_N fragment comprising amino acid of about 1-892 (SEQ ID NO: 2) is fused to the receptor binding domain of any one of BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/E, BoNT/F, and BoNT/G. The receptor binding domains of different BoNTs correspond to amino acids of about 860-1291 of BoNT/B1. It is to be understood that the border of the X-LC-H_N fragment and/or the receptor binding domains may vary by 1-25 amino acids. For example, the X-LC-H_N fragment that may be used for the chimeric toxin may comprise amino acids 1- 917 or 1-867 of BoNT/X. In some embodiments, the X-LC-H_N fragment that may be used for the chimeric toxin may comprise amino acids 1-893, 1-894, 1-895, 1-896, 1-897, 1-898, 1-899, 1-900, 1-901, 1-902, 1-892, 1-891, 1-890, 1-889, 1-888, 1-887, 1-886, 1-885, 1-884, or 1-883 of BoNT/X. Similarly, the receptor binding that may be used for the chimeric toxin may comprise amino acid corresponding to 885-1291 or 835-1291 of BoNT/X. In some embodiments, the receptor binding that may be used for the chimeric toxin may comprise amino acid corresponding to 860-1291, 861-1291, 862-1291, 863-1291, 864-1291, 865-1291, 866-1291, 867-1291, 868-1291, 869-1291, 870-1291, 860-1291, 859-1291, 858-1291, 857-1291, 856-1291, 855-1291, 854-1291, 853-1291, 852-1291, or 851-1291 of BoNT/B. The skilled artisan is able to identified the domains that may be used for the chimeric toxin of the present disclosure, based on his/her knowledge in protein homology, with or without the assistance of a sequence alignment software. The methods of fusing the fragments are standard recombinant techniques that are well known to one skilled in the art.

[0126] Further contemplated herein are modified BoNT/X polypeptides comprising a modified linker region, wherein the linker region comprises a specific protease cleavage site. A "specific protease cleavage site," as used herein, refers to a recognition and cleavage site for a specification protease, as opposed to a sequence that is recognized and cleavage by more than one non-specific proteases. Such specific proteases include, without limitation: thrombin, TEV, PreScission, Factor Xa, MMP-12, MMP-13, MMP-17, MMP-20, Granzyme-B, and Enterokinase. The cleavage site of the specific proteases may be added to the linker region of the BoNT/X polypeptide via insertion or replacement of the existing amino acids in the linker region *(e.g.,* replace amino acids 424-460 of the BoNT/X polypeptide). The sequences of the specific protease cleavage sites sequences are also provided: LVPR|GS (thrombin, SEQ ID NO: 50), ENLYFQ|G (TEV, SEQ ID NO: 51), LEVLFQ|GP (PreScission, SEQ ID NO: 52), IEGR| or IDGR| (Factor Xa, SEQ ID NO: 53 or 54), DDDDK| (Enterokinase, SEQ ID NO: 55) and AHREQIGG| (SUMO protease, SEQ ID NO: 56). "|" indicates where cleavage occurs.

[0127] Other aspects of the present disclosure provide the functional characterization of the BoNT/X polypeptides. The BoNT/X polypeptides, modified BoNT/X polypeptides, and chimeric BoNT polypeptides of the present disclosure can bind and enter target cells, *e.g.,* neurons, and cleave its substrate proteins, e.g. SNARE proteins. The term "SNARE proteins," as used herein, refers to SNAP (Soluble NSF Attachment Protein) Receptors, which is a large protein superfamily consisting of more than 60 members in yeast and mammalian cells. The primary role of SNARE proteins is to mediate vesicle fusion, *i.e.,* the fusion of vesicles with their target membrane bound compartments (such as a lysosome). The best studied SNARE proteins are those that mediate docking of synaptic vesicles with the presynaptic membrane in neurons, e.g., SNAP-25, VAMP1, VAMP2, VAMP3, VAMP4, VAMP5, VAMP7, VAMP8, syntaxin1, and

Ykt6. Several of these SNARE proteins are substrates of BoNTs. For example, VAMP1, VAMP2, VAMP3, SNAP-25, and syntaxin 1 have been shown to be cleaved by known BoNTs, e.g., BoNT/A and BoNT/B.

[0128] Provided herein are data showing that BoNT/X cleaves the SNARE proteins that are known substrates of BoNTs. One surprising finding of the present disclosure is that BoNT/X is able to cleave several SNARE proteins that other BoNTs are not able to cleave, e.g., VAMP4, VAMP5, and Ykt6. VAMP4 is widely expressed and is known to mediate vesicle fusion between trans-Golgi network (TGN) and endosomes, as well as homotypic fusion of endosomes. BoNTs are traditionally known to be limited to target SNAREs that mediate vesicle exocytosis onto plasma membranes. BoNT/X is the first BoNT that is capable of cleaving SNAREs mediating other type fusion events inside cells that is not with plasma membrane as the destine. VAMP4 may also contribute to asynchronous synaptic vesicle exocytosis, enlargeosome exocytosis, and activity-dependent bulk endocytosis (ADBE) in neurons. In addition, VAMP4 has been implicated in granule release in immune cells. Thus, BoNT/X might have a unique potential among all BoNTs to modulate inflammatory secretion in immune cells, which can be exploited therapeutically. VAMP5 is mainly expressed in muscles and its function remains to be established. BoNT/X will be a unique tool for investigating the function of VAMP4 and VAMP5. Ykt6 functions in endoplasmic reticulum to Golgi transport. It also functions in early/recycling endosome to TGN transport. The identification of Ykt6 as a substrate of the BoNT polypeptides described herein is significant because it opens up new therapeutic application for blocking secretion in a wide range of cells by BoNTs.

[0129] Another surprising finding of the present disclosure is that BoNT/X cleaves the SNARE proteins at a novel site what was not previously described. As illustrated in the Examples and Figures of the present disclosure, BoNT/X cleaves between amino acids R66-S67 in VAMP1, VAMP2, and VAMP3. R66-A67 is a novel cleavage site distinct from established target sites for all other BoNTs (FIG. 2F). It is also the only BoNT cleavage site located within a region previously known as the SNARE motif (FIG. 2F,).

[0130] Accordingly, the BoNT polypeptides of the present disclosure have expanded profile of target cells and substrates. In some embodiments, the BoNT polypeptide cleaves a SNARE protein in the cell. In some embodiments, the BoNT polypeptide cleaves a SNARE protein selected from the group consisting of: SNAP-25, VAMP1, VAMP2, VAMP3, VAMP4, VAMP5, Ykt6, and syntaxin 1. In some embodiments, the BoNT polypeptide cleaves VAMP 1 (SEQ ID NO: 39). In some embodiments, the BoNT polypeptide cleaves VAMP1 between amino acid residues corresponding to R66 and A67 of SEQ ID NO: 39. In some embodiments, the BoNT polypeptide cleaves VAMP2 (SEQ ID NO: 40). In some embodiments, the BoNT polypeptide cleaves VAMP2 between amino acid residues corresponding to R66 and A67 of SEQ ID NO: 40. In some embodiments, the BoNT polypeptide cleaves VAMP3 (SEQ ID NO: 31). In some embodiments, the BoNT polypeptide cleaves VAMP3 between amino acid residues corresponding to R66 and A67 of SEQ ID NO: 41. In some embodiments, the BoNT polypeptide cleaves VAMP4 (SEQ ID NO: 42). In some embodiments, the BoNT polypeptide cleaves VAMP4 between amino acid residues corresponding to K87 and S88 of SEQ ID NO:

42. In some embodiments, the BoNT polypeptide cleaves VAMP5 (SEQ ID NO: 43). In some embodiments, the BoNT polypeptide cleaves VAMP5 between amino acid residues corresponding to R40 and S41 of SEQ ID NO: 43. In some embodiments, the BoNT polypeptide cleaves Ykt6 (SEQ ID NO: 44). In some embodiments, the BoNT polypeptide cleaves Ykt6 between amino acid residues corresponding to K173 and S174 of SEQ ID NO: 44.

[0131] In some embodiments, the BoNT polypeptide of the present disclosure cleaves a SNARE protein in a target cell. As used herein, a "target cell" means a cell that is a naturally occurring cell that BoNT is capable of entering or intoxicating. In some embodiments, a target cell is a secretory cell, *e.g.,* a neuron or a secretory immune cell. Examples of neurons that may be BoNT target cells include, without limitation, motor neurons; sensory neurons; autonomic neurons; such as, e.g., sympathetic neurons and parasympathetic neurons; non-peptidergic neurons, such as, e.g., cholinergic neurons, adrenergic neurons, noradrenergic neurons, serotonergic neurons, GABAergic neurons; and peptidergic neurons, such as, e.g., Substance P neurons, Calcitonin Gene Related Peptide neurons, vasoactive intestinal peptide neurons, Neuropeptide Y neurons, cholecystokinin neurons.

[0132] The BoNT polypeptide of the present disclosure, e.g., the BoNT/X or the modified BoNT/X polypeptide, is able to target other types of secretory cells other than neurons, due to its ability to cleave VAMP4 or Ykt6. In some embodiments, the secretory cell targeted by the BoNT polypeptide is a secretory immune cell. A "secretory immune cell," as used herein, refers to immune cells that secrets cytokines, chemokines, or antibodies. Such secretory immune cells may be innate immune cells including, without limitation, natural killer cells, mast cells, eosinophils, basophils, macrophages, neutrophils, and dendritic cells. Secretory immune cells that secret antibodies (e.g., white blood cells) may also be targeted by the BoNT polypeptides of the present disclosure. Non-limiting examples of antibody secreting cells include, without limitation, plasma B cells, plasmocytes, plasmacytes, and effector B cells. In some embodiments, the target cell is a cultured cell, *e.g.,* a cultured neuron or a cultured secretory immune cell. In some embodiments, the target cell is *in vivo.* In some embodiments, target cell is from a mammal. In some embodiments, the mammal is a human. In embodiments, the mammal is a rodent, *e.g.,* a mouse or a rat.

[0133] In some embodiments, the BoNT polypeptide suppresses neuronal activity. In some embodiments, the BoNT polypeptide modulates immune response. In some embodiments, the BoNT polypeptide induces flaccid paralysis. "Flaccid paralysis" refers to a clinical manifestation characterized by weakness or paralysis and reduced muscle tone without other obvious cause (e.g., trauma).

[0134] Other aspects of the present disclosure provide modified BoNT/X polypeptides comprising an inactive protease domain. Such BoNT/X polypeptides (also referred to herein as "inactive BoNT/X") can enter the target cells but cannot cleave the substrate proteins *(e.g.,* a SNARE protein) due to the inactivation of the protease domain. In some embodiments, the inactive BoNT/X is an X-LC-H_N fragment comprising: a) an inactive protease domain; b) a linker region; and c) a translocation domain. In some embodiments, the inactive BoNT/X is a full length BoNT/X polypeptide comprising: a) an inactive protease domain; b) a linker region; c) a translocation domain; and d) a receptor binding domain. In some embodiments, the inactive protease domain comprises one or more substitution mutation(s) in a position corresponding to R360, Y363, H227, E228, or H231 of SEQ ID NO: 1. In some embodiments, the one or more substitution mutation(s) corresponds to R360A/Y363F, H227Y, E228Q, or H231Y in SEQ ID NO: 1. It is to be understood that the inactive BoNT/X polypeptide may comprise any mutation(s) that inactivates the protease domain.

[0135] In some embodiments, the inactive BoNT/X polypeptide comprises an amino acid sequence of any one of SEQ ID NOs: 31-38. In some embodiments, the inactive BoNT/X polypeptide comprises an amino acid sequence that has at least 85% identity to any one of SEQ ID NOs: 31-38. For example, the inactive BoNT/X polypeptide may comprise an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to any one of SEQ ID NOs: 31-38. In some embodiments, the inactive BoNT/X polypeptide comprises an amino acid sequence that has 85%, 86%, 87%, 88%, 89% ,90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to any one of SEQ ID NOs: 31-38. In some embodiments, the inactive BoNT/X polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 31-38.

[0136] In some embodiments, the inactive BoNT/X (e.g., inactive X-LC-H_N or inactive full length BoNT/X) further comprises mutations in the linker region. In some embodiments, the modification in the linker region comprises one single substitution mutation in a position corresponding to C461 or C467 of SEQ ID NO: 1. In some embodiments, the single substitution mutation corresponds to C461A, C461S, C467A, or C467S in SEQ ID NO: 1. In some embodiments, the inactive BoNT/X *(e.g.,* the inactive full length BoNT/X) further comprises a modification in the receptor binding domain. In some embodiments, the modification in the receptor binding domain comprises a substitution mutation in a position corresponding to C1240 of SEQ ID NO: 1.

[0137] It is also envisioned that the modified BoNT/X polypeptide comprising an inactive protease domain described herein can be utilized as a delivery tool to target cells (*e.g.*, neurons) in humans. For example, the modified BoNT/X can be linked to other therapeutic agents, covalently or non-covalently, and acts as the targeting vehicle to deliver the therapeutic agents to target cells in humans.

[0138] As such, another aspect of the disclosure relates to a chimeric polypeptide molecule comprising a first portion that is an inactive BoNT/X, comprising one or more substitution mutations that inactivates the protease domain, linked to a second portion. The second portion of the molecule can be a bioactive molecule such as a therapeutic agent *(e.g.,* a polypeptide or non-polypeptide drug). Linkage of the first and second portions of the molecule can be covalent (e.g., in the form of a fusion protein) or non-covalent. Methods of such linkage are known in the art and can readily be applied by the skilled practitioner. When the second portion of the chimeric molecule is a polypeptide and the chimeric molecule is in the form of a protein, nucleic acids and nucleic acid vectors encoding such chimeric molecules are provided.

[0139] Also provided are cells comprising the nucleic acids or nucleic acid vectors, and cells expressing such chimeric molecules. The chimeric molecules in a fusion protein form may be expressed and isolated using the methods disclosed herein.

[0140] The modified BoNT/X polypeptides, the chimeric BoNT polypeptides, or the chimeric molecules comprising a second portion that is a polypeptide of the present disclosure (e.g., without limitation, polypeptides comprising amino acid sequence of any one of SEQ ID NOs: 1-38), will generally be produced by expression form recombinant nucleic acids in appropriate cells (*e.g.*, *E. coli*, or insect cells) and isolated. The nucleic acids encoding the polypeptides described herein may be obtained, and the nucleotide sequence of the nucleic acids determined, by any method known in the art.

[0141] Further provided herein are isolated and/or recombinant nucleic acids encoding any of the BoNT polypeptides disclosed herein. The nucleic acids encoding the isolated polypeptide fragments of the present disclosure, may be DNA or RNA, double-stranded or single stranded. In certain aspects, the subject nucleic acids encoding the isolated polypeptide fragments are further understood to include nucleic acids encoding polypeptides that are variants of any one of the modified BoNT polypeptides described herein.

[0142] Variant nucleotide sequences include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants. In some embodiments, the isolated nucleic acid molecule of the present disclosure comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence that has at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity of any one of SEQ ID NOs: 1-38. In some embodiments, the isolated nucleic acid molecule of the present disclosure comprising a polynucleotide encoding a polypeptide comprising an amino acid sequence that has 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity of any one of SEQ ID NOs: 1-38.

[0143] In some embodiments, the nucleic acid is comprised within a vector, such as an expression vector. In some embodiments, the vector comprises a promoter operably linked to the nucleic acid.

[0144] A variety of promoters can be used for expression of the polypeptides described herein, including, cytomegalovirus (CMV) intermediate early promoter, a viral LTR such as the Rous sarcoma virus LTR, HIV-LTR, HTLV-1 LTR, the simian virus 40 (SV40) early promoter, E. coli lac UV5 promoter, and the herpes simplex tk virus promoter. Regulatable promoters can also be used. Such regulatable promoters include those using the lac repressor from E. coli as a transcription modulator to regulate transcription from lac operator-bearing mammalian cell promoters [Brown, M. et al., Cell, 49:603-612 (1987)], those using the tetracycline repressor (tetR) [Gossen, M., and Bujard, H., Proc. Natl. Acad. Sci. USA 89:5547-5551 (1992); Yao, F. et al., Human Gene Therapy, 9:1939-1950 (1998); Shockelt, P., et al., Proc. Natl. Acad. Sci. USA, 92:6522-6526 (1995)].

[0145] Other systems include FK506 dimer, VP16 or p65 using astradiol, RU486, diphenol murislerone, or rapamycin. Inducible systems are available from Invitrogen, Clontech and Ariad. Regulatable promoters that include a repressor with the operon can be used. In one embodiment, the lac repressor from Escherichia coli can function as a transcriptional modulator to regulate transcription from lac operator-bearing mammalian cell promoters [M. Brown et al., Cell, 49:603-612 (1987)]; Gossen and Bujard (1992); [M. Gossen et al., Natl. Acad. Sci. USA, 89:5547-5551 (1992)] combined the tetracycline repressor (tetR) with the transcription activator (VP 16) to create a tetR-mammalian cell transcription activator fusion protein, tTa (tetR-VP 16), with the tetO-bearing minimal promoter derived from the human cytomegalovirus (HCMV) major immediate-early promoter to create a tetR-tet operator system to control gene expression in mammalian cells. In one embodiment, a tetracycline inducible switch is used (Yao et al., Human Gene Therapy; Gossen et al., Natl. Acad. Sci. USA, 89:5547-5551 (1992); Shockett et al., Proc. Natl. Acad. Sci. USA, 92:6522-6526 (1995)).

[0146] Additionally, the vector can contain, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; internal ribosome binding sites (IRESes), versatile multiple cloning sites; and T7 and SP6 RNA promoters for *in vitro* transcription of sense and antisense RNA. Suitable vectors and methods for producing vectors containing transgenes are well known and available in the art.

[0147] An expression vector comprising the nucleic acid can be transferred to a host cell by conventional techniques (e.g., electroporation, liposomal transfection, and calcium phosphate precipitation) and the transfected cells are then cultured by conventional techniques to produce the polypeptides described herein. In some embodiments, the expression of the polypeptides described herein is regulated by a constitutive, an inducible or a tissue-specific promoter.

[0148] The host cells used to express the isolated polypeptides described herein may be either bacterial cells such as Escherichia coli, or, preferably, eukaryotic cells. In particular, mammalian cells, such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for immunoglobulins (Foecking et al. (1986) "Powerful And Versatile Enhancer-Promoter Unit For Mammalian Expression Vectors," Gene 45:101-106; Cockett et al. (1990) "High Level Expression Of Tissue Inhibitor Of Metalloproteinases In Chinese Hamster Ovary Cells Using Glutamine Synthetase Gene Amplification," Biotechnology 8:662-667). A variety of host-expression vector systems may be utilized to express the isolated polypeptides described herein. Such host-expression systems represent vehicles by which the coding sequences of the isolate d polypeptides described herein may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the isolated polypeptides described herein in situ. These include, microorganisms such as bacteria *(e.g.,* E. coli and B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing coding sequences for the isolated polypeptides described herein; yeast (e.g., Saccharomyces pichia) transformed with recombinant yeast expression vectors containing sequences encoding the isolated polypeptides described herein; insect cell systems infected with recombinant virus expression vectors (e.g., baclovirus) containing the sequences encoding the isolated polypeptides described herein; plant cell systems infected with recombinant virus expression vectors (*e.g*., cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV) or transformed with recombinant plasmid expression vectors *(e.g.,* Ti plasmid) containing sequences encoding the isolated polypeptides described herein; or mammalian cell systems (*e.g*., COS, CHO, BHK, 293, 293T, 3T3 cells, lymphotic cells (see U.S. Pat. No. 5,807,715), Per C.6 cells (human retinal cells developed by Crucell) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells *(e.g.,* metallothionein promoter) or from mammalian viruses *(e.g.,* the adenovirus late promoter; the vaccinia virus 7.5K promoter).

[0149] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the polypeptides being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of polypeptides described herein, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, the E. coli expression vector pUR278 (Rüther et al. (1983) "Easy Identification Of cDNA Clones," EMBO J. 2:1791-1794), in which the coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye et al. (1985) "Up-Promoter Mutations In The lpp Gene Of Escherichia Coli," Nucleic Acids Res. 13:3101-3110; Van Heeke et al. (1989) "Expression Of Human Asparagine Synthetase In Escherichia Coli," J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione.

[0150] The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety. In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The coding sequence may be cloned individually into non-essential regions *(e.g.,* the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (e.g., the polyhedrin promoter).

[0151] In mammalian host cells, a number of viral-based expression systems may be utilized.

In cases where an adenovirus is used as an expression vector, the coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the immunoglobulin molecule in infected hosts *(e.g.,* see Logan et al. (1984) "Adenovirus Tripartite Leader Sequence Enhances Translation Of mRNAs Late After Infection," Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic.

[0152] The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bitter et al. (1987) "Expression And Secretion Vectors For Yeast," Methods in Enzymol. 153:516-544). In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications *(e.g.,* glycosylation) and processing *(e.g.,* cleavage) of protein products may be important for the function of the protein. For example, in certain embodiments, the polypeptides described herein may be expressed as a single gene product *(e.g.,* as a single polypeptide chain, *i.e.,* as a polyprotein precursor), requiring proteolytic cleavage by native or recombinant cellular mechanisms to form separate polypeptides described herein.

[0153] The disclosure thus encompasses engineering a nucleic acid sequence to encode a polyprotein precursor molecule comprising the polypeptides described herein, which includes coding sequences capable of directing post translational cleavage of said polyprotein precursor. Post-translational cleavage of the polyprotein precursor results in the polypeptides described herein. The post translational cleavage of the precursor molecule comprising the polypeptides described herein may occur *in vivo* (*i.e.*, within the host cell by native or recombinant cell systems/mechanisms, *e.g.* furin cleavage at an appropriate site) or may occur *in vitro* (*e.g.* incubation of said polypeptide chain in a composition comprising proteases or peptidases of known activity and/or in a composition comprising conditions or reagents known to foster the desired proteolytic action).

[0154] Purification and modification of recombinant proteins is well known in the art such that the design of the polyprotein precursor could include a number of embodiments readily appreciated by a skilled worker. Any known proteases or peptidases known in the art can be used for the described modification of the precursor molecule, e.g., thrombin or factor Xa (Nagai et al. (1985) "Oxygen Binding Properties Of Human Mutant Hemoglobins Synthesized In Escherichia Coli," Proc. Nat. Acad. Sci. USA 82:7252-7255, and reviewed in Jenny et al. (2003) "A Critical Review Of The Methods For Cleavage Of Fusion Proteins With Thrombin And Factor Xa," Protein Expr. Purif. 31:1-11)), enterokinase (Collins-Racie et al. (1995)

"Production Of Recombinant Bovine Enterokinase Catalytic Subunit In Escherichia Coli Using The Novel Secretory Fusion Partner DsbA," BiotecHNology 13:982-987)), furin, and AcTEV (Parks et al. (1994) "Release Of Proteins And Peptides From Fusion Proteins Using A Recombinant Plant Virus Proteinase," Anal. Biochem. 216:413-417)) and the Foot and Mouth Disease Virus Protease C3.

[0155] Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include CHO, VERY, BHK, HeLa, COS, MDCK, 293, 293T, 3T3, WI38, BT483, Hs578T, HTB2, BT20 and T47D, CRL7030 and Hs578Bst.

[0156] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express polypeptides described herein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the polypeptides described herein. Such engineered cell lines may be particularly useful in screening and evaluation of polypeptides that interact directly or indirectly with the polypeptides described herein.

[0157] A number of selection systems may be used, including the herpes simplex virus thymidine kinase (Wigler et al. (1977) "Transfer Of Purified Herpes Virus Thymidine Kinase Gene To Cultured Mouse Cells," Cell 11: 223-232), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al. (1992) "Use Of The HPRT Gene And The HAT Selection TecHNique In DNA-Mediated Transformation Of Mammalian Cells First Steps Toward Developing Hybridoma TecHNiques And Gene Therapy," Bioessays 14: 495-500), and adenine phosphoribosyltransferase (Lowy et al. (1980) "Isolation Of Transforming DNA: Cloning The Hamster aprt Gene," Cell 22: 817-823) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al. (1980) "Transformation Of Mammalian Cells With An Amplifiable Dominant-Acting Gene," Proc. Natl. Acad. Sci. USA 77:3567-3570; O'Hare et al. (1981) "Transformation Of Mouse Fibroblasts To Methotrexate Resistance By A Recombinant Plasmid Expressing A Prokaryotic Dihydrofolate Reductase," Proc. Natl. Acad. Sci. USA 78: 1527-1531); gpt, which confers resistance to mycophenolic acid (Mulligan et al. (1981) "Selection For Animal Cells That Express The

Escherichia coli Gene Coding For Xanthine-Guanine Phosphoribosyltransferase," Proc. Natl. Acad. Sci. USA 78: 2072-2076); neo, which confers resistance to the aminoglycoside G-418 (Tolstoshev (1993) "Gene Therapy, Concepts, Current Trials And Future Directions," Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan (1993) "The Basic Science Of Gene Therapy," Science 260:926-932; and Morgan et al. (1993) "Human Gene Therapy," Ann. Rev. Biochem. 62:191-217) and hygro, which confers resistance to hygromycin (Santerre et al. (1984) "Expression Of Prokaryotic Genes For Hygromycin B And G418 Resistance As Dominant-Selection Markers In Mouse L Cells," Gene 30:147-156). Methods commonly known in the art of recombinant DNA tec$H_N$ology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, JoHN Wiley & Sons, NY; Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, Current Protocols in Human Genetics, JoHN Wiley & Sons, NY.; Colberre-Garapin et al. (1981) "A New Dominant Hybrid Selective Marker For Higher Eukaryotic Cells," J. Mol. Biol. 150:1-14.

[0158] The expression levels of polypeptides described herein can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning, Vol. 3 (Academic Press, New York, 1987). When a marker in the vector system expressing a polypeptide described herein is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the nucleotide sequence of a polypeptide described herein or a polypeptide described herein, production of the polypeptide will also increase (Crouse et al. (1983) "Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," Mol. Cell. Biol. 3:257-266).

[0159] Once a polypeptide described herein has been recombinantly expressed, it may be purified by any method known in the art for purification of polypeptides, polyproteins or antibodies (e.g., analogous to antibody purification schemes based on antigen selectivity) for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen (optionally after Protein A selection where the polypeptide comprises an Fc domain (or portion thereof)), and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of polypeptides or antibodies. Other aspects of the present disclosure relate to a cell comprising a nucleic acid described herein or a vector described herein.

[0160] The cell may be a prokaryotic or eukaryotic cell. In some embodiments, the cell in a mammalian cell. Exemplary cell types are described herein. Other aspects of the present disclosure related to a cell expressing the modified BoNT polypeptides described herein. The cell may be a prokaryotic or eukaryotic cell. In some embodiments, the cell in a mammalian cell. Exemplary cell types are described herein. The cell can be for propagation of the nucleic acid or for expression of the nucleic acid, or both. Such cells include, without limitation, prokaryotic cells including, without limitation, strains of aerobic, microaerophilic, capnophilic, facultative, anaerobic, gram-negative and gram-positive bacterial cells such as those derived from, e.g., Escherichia coli, Bacillus subtilis, Bacillus licheniformis, Bacteroides fragilis, Clostridia perfringens, Clostridia difficile, Caulobacter crescentus, Lactococcus lactis, Methylobacterium extorquens, Neisseria meningirulls, Neisseria meningitidis, Pseudomonas fluorescens and Salmonella typhimurium; and eukaryotic cells including, without limitation, yeast strains, such as, *e.g.,* those derived from Pichia pastoris, Pichia methanolica, Pichia angusta, Schizosaccharomyces pombe, Saccharomyces cerevisiae and Yarrowia lipolytica; insect cells and cell lines derived from insects, such as, *e.g.,* those derived from Spodoptera frugiperda, Trichoplusia ni, Drosophila melanogaster and Manduca sexta; and mammalian cells and cell lines derived from mammalian cells, such as, *e.g.,* those derived from mouse, rat, hamster, porcine, bovine, equine, primate and human. Cell lines may be obtained from the American Type Culture Collection, European Collection of Cell Cultures and the German Collection of Microorganisms and Cell Cultures. Non-limiting examples of specific protocols for selecting, making and using an appropriate cell line are described in *e.g.,* INSECT CELL CULTURE ENGINEERING (Mattheus F. A. Goosen et al. eds., Marcel Dekker, 1993); INSECT CELL CULTURES: FUNDAMENTAL AND APPLIED ASPECTS (J. M. Vlak et al. eds., Kluwer Academic Publishers, 1996); Maureen A. Harrison & Ian F. Rae, GENERAL TECHNIQUES OF CELL CULTURE (Cambridge University Press, 1997); CELL AND TISSUE CULTURE: LABORATORY PROCEDURES (Alan Doyle et al eds., JoHN Wiley and Sons, 1998); R. Ian FresHNey, CULTURE OF ANIMAL CELLS: A MANUAL OF BASIC TECHNIQUE (Wiley-Liss, 4.sup.th ed. 2000); ANIMAL CELL CULTURE: A PRACTICAL APPROACH (JoHN R. W. Masters ed., Oxford University Press, 3.sup.rd ed. 2000); MOLECULAR CLONING A LABORATORY MANUAL, supra, (2001); BASIC CELL CULTURE: A PRACTICAL APPROACH (JoHN M. Davis, Oxford Press, 2.sup.nd ed. 2002); and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, supra, (2004).

[0161] These protocols are routine procedures within the scope of one skilled in the art and from the teaching herein. Yet other aspects of the present disclosure relate to a method of producing a polypeptide described herein, the method comprising obtaining a cell described herein and expressing nucleic acid described herein in said cell. In some embodiments, the method further comprises isolating and purifying a polypeptide described herein.

[0162] In some embodiments, botulinum neurotoxin can be obtained by establishing and growing cultures of Clostridium botulinum in a fermenter and then harvesting and purifying the fermented mixture in accordance with known procedures. All the botulinum toxin serotypes are initially synthesized as inactive single chain proteins which must be cleaved or nicked by proteases to become neuroactive.

[0163] The bacterial strains that make botulinum toxin serotypes A and G possess endogenous proteases and serotypes A and G can therefore be recovered from bacterial cultures in predominantly their active form. In contrast, botulinum toxin serotypes C, D and E are synthesized by non-proteolytic strains and are therefore typically inactive when recovered from culture. Serotypes B and F are produced by both proteolytic and non-proteolytic strains and therefore can be recovered in either the active or inactive form. The proteolytic strains that produce, for example, the botulinum toxin type B serotype may only cleave a portion of the toxin produced. The production of BoNT/X polypeptides using these strains are contemplated herein.

[0164] The exact proportion of nicked to un-nicked molecules depends on the length of incubation and the temperature of the culture. Therefore, a certain percentage of a preparation of, for example, the botulinum toxin type B toxin may be inactive. In one embodiment, the neurotoxin of the present disclosure is in an active state. In one embodiment, the neurotoxin is in an inactive state. In one embodiment, a combination of active and inactive neurotoxin is envisioned.

[0165] One aspect of the present disclosure provides novel methods of producing BoNTs via an *in vitro* transpeptidase reaction that ligates two non-toxic fragments of BoNTs. Such methods comprise the steps of: (i) obtaining a first BoNT fragment comprising a light chain (LC) and a N-terminal domain of a heavy chain (H_N), wherein the first BoNT fragment comprises a C-terminal LPXTGG (SEQ ID NO: 60) motif; (ii) obtaining a second BoNT fragment comprising a C-terminal domain of the heavy chain (HC); wherein the second BoNT fragment comprise a specific protease cleavage site at its N-terminus; (iii) cleaving the second BoNT fragment with a specific protease, wherein the cleavage results in a free glycine residue at the N-terminus; and (iv) contacting the first BoNT fragment and the second BoNT fragment in the presence of a transpeptidase, thereby ligating the first BoNT fragment and the second BoNT fragment to form a ligated BoNT.

[0166] In some embodiments, the first BoNT fragment comprises the X-LC-H_N polypeptide described herein fused to a C-terminal LPXTGG (SEQ ID NO: 60) motif *(e.g.,* SEQ ID NO: 45), or any variants thereof. In some embodiments, the second BoNT fragment comprises the H_C polypeptide described herein, or any variants thereof *(e.g.,* SEQ ID NO: 46). It is to be understood that any BoNT fragments or domains may be ligated using the methods described herein.

[0167] The methods described herein may also be used to generate chimeric BoNTs. For example, the first BoNT fragment may be from BoNT serotype A, B, C, D, E, F, G, or X. Similarly, the second BoNT fragment may be from BoNT serotype A, B, C, D, E, F, G, or X. One skilled in the art will be able to discern the combinations that may be made. In some embodiments, the chimeric BoNT polypeptides described herein (e.g., BoNT/X-LC-H_N-A1-H_C, BoNT/X-LC-H_N-B1-H_C, or BoNT/X-LC-H_N-C1-H_C) are made using this method.

[0168] In some embodiments, the transpeptidase is a sortase. In some embodiments, the sortase is from Staphylococcus aureus (SrtA).

[0169] Other peptide ligation systems available in the art may also be used to ligate two non-toxic BoNT fragments. For example, an intein-mediated protein ligation reaction allows the ligation of a synthetic peptide or a protein with an N-terminal cysteine residue to the C-terminus of a bacterially expressed protein through a native peptide bond (Evans et al., (1998) Protein

Sci.7, 2256-2264, Dawson et al.,(1994)Science266, 776-779; Tam et al., (1995) Proc. Natl. Acad. Sci. USA92, 12485-12489, Muir et al.,(1998)Proc. Natl. Acad. Sci. USA95,6705-6710; Severinov and Muir(1998)J. Biol. Chem.273, 16205-16209). Kits are commercially available (e.g., from New England Biolabs) for intern-mediated protein ligation reactions.

[0170] In some embodiments, the first BoNT fragment further comprises an affinity tag. In some embodiments, the affinity tag is fused to first BoNT fragment at the N-terminus. In some embodiments, the affinity tag is fused to the first BoNT fragment at the C-terminus. In the event that the affinity tag is fused to the C-terminus of the first BoNT fragment, the transpeptidase cleaves between the T and G in the LPXTGG (SEQ ID NO: 60) motif and removes the affinity tag before ligating the first BoNT fragment and the second BoNT fragment.

[0171] In some embodiments, the second BoNT fragment further comprises an affinity tag. In some embodiments, the affinity tag is fused to the first BoNT fragment at the N-terminus. In some embodiments, the affinity tag is fused to the second BoNT fragment at the C-terminus. In the event that the affinity tag is fused to the N-terminus of the first BoNT fragment, the specific protease cleaves in the specific protease cleavage site and removes the affinity tag before ligating the first BoNT fragment and the second BoNT fragment by the transpeptidase.

[0172] An "affinity tag," as used herein, refers to a polypeptide sequence that can bind specifically to a substance or a moiety, *e.g.,* a tag comprising six Histidines bind specifically to Ni^{2+}. Affinity tags may be appended to proteins to facilitate their isolation. The affinity tags are typically fused to proteins via recombinant DNA techniques known by those skilled in the art. The use of affinity tags to facilitate protein isolate is also well known in the art. Suitable affinity tags that may be used in accordance with the present disclosure include, without limitation, His6, GST, Avi, Strep, S, MBP, Sumo, FLAG, HA, Myc, SBP, E, Calmodulin, Softag 1, Softag 3, TC, V5, VSV, Xpress, Halo, and Fc.

[0173] The second BoNT fragment has a specific protease cleavage at the N-terminus. Cleavage of the site by the specific protease results to a free glycine residue at the N-terminus of the second BoNT fragment. Suitable specific protease that may be used in accordance with the present disclosure include, without limitation: thrombin, TEV, PreScission, Enterokinase, and SUMO protease. In some embodiments, the specific protease is thrombin, and the cleavage site is : LVPR|GS (SEQ ID NO: 50).

[0174] The BoNT/X polypeptides described herein affords potential for therapeutic use. For example, BoNT/X might be more potent compared to other BoNT serotypes. BoNT/X is more versatile and may be more effective in a wide range of cells due to its ability to cleave more substrates than other BoNT serotypes.

[0175] Thus, the present disclosure also contemplates pharmaceutically compositions comprising the BoNT/X polypeptides or the chimeric molecules of the present disclosure. As it may also become clear later in the present disclosure, the pharmaceutical composition of the present disclosure, may further comprise other therapeutic agents suitable for the specific disease such composition is designed to treat. In some embodiments, the pharmaceutically composition of the present disclosure further comprises pharmaceutically-acceptable carriers.

[0176] The term "pharmaceutically-acceptable carrier", as used herein, means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (e.g., lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the polypeptide from one site *(e.g.,* the delivery site) of the body, to another site *(e.g.,* organ, tissue or portion of the body).

[0177] A pharmaceutically acceptable carrier is "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the tissue of the subject (*e.g.*, physiologically compatible, sterile, physiologic pH, etc.). Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethylcellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol (PEG); (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; (22) C2-C12 alcohols, such as ethanol; and (23) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, coloring agents, release agents, coating agents, sweetening agents, flavoring agents, perfuming agents, preservative and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable carrier" or the like are used interchangeably herein. In some embodiments, a BoNT polypeptide of the present disclosure in a composition is administered by injection, by means of a catheter, by means of a suppository, or by means of an implant, the implant being of a porous, non-porous, or gelatinous material, including a membrane, such as a sialastic membrane, or a fiber.

[0178] Typically, when administering the composition, materials to which the polypeptide of the disclosure does not absorb are used. In other embodiments, the BoNT polypeptides of the present disclosure for use are delivered in a controlled release system. Such compositions and methods for administration are provides in U.S. Patent publication No. 2007/0020295. In one embodiment, a pump may be used (see, *e.g.,* Langer, 1990, Science 249:1527-1533; Sefton, 1989, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980, Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used. (See, *e.g.,* Medical Applications of Controlled Release (Langer and Wise eds., CRC Press, Boca Raton, Fla., 1974); Controlled Drug Bioavailability, Drug Product Design and Performance (Smolen and Ball eds., Wiley, New York, 1984); Ranger and Peppas, 1983, Macromol. Sci. Rev. Macromol. Chem. 23:61. See also Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351; Howard et al., 1989, J. Neurosurg. 71:105.) Other controlled release systems are discussed, for example, in Langer, supra.

[0179] The BoNT polypeptides for use of the present disclosure can be administered as pharmaceutical compositions comprising a therapeutically effective amount of a binding agent and one or more pharmaceutically compatible ingredients. In typical embodiments, the pharmaceutical composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous or subcutaneous administration to a subject, *e.g.,* a human being.

[0180] Typically, compositions for administration by injection are solutions in sterile isotonic aqueous buffer. Where necessary, the pharmaceutical can also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the pharmaceutical is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the pharmaceutical is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients can be mixed prior to administration. A pharmaceutical composition for systemic administration may be a liquid, e.g., sterile saline, lactated Ringer's or Hank's solution. In addition, the pharmaceutical composition can be in solid forms and re-dissolved or suspended immediately prior to use. Lyophilized forms are also contemplated. The pharmaceutical composition can be contained within a lipid particle or vesicle, such as a liposome or microcrystal, which is also suitable for parenteral administration. The particles can be of any suitable structure, such as unilamellar or plurilamellar, so long as compositions are contained therein.

[0181] The polypeptides of the present disclosure can be entrapped in 'stabilized plasmid-lipid particles' (SPLP) containing the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE), low levels (5-10 mol %) of cationic lipid, and stabilized by a polyethyleneglycol (PEG) coating (Zhang Y. P. et al., Gene Ther. 1999, 6:1438-47). Positively charged lipids such as N-[1-(2,3-dioleoyloxi)propyl]-N,N,N-trimethyl-amoniummethylsulfate, or "DOTAP," are particularly preferred for such particles and vesicles. The preparation of such lipid particles is well known. See, *e.g.,* U.S. Patent Nos. 4,880,635; 4,906,477; 4,911,928; 4,917,951; 4,920,016; and 4,921,757. The pharmaceutical compositions of the present disclosure may be administered or packaged as a unit dose, for example.

[0182] The term "unit dose" when used in reference to a pharmaceutical composition of the present disclosure refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; *i.e.,* carrier, or vehicle. In some embodiments, the BoNT/X polypeptides described herein may be conjugated to a therapeutic moiety, *e.g.,* an antibiotic. Tec$H_N$iques for conjugating such therapeutic moieties to polypeptides, including *e.g.,* Fc domains, are well known; see, *e.g.,* Amon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), 1985, pp. 243-56, Alan R. Liss, Inc.); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), 1987, pp. 623-53, Marcel Dekker, Inc.); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), 1985, pp. 475-506); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), 1985, pp. 303-16, Academic Press; and Thorpe et al. (1982) "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates," Immunol. Rev., 62:119-158. Further, the pharmaceutical composition can be provided as a pharmaceutical kit comprising (a) a container containing a polypeptide of the disclosure in lyophilized form and (b) a second container containing a pharmaceutically acceptable diluent (e.g., sterile water) for injection. The pharmaceutically acceptable diluent can be used for reconstitution or dilution of the lyophilized polypeptide of the disclosure. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In another aspect, an article of manufacture containing materials useful for the treatment of the diseases described above is included. In some embodiments, the article of manufacture comprises a container and a label.

[0183] Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. In some embodiments, the container holds a composition that is effective for treating a disease described herein and may have a sterile access port. For example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle. The active agent in the composition is an isolated polypeptide of the disclosure. In some embodiments, the label on or associated with the container indicates that the composition is used for treating the disease of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution, or dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

[0184] The BoNT polypeptides (e.g., BoNT/X polypeptides), the chimeric molecules, and the pharmaceutical compositions of the present disclosure may be used for the treatment of conditions associated with unwanted neuronal activities. Thus, further provided herein are the BoNT polypeptide, the chimeric molecule, or the pharmaceutical composition described herein for use in methods of treating a condition associated with unwanted neuronal activity, the method comprising administering a therapeutically effective amount of the BoNT polypeptide, the chimeric molecule, or the pharmaceutical composition described herein to thereby treat the condition. In some embodiments, the BoNT polypeptides, the chimeric molecules, and the pharmaceutic compositions of the present disclosure contact one or more neuron(s) exhibiting unwanted neuronal activity,

[0185] Conditions typically treated with a neurotoxin (e.g., skeletal muscle conditions, smooth muscle conditions, glandular conditions, a neuromuscular disorder, an autonomic disorder, pain, or an aesthetic/cosmetic condition) are associated with unwanted neuronal activity, as determined by the skilled practitioner. Administration is by a route that contacts an effective amount of the composition to neurons exhibiting the unwanted activity. In some embodiments, the condition may be associated with overactive neurons or glands. Specific conditions envisioned for the BoNT polypeptide, the chimeric molecule, or the pharmaceutical composition described herein for use in treatment by the methods discussed herein include, without limitation, spasmodic dysphonia, spasmodic torticollis, laryngeal dystonia, oromandibular dysphonia, lingual dystonia, cervical dystonia, focal hand dystonia, blepharospasm, strabismus, hemifacial spasm, eyelid disorder, cerebral palsy, focal spasticity and other voice disorders, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia and other muscle tone disorders and other disorders characterized by involuntary movements of muscle groups, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions as well as other secretory disorders, pain from muscle spasms, headache pain. In addition, the present disclosure can be used to treat dermato logical or aesthetic/cosmetic conditions, for example, reduction of brow furrows, reduction of skin wrinkles.

[0186] One unique property of the BoNT/X polypeptides of the present disclosure is its ability to cleave VAMP4, VAMP5, and Ykt6. Thus, further contemplated herein are therapeutic use of the BoNT/X polypeptides in conditions associated with unwanted secretion activities in a wide range of cells. In some embodiments, the unwanted secretion is immune secretion. Conditions associated with unwanted immune secretion include, without limitation: inflammation, psoriasis, allergy, haemophagocytic lymphohistiocytosis, and alcoholic pancreatic disease.

[0187] The present disclosure can also be used in the treatment of sports injuries. Borodic U.S. Pat. No. 5,053,005 discloses methods for treating juvenile spinal curvature, *i.e.* scoliosis, using botulinum type A. In one embodiment, a BoNT polypeptide described herein is for use in substantially similar methods as disclosed by Borodic, a BoNT polypeptide can be administered to a mammal, preferably a human, to treat spinal curvature. In a suitable embodiment, a BoNT polypeptide comprising botulinum type E fused with a leucine-based motif is administered. Even more preferably, a BoNT polypeptide comprising botulinum type A-E with a leucine-based motif fused to the carboxyl terminal of its light chain is administered to the mammal, preferably a human, to treat spinal curvature.

[0188] In addition, the BoNT polypeptides can be administered to treat neuromuscular disorders using well known techniques that are commonly performed with botulinum type A. For example, the present disclosure can be used to treat pain, for example, headache pain, pain from muscle spasms and various forms of inflammatory pain. For example, Aoki U.S. Pat.

No. 5,721,215 and Aoki U.S. Pat. No. 6,113,915 disclose methods of using botulinum toxin type A for treating pain.

[0189] Autonomic nervous system disorders can also be treated with a modified neurotoxin. For example, glandular malfunctioning is an autonomic nervous system disorder. Glandular malfunctioning includes excessive sweating and excessive salivation. Respiratory malfunctioning is another example of an autonomic nervous system disorder. Respiratory malfunctioning includes chronic obstructive pulmonary disease and asthma. Sanders *et al.* disclose methods for treating the autonomic nervous system; for example, treating autonomic nervous system disorders such as excessive sweating, excessive salivation, asthma, etc., using naturally existing botulinum toxins.

[0190] In one embodiment, a BoNT polypeptide described herein is for use in substantially similar methods to that of Sanders *et al.* but using a BoNT polypeptide, to treat autonomic nervous system disorders such as the ones discussed above. For example, a BoNT polypeptide can be locally applied to the nasal cavity of the mammal in an amount sufficient to degenerate cholinergic neurons of the autonomic nervous system that control the mucous secretion in the nasal cavity. Pain that can be treated by a modified neurotoxin includes pain caused by muscle tension, or spasm, or pain that is not associated with muscle spasm. For example, Binder in U.S. Pat. No. 5,714,468 discloses that headache caused by vascular disturbances, muscular tension, neuralgia and neuropathy can be treated with a naturally occurring botulinum toxin, for example Botulinum type A.

[0191] In one embodiment, a BoNT polypeptide described herein is for use in substantially similar methods to that of Binder, but using a BoNT polypeptide described herein, to treat headache, especially the ones caused by vascular disturbances, muscular tension, neuralgia and neuropathy. Pain caused by muscle spasm can also be treated by an administration of a BoNT polypeptide described herein. For example, a botulinum type E fused with a leucine-based motif, preferably at the carboxyl terminal of the botulinum type E light chain, can be administered intramuscularly at the pain/spasm location to alleviate pain. Furthermore, a modified neurotoxin can be administered to a mammal to treat pain that is not associated with a muscular disorder, such as spasm.

[0192] In one broad embodiment, BoNT polypeptide, the chimeric molecule, or the pharmaceutical composition described herein for use in methods of the present disclosure to treat non-spasm related pain include central administration or peripheral administration of the BoNT polypeptide. For example, Foster et al. in U.S. Pat. No. 5,989,545 discloses that a botulinum toxin conjugated with a targeting moiety can be administered centrally (intrathecally) to alleviate pain.

[0193] In one embodiment, compositions described herein are for use in substantially similar methods to that of Foster *et al.* but using the compositions described herein to treat pain. The pain to be treated can be an acute pain or chronic pain. An acute or chronic pain that is not associated with a muscle spasm can also be alleviated with a local, peripheral administration of the modified neurotoxin to an actual or a perceived pain location on the mammal.

[0194] In one embodiment, the BoNT polypeptide for use is administered subcutaneously at or near the location of pain, for example, at or near a cut. In some embodiments, the modified neurotoxin for use is administered intramuscularly at or near the location of pain, for example, at or near a bruise location on the mammal. In some embodiments, the BoNT polypeptide for use is injected directly into a joint of a mammal, for treating or alleviating pain caused by arthritic conditions. Also, frequent repeated injection or infusion of the modified neurotoxin to a peripheral pain location is within the scope of the present disclosure. Routes of administration for such methods are known in the art and easily adapted to the methods described herein by the skilled practitioner (e.g., see for example, Harrison's Principles of Internal Medicine (1998), edited by Anthony Fauci et al., 14.sup.th edition, published by McGraw Hill).

[0195] By way of non-limiting example, the treatment of a neuromuscular disorder can comprise a step of locally administering an effective amount of the molecule to a muscle or a group of muscles, the treatment of an autonomic disorder can comprise a step of locally administering an effective of the molecule to a gland or glands, and the treatment of pain can comprise a step of administering an effective amount of the molecule the site of the pain. In addition, the treatment of pain can comprise a step of administering an effective amount of a modified neurotoxin to the spinal cord.

[0196] "A therapeutically effective amount" as used herein refers to the amount of each therapeutic agent of the present disclosure required to confer therapeutic effect on the subject, either alone or in combination with one or more other therapeutic agents. Effective amounts vary, as recognized by those skilled in the art, depending on the particular condition being treated, the severity of the condition, the individual subject parameters including age, physical condition, size, gender and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a subject may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons. Empirical considerations, such as the half-life, generally will contribute to the determination of the dosage. For example, therapeutic agents that are compatible with the human immune system, such as polypeptides comprising regions from humanized antibodies or fully human antibodies, may be used to prolong half-life of the polypeptide and to prevent the polypeptide being attacked by the host's immune system.

[0197] Frequency of administration may be determined and adjusted over the course of therapy, and is generally, but not necessarily, based on treatment and/or suppression and/or amelioration and/or delay of a disease. Alternatively, sustained continuous release formulations of a polypeptide may be appropriate. Various formulations and devices for achieving sustained release are known in the art. In some embodiments, dosage is daily, every other day, every three days, every four days, every five days, or every six days. In some embodiments, dosing frequency is once every week, every 2 weeks, every 4 weeks, every 5 weeks, every 6 weeks, every 7 weeks, every 8 weeks, every 9 weeks, or every 10 weeks; or once every month, every 2 months, or every 3 months, or longer. The progress of this therapy is easily monitored by conventional techniques and assays.

[0198] The dosing regimen (including the polypeptide used) can vary over time. In some embodiments, for an adult subject of normal weight, doses ranging from about 0.01 to 1000 mg/kg may be administered. In some embodiments, the dose is between 1 to 200 mg. The particular dosage regimen, *i.e.,* dose, timing and repetition, will depend on the particular subject and that subject's medical history, as well as the properties of the polypeptide (such as the half-life of the polypeptide, and other considerations well known in the art).

[0199] For the purpose of the present disclosure, the appropriate dosage of a therapeutic agent as described herein will depend on the specific agent (or compositions thereof) employed, the formulation and route of administration, the type and severity of the disease, whether the polypeptide is administered for preventive or therapeutic purposes, previous therapy, the subject's clinical history and response to the antagonist, and the discretion of the attending physician. Typically the clinician will administer a polypeptide until a dosage is reached that achieves the desired result.

[0200] Administration of one or more polypeptides can be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of a polypeptide may be essentially continuous over a preselected period of time or may be in a series of spaced dose, *e.g.,* either before, during, or after developing a disease. As used herein, the term "treating" refers to the application or administration of a polypeptide or composition including the polypeptide to a subject in need thereof.

[0201] "A subject in need thereof", refers to an individual who has a disease, a symptom of the disease, or a predisposition toward the disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptom of the disease, or the predisposition toward the disease. In some embodiments, the subject has CDI. In some embodiments, the subject has cancer. In some embodiments, the subject is a mammal. In some embodiments, the subject is a non-human primate. In some embodiments, the subject is human. Alleviating a disease includes delaying the development or progression of the disease, or reducing disease severity. Alleviating the disease does not necessarily require curative results.

[0202] As used therein, "delaying" the development of a disease means to defer, hinder, slow, retard, stabilize, and/or postpone progression of the disease. This delay can be of varying lengths of time, depending on the history of the disease and/or individuals being treated. A method that "delays" or alleviates the development of a disease, or delays the onset of the disease, is a method that reduces probability of developing one or more symptoms of the disease in a given time frame and/or reduces extent of the symptoms in a given time frame, when compared to not using the method. Such comparisons are typically based on clinical studies, using a number of subjects sufficient to give a statistically significant result.

[0203] "Development" or "progression" of a disease means initial manifestations and/or ensuing progression of the disease. Development of the disease can be detectable and assessed using standard clinical techniques as well known in the art. However, development also refers to progression that may be undetectable. For purpose of this disclosure, development or progression refers to the biological course of the symptoms. "Development" includes occurrence, recurrence, and onset.

[0204] As used herein "onset" or "occurrence" of a disease includes initial onset and/or recurrence. Conventional methods, known to those of ordinary skill in the art of medicine, can be used to administer the isolated polypeptide or pharmaceutical composition to the subject, depending upon the type of disease to be treated or the site of the disease. This composition can also be administered via other conventional routes, e.g., administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir.

[0205] The term "parenteral" as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional, and intracranial injection or infusion techniques. In addition, it can be administered to the subject via injectable depot routes of administration such as using 1-, 3-, or 6-month depot injectable or biodegradable materials and methods.

[0206] As used herein, a "subject" refers to a human or animal. Usually the animal is a vertebrate such as a primate, rodent, domestic animal or game animal. Primates include chimpanzees, cynomologous monkeys, spider monkeys, and macaques, *e.g.*, Rhesus. Rodents include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include cows, horses, pigs, deer, bison, buffalo, feline species, *e.g.*, domestic cat, canine species, *e.g.,* dog, fox, wolf, avian species, *e.g.,* chicken, emu, ostrich, and fish, *e.g.,* trout, catfish and salmon. Patient or subject includes any subset of the foregoing, *e.g.,* all of the above, but excluding one or more groups or species such as humans, primates or rodents. In certain embodiments of the aspects described herein, the subject is a mammal, *e.g.*, a primate, *e.g.,* a human.

[0207] The terms, "patient" and "subject" are used interchangeably herein. A subject can be male or female. A subject can be a fully developed subject (*e.g.*, an adult) or a subject undergoing the developmental process (*e.g.,* a child, infant or fetus). Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow. Mammals other than humans can be advantageously used as subjects that represent animal models of disorders associated with unwanted neuronal activity. In addition, the methods and compositions described herein can be used to treat domesticated animals and/or pets.

[0208] The following examples are intended to be illustrative of certain embodiments and are non-limiting.

EXAMPLES

[0209]

Table 1 BoNT Polypeptide Sequences

SEQ ID NO.	Description	Sequence
1	WT BoNT/X	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP
		DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIK IVTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
2	WT BoNT/X LC-H_N	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN

SEQ ID NO.	Description	Sequence
		LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEI
3	WT BoNT/X LC	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKN
4	WT BoNT/X C461S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPSSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI
		GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
5	WT BoNT/X C461A	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPASLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI

SEQ ID NO.	Description	Sequence
		VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
6	WT BoNT/X C467S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGSIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA
		WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
7	WT BoNT/X C467A	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGAIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED

SEQ ID NO.	Description	Sequence
8	WT BoNT/X C1240S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYSQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
9	WT BoNT/X C1240S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY
		VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYAQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED

SEQ ID NO.	Description	Sequence
10	WT BoNT/X C461S/C1240 A	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPSSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYAQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
11	WT BoNT/X C461S/C1240 S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPSSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS
		SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYSQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
12	WT BoNT/X	

SEQ ID NO.	Description	Sequence
	C461A/C124 OS	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPASLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYSQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
13	WT BoNT/X C461A/C124 0A	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPASLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK
		IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYAQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
14	WT BoNT/X C467S/C1240 A	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP

SEQ ID NO.	Description	Sequence
		EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGSIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYAQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
15	WT BoNT/X C467S/C1240 S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGSIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK
		VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYSQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
16	WT BoNT/X C467A/C124 OS	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP

SEQ ID NO.	Description	Sequence
		DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGAIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYSQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
17	WT BoNT/X C467A/C124 0A	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGAIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGK IKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNF SISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKI VTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRL KNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYN KKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSK VLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDL TTDFEIIQRQEKYRNYAQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSA WYFQNYENLNLRKHTKTNWYFIPKDEGWDED
18	WT BoNT/X LC-H_N C461A	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP
		DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP

SEQ ID NO.	Description	Sequence
		ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPASLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEI
19	WT BoNT/X LC-H_N C461S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPSSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEI
20	WT BoNT/X LC-H_N C467A	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGAIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEI
21	WT BoNT/X LC-H_N C467S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGSIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS

SEQ ID NO.	Description	Sequence
		SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI
		GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEI
22	BoNT/X-LC-H_N-A1-H_C	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIIINTSILNLRYESNHL IDLSRYASKINIGSKVNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFS TSFWIRIPKYFNSISLNNEYTIINCMENNSGWKVSLNYGEIIWTLQDTQEIKQ RVVFKYSQMINISDYINRWIFVTITNNRLNNSKIYINGRLIDQKPISNLGNIHA SNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFW GDYLQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYL NSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAG VEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGF HQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWGERPL
23	BoNT/X-LC-H_N-B1-Hc	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIILNNIILNLRYKDNN LIDLSGYGAKVEVYDGVELNDKNQFKLTSSANSKIRVTQNQNIIFNSVFLDF SVSFWIRIPKYKNDGIQNYIHNEYTIINCMKNNSGWKISIRGNRIIWTLIDING KTKSVFFEYNIREDISEYINRWFFVTITNNLNNAKIYINGKLESNTDIKDIREVI ANGEIIFKLDGDIDRTQFIWMKYFSIFNTELSQSNIEERYKIQSYSEYLKDFW GNPLMYNKEYYMFNAGNKNSYIKLKKDSPVGEILTRSKYNQNSKYINYRDL YIGEKFIIRRKSNSQSINDDIVRKEDYIYLDFFNLNQEWRVYTYKYFKKEEEK LFLAPISDSDEFYNTIQIKEYDEQPTYSCQLLFKKDEESTDEIGLIGIHRFYESG IVFEEYKDYFCISKWYLKEVKRKPYNLKLGCNWQFIPKDEGWTE

SEQ ID NO.	Description	Sequence
24	BoNT/X-LC-H_N-C1-H_C	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY
		VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIINDSKILSLQNRKNT LVDTSGYNAEVSEEGDVQLNPIFPFDFKLGSSGEDRGKVIVTQNENIVYNSM YESFSISFWIRINKWVSNLPGYTIIDSVKNNSGWSIGIISNFLVFTLKQNEDSE QSINFSYDISNNAPGYNKWFFVTVTNNMMGNMKIYINGKLIDTIKVKELTGI NFSKTITFEINKIPDTGLITSDSDNINMWIRDFYIFAKELDGKDINILFNSLQYT NVVKDYWGNDLRYNKEYYMVNIDYLNRYMYANSRQIVFNTRRNNNDFNE GYKIIIKRIRGNTNDTRVRGGDILYFDMTINNKAYNLFMKNETMYADNHST EDIYAIGLREQTKDINDNIIFQIQPMNNTYYYASQIFKSNFNGENISGICSIGTY RFRLGGDWYRHNYLVPTVKQGNYASLLESTSTHWGFVPVSE
25	BoNT/X-LC-H_N-A1-H_C C461S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPSSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIIINTSILNLRYESNHL IDLSRYASKINIGSKVNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFS TSFWIRIPKYFNSISLNNEYTIINCMENNSGWKVSLNYGEIIWTLQDTQEIKQ RVVFKYSQMINISDYINRWIFVTITNNRLNNSKIYINGRLIDQKPISNLGNIHA SNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFW GDYLQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYL NSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAG VEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGF HQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWGERPL
26	BoNT/X-LC-H_N-B1-Hc C461S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP

SEQ ID NO.	Description	Sequence
		DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIP VQGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIY VNILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNG LLYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPSSLLNGCIEVENKDLFLISN KDSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILER NEELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEAL SNPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKS SDTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVI GGELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHT YKALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAM KNTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTN
		LSSSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIILNNIILNLRYKDNN LIDLSGYGAKVEVYDGVELNDKNQFKLTSSANSKIRVTQNQNIIFNSVFLDF SVSFWIRIPKYKNDGIQNYIHNEYTIINCMKNNSGWKISIRGNRIIWTLIDING KTKSVFFEYNIREDISEYINRWFFVTITNNLNNAKIYINGKLESNTDIKDIREVI ANGEIIFKLDGDIDRTQFIWMKYFSIFNTELSQSNIEERYKIQSYSEYLKDFW GNPLMYNKEYYMFNAGNKNSYIKLKKDSPVGEILTRSKYNQNSKYINYRDL YIGEKFIIRRKSNSQSINDDIVRKEDYIYLDFFNLNQEWRVYTYKYFKKEEEK LFLAPISDSDEFYNTIQIKEYDEQPTYSCQLLFKKDEESTDEIGLIGIHRFYESG IVFEEYKDYFCISKWYLKEVKRKPYNLKLGCNWQFIPKDEGWTE
27	BoNT/X-LC-H_N-C1-H_C C461S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPSSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIINDSKILSLQNRKNTLV DTSGYNAEVSEEGDVQLNPIFPFDFKLGSSGEDRGKVIVTQNENIVYNSMYE SFSISFWIRINKWVSNLPGYTIIDSVKNNSGWSIGIISNFLVFTLKQNEDSEQSI NFSYDISNNAPGYNKWFFVTVTNNMMGNMKIYINGKLIDTIKVKELTGINFS KTITFEINKIPDTGLITSDSDNINMWIRDFYIFAKELDGKDINILFNSLQYTNVV KDYWGNDLRYNKEYYMVNIDYLNRYMYANSRQIVFNTRRNNNDFNEGYK IIKRIRGNTNDTRVRGGDILYFDMTINNKAYNLFMKNETMYADNHSTEDIY AIGLREQTKDINDNIIFQIQPMNNTYYYASQIFKSNFNGENISGICSIGTYRFRL GGDWYRHNYLVPTVKQGNYASLLESTSTHWGFVPVSE
28	BoNT/X-LC-H_N-A1-H_C C467S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV

SEQ ID NO.	Description	Sequence
		NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGSIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIIINTSILNLRYESNHLID LSRYASKINIGSKVNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTS FWIRIPKYFNSISLNNEYTIINCMENNSGWKVSLNYGEIIWTLQDTQEIKQRV VFKYSQMINISDYINRWIFVTITNNRLNNSKIYINGRLIDQKPISNLGNIHASN NIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFWG DYLQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYLN SSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFH
		QFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWGERPL
29	BoNT/X-LC-H_N-B1-Hc C467S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGSIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIILNNIILNLRYKDNNLID LSGYGAKVEVYDGVELNDKNQFKLTSSANSKIRVTQNQNIIFNSVFLDFSVS FWIRIPKYKNDGIQNYIHNEYTIINCMKNNSGWKISIRGNRIIWTLIDINGKTK SVFFEYNIREDISEYINRWFFVTITNNLNNAKIYINGKLESNTDIKDIREVIANG EIIFKLDGDIDRTQFIWMKYFSIFNTELSQSNIEERYKIQSYSEYLKDFWGNPL MYNKEYYMFNAGNKNSYIKLKKDSPVGEILTRSKYNQNSKYINYRDLYIGE KFIIRRKSNSQSINDDIVRKEDYIYLDFFNLNQEWRVYTYKYFKKEEEKLFLA PISDSDEFYNTIQIKEYDEQPTYSCQLLFKKDEESTDEIGLIGIHRFYESGIVFE EYKDYFCISKWYLKEVKRKPYNLKLGCNWQFIPKDEGWTE
30	BoNT/X-LC-H_N-C1-H_C C467S	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGSIEVENKDLFLISNK

SEQ ID NO.	Description	Sequence
		DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIINDSKILSLQNRKNTLV DTSGYNAEVSEEGDVQLNPIFPFDFKLGSSGEDRGKVIVTQNENIVYNSMYE SFSISFWIRINKWVSNLPGYTIIDSVKNNSGWSIGIISNFLVFTLKQNEDSEQSI NFSYDISNNAPGYNKWFFVTVTNNMMGNMKIYINGKLIDTIKVKELTGINFS KTITFEINKIPDTGLITSDSDNINMWIRDFYIFAKELDGKDINILFNSLQYTNVV KDYWGNDLRYNKEYYMVNIDYLNRYMYANSRQIVFNTRRNNNDFNEGYK IIIKRIRGNTNDTRVRGGDILYFDMTINNKAYNLFMKNETMYADNHSTEDIY AIGLREQTKDINDNIIFQIQPMNNTYYYASQIFKSNFNGENISGICSIGTYRFRL GGDWYRHNYLVPTVKQGNYASLLESTSTHWGFVPVSE
31	BoNT/X R360A/Y363 F	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV
		KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVAKHFLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGKIK DLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNFSIS FWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKIVT PDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRLKN NRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYNKK YYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSKVLI KNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDLTTD FEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSAWYF QNYENLNLRKHTKTNWYFIPKDEGWDED
32	BoNT/X H227Y	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMYELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS

SEQ ID NO.	Description	Sequence
		DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGKIK DLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNFSIS FWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKIVT PDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRLKN NRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYNKK YYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSKVLI KNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDLTTD FEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSAWYF QNYENLNLRKHTKTNWYFIPKDEGWDED
33	BoNT/X E228Q	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHQLVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS
		NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGKIK DLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNFSIS FWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKIVT PDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRLKN NRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYNKK YYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSKVLI KNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDLTTD FEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSAWYF QNYENLNLRKHTKTNWYFIPKDEGWDED
34	BoNT/X H231Y	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVYVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS

SEQ ID NO.	Description	Sequence
		NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGKIK DLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNFSIS FWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKIVT PDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRLKN NRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYNKK YYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTITFPNNIRYGALYNGSKVLI KNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNEDTNYIGTTYGTTHDLTTD FEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSKPTFHDYRDWVYSSAWYF QNYENLNLRKHTKTNWYFIPKDEGWDED
35	BoNT/X-LC-H_N R360A/Y363 F	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVAKHFLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEI
36	BoNT/X-LC-H_N H227Y	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP
		EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMYELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEI
37	BoNT/X-LC-H_N E228Q	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHQLVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFE ELLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV

SEQ ID NO.	Description	Sequence
		NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEI
38	BoNT/X-LC-H_N H231Y	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVYVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEI
39	VAMP 1	MSAPAQPPAEGTEGTAPGGGPPGPPPNMTSNRRLQQTQAQVEEVVDIIRVN VDKVLERDQKLSELDDRADALQAGASQFESSAAKLKRKYWWKNCKMMIM LGAICAIIVVVIVIYFFT
40	VAMP2	MSATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNV DKVLERDQKLSELDDRADALQAGASQFETSAAKLKRKYWWKNLKMMIIL GVICAIILIIIIVYFSS
41	VAMP3	MSTGVPSGSSAATGSNRRLQQTQNQVDEVVDIMRVNVDKVLERDQKLSEL DDRADALQAGASQFETSAAKLKRKYWWKNCKMWAIGISVLVIIVIIIIVWC VS
42	VAMP4	MPPKFKRHLNDDDVTGSVKSERRNLLEDDSDEEEDFFLRGPSGPRFGPRND KIKHVQNQVDEVIDVMQENITKVIERGERLDELQDKSESLSDNATAFSNRSK QLRRQMWWRGCKIKAIMALVAAILLLVIIILIVMKYRT
43	VAMP5	MAGIELERCQQQANEVTEIMRNNFGKVLERGVKLAELQQRSDQLLDMSSTF NKTTQNLAQKKCWENIRYRICVGLVVVGVLLIILIVLLVVFLPQSSDSSSAPR TQDAGIASGPGN
44	Ykt6	

SEQ ID NO.	Description	Sequence
		MKLYSLSVLYKGEAKVVLLKAAYDVSSFSFFQRSSVQEFMTFTSQLIVERSS KGTRASVKEQDYLCHVYVRNDSLAGVVIADNEYPSRVAFTLLEKVLDEFSK QVDRIDWPVGSPATIHYPALDGHLSRYQNPREADPMTKVQAELDETKIILHN TMESLLERGEKLDDLVSKSEVLGTQSKAFYKTARKQNSCCAII
45	BoNT/X-LC-H_N-LPETGG	MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPER YNFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKP EGEKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGP DIANNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFV KREFAPDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEE LLTFGGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPV QGRLGNFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYV NILDDNSYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGL LYNAIYRNSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNK DSLNDINLSEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERN EELYEPIRNSLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALS NPNKVYSPFKNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSS DTLAIVPYIGPLLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIG GELAREQVEAIVNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTY KALSRQANAIKMNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMK NTEKFMIKLSNSYLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLS SSLRRKVSIRLNKNIAFDINDIPFSEFDDLINQYKNEILPETGG
46	G-BoNT/X-Hc	GEDYEVLNLGAEDGKIKDLSGTTSDINIGSDIELADGRENKAIKIKGSENSTIK IAMNKYLRFSATDNFSISFWIKHPKPTNLLNNGIEYTLVENFNQRGWKISIQD SKLIWYLRDHNNSIKIVTPDYIAFNGWNLITITNNRSKGSIVYVNGSKIEEKDI SSIWNTEVDDPIIFRLKNNRDTQAFTLLDQFSIYRKELNQNEVVKLYNYYFN SNYIRDIWGNPLQYNKKYYLQTQDKPGKGLIREYWSSFGYDYVILSDSKTIT FPNNIRYGALYNGSKVLIKNSKKLDGLVRNKDFIQLEIDGYNMGISADRFNE DTNYIGTTYGTTHDLTTDFEIIQRQEKYRNYCQLKTPYNIFHKSGLMSTETSK PTFHDYRDWVYSSAWYFQNYENLNLRKHTKTNWYFIPKDEGWDED
47	BoNT/A1-Hc	IINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQLFNLESSKIEVIL KNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIINCMENNSGWKVSLNYG EIIWTLQDTQEIKQRVVFKYSQMINISDYINRWIFVTITNNRLNNSKIYINGRLI DQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLY DNQSNSGILKDFWGDYLQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLK GPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNK EYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQ DNNGNDIGFIGFHQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWG ERPL
48	BoNT/B1-Hc	ILNNIILNLRYKDNNLIDLSGYGAKVEVYDGVELNDKNQFKLTSSANSKIRV TQNQNIIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIINCMKNNSGWKIS IRGNRIIWTLIDINGKTKSVFFEYNIREDISEYINRWFFVTITNNLNNAKIYING KLESNTDIKDIREVIANGEIIFKLDGDIDRTQFIWMKYFSIFNTELSQSNIEERY KIQSYSEYLKDFWGNPLMYNKEYYMFNAGNKNSYIKLKKDSPVGEILTRSK YNQNSKYINYRDLYIGEKFIIRRKSNSQSINDDIVRKEDYIYLDFFNLNQEWR VYTYKYFKKEEEKLFLAPISDSDEFYNTIQIKEYDEQPTYSCQLLFKKDEEST DEIGLIGIHRFYESGIVFEEYKDYFCISKWYLKEVKRKPYNLKLGCNWQFIPK DEGWTE
49	BoNT/C1-Hc	INDSKILSLQNRKNTLVDTSGYNAEVSEEGDVQLNPIFPFDFKLGSSGEDRGK VIVTQNENIVYNSMYESFSISFWIRINKWVSNLPGYTIIDSVKNNSGWSIGIIS

SEQ ID NO.	Description	Sequence
		NFLVFTLKQNEDSEQSINFSYDISNNAPGYNKWFFVTVTNNMMGNMKIYIN GKLIDTIKVKELTGINFSKTITFEINKIPDTGLITSDSDNINMWIRDFYIFAKEL DGKDINILFNSLQYTNVVKDYWGNDLRYNKEYYMVNIDYLNRYMYANSR QIVFNTRRNNNDFNEGYKIIIKRIRGNTNDTRVRGGDILYFDMTINNKAYNLF
		MKNETMYADNHSTEDIYAIGLREQTKDINDNIIFQIQPMNNTYYYASQIFKS NFNGENISGICSIGTYRFRLGGDWYRHNYLVPTVKQGNYASLLESTSTHWGF VPVSE
50	Thrombin cleavage site	LVPR|GS
51	TEV	ENLYFQ|G
52	PreScission cleavage site	LEVLFQ|GP
53	Factor Xa cleavage site	IEGR|
54	Factor Xa cleavage site	IDGR|
55	Enterokinase cleavage site	DDDDK|
56	SUMO protease cleavage site	AHREQIGGI
*mutations are indicated by underlining		

A Novel Botulinum Neurotoxin and Its Derivatives

[0210] Botulinum neurotoxins (BoNTs) are among the most dangerous potential bioterrorism agents and are also used clinically to treat a growing list of medical conditions. There are seven serotypes of BoNTs (BoNT/A-G) known to date, and no new types have been recognized for the past 45 years. Ggenomic database searching of *clostridium botulinum* strains revealed a novel BoNT type, named BoNT/X. This toxin showed the lowest sequence identity with other BoNTs and it is not recognized by antisera raised against known BoNT types. It cleaves vesicle associated membrane protein (VAMP) in neurons, which is also the target of BoNT/B/D/F/G, but BoNT/X cleaves at a site (between Arg66-Ala67 on VAMP2) unique to this toxin. To validate the activity of BoNT/X, a limited amount of full-length BoNT/X were assembled by covalently linking two non-toxic fragments of BoNT/X using a transpeptidase (sortase). Assembled BoNT/X entered cultured neurons and cleaved VAMP2, and caused flaccid paralysis in mice measured by Digit Abduction Score assay. Together, these data established BoNT/X as a novel BoNT type. Its discovery poses an urgent challenge for developing effective countermeasures and also presents a novel tool for potential therapeutic applications.

Searching genomic databases revealed a novel BoNT gene

[0211] In an attempt to survey the evolutionary landscape of BoNTs, iterative Hidden Markov model searches of the PubMed sequence database were performed, utilizing sequences of the seven BoNTs as probes. The search successfully identified major BoNT serotypes, subtypes, and mosaic toxins, as well as related tetanus neurotoxin (TeNT) (FIG. 5). Unexpectedly, it also revealed a novel BoNT gene (GenBankNo. BAQ 12790.1), from the recently reported genomic sequence of *Clostridium botulinum* strain 111. This toxin gene is herein designated as BoNT/X.

[0212] Phylogenetic analysis revealed that BoNT/X is clear distinct from all other BoNTs and TeNT (FIG. 1A). It has the least protein sequence identity (<31%) from any other BoNTs among pair-wise comparisons within BoNT/TeNT family (FIG. 1A). For instance, BoNT/A and BoNT/B share 39% sequence identity, and BoNT/B and BoNT/G have 58% sequence identity. Furthermore, a sliding sequence comparison window demonstrated that the low similarity is evenly distributed along BoNT/X sequence as compared to the other seven BoNTs and TeNT (FIG. 1B), indicating that it is not a mosaic toxin.

[0213] Despite the low sequence identity, the overall domain arrangement and a few key features of BoNTs appear to be conserved in BoNT/X (FIG. 1B), including: (1) a conserved zinc-dependent protease motif HExxH (residues 227-231, HELVH (SEQ ID NO: 92)) is located in the putative LC; (2) there are two conserved cysteines located at the border between the putative LC and HC, which may form the essential inter-chain disulfide bond; (3) a conserved receptor binding motif SxWY exists in the putative H_C (residues 1274-1277, SAWY (SEQ ID NO: 93)), which recognizes lipid co-receptor gangliosides [43,44].

[0214] As expected, BoNT/X gene is preceded with a putative NTNHA gene (FIG. 1C). They are located in an OrfX gene cluster. However, the OrfX gene cluster of BoNT/X has two unique features compared to the other two known OrfX clusters (FIG. 1C): (1) there is an additional OrfX2 protein (designated as OrfX2b) located next to the BoNT/X gene, which has not been reported for any other OrfX clusters; (2) the reading frame of OrfX genes has the same direction with the BoNT/X gene, while they are usually opposite to the direction of BoNT gene in other OrfX clusters (FIG. 1C). Together, these features suggest that BoNT/X may constitute a unique evolutionary branch of the BoNT family.

The LC of BoNT/X cleaves VAMP2 at a novel site

[0215] Whether BoNT/X is a functional toxin was next examined. First, the LC of BoNT/X (X-LC) was investigated. The border of the LC (residues 1-439) was determined by sequence alignment with other BoNTs. The cDNA encoding the LC was synthesized and the LC was produced as a His6-tagged recombinant protein in *E.coli.* X-LC was incubated with rat brain detergent extracts (BDE) and immunoblot analysis was used to examine whether the three dominant SNARE proteins in the brain, SNAP-25, VAMP2, and syntaxin 1, were cleaved. LCs of BoNT/A (A-LC) and BoNT/B (B-LC) were assayed in parallel as controls. Cleavage of SNAP-25 by BoNT/A generates a smaller fragment that can still be recognized on immunoblot, while cleavage of VAMP2 by BoNT/B abolishes the immunoblot signal of VAMP2 (FIG. 2A). Synaptophysin (Syp), a synaptic vesicle protein, was also detected as an internal loading control. Incubation of X-LC with rat brain DTE did not affect syntaxin 1 or SNAP-25, but abolished VAMP2 signals (FIG. 2A). LCs of BoNTs are zinc-dependent proteases [25]. As expected, EDTA prevented cleavage of SNARE proteins by X-, A-, and B-LCs (FIG. 2A). To further confirm that X-LC cleaves VAMP2, the cytosolic domain of VAMP2 (residues 1-96) as a His6-tagged protein was purified. Incubation of VAMP2 (1-96) with X-LC converted the VAMP2 band into two lower molecular weight bands on SDS-PAGE gel (FIG. 2B), confirming that X-LC cleaves VAMP2.

[0216] To identify the cleavage site on VAMP2, the VAMP2 (1-96) protein was analyzed with or without pre-incubation with X-LC, by liquid chromatography-tandem mass spectrometry (LC-MS/MS, FIGs. 2C-2E, see below for detail). A single dominant peptide peak appeared after incubation with X-LC (FIGs. 2C, 2E, and 6). Its molecular weight was determined to be 3081.7, which fits only the peptide sequence of residues A67-L96 of VAMP2 (FIGs. 2C, 2E). Consistently, the other fragment from the beginning of the His6-tag to the residue R66 of VAMP2 was also detected (FIG. 2D). To further confirm this result, the assay was repeated with a different VAMP2 fragment: GST-tagged recombinant VAMP2 (33-86) (FIG. 7). Incubation with X-LC generated a single dominant peak, with a molecular weight of 2063.1, which fits only the peptide sequence of residues A67-R86 of VAMP2 (FIGs. 7D-7E). As expected, the other fragment from the beginning of the GST tag to the residue R66 of VAMP2 was also detected (FIG. 7F). Together, these results demonstrated that X-LC has a single cleavage site on VAMP2 between R66 and A67.

[0217] R66-A67 is a novel cleavage site distinct from established target sites for all other BoNTs (FIG. 2F). It is also the only BoNT cleavage site located within a region previously known as SNARE motif (FIG. 2F, shaded regions) [45]. VAMP family proteins include VAMP1, 2, 3, 4, 5, 7, 8, as well as related Sec22b and Ykt6. R66-A67 is conserved in VAMP1 and VAMP3, which are highly homologous to VAMP2, but not in other VAMP homologs such as VAMP7 and VAMP8. To validate the specificity of X-LC, HA-tagged full-length VAMP1, 3, 7, 8 and myc-tagged Sec22b and Ykt6 were expressed in HEK293 cells via transient transfection. Cell lysates were incubated with X-LC (FIG. 2G). Both VAMP1 and 3 were cleaved by X-LC, as evidenced by the shift of immunoblot signal to lower molecular weight, while VAMP7, VAMP8, and Sec22B were resistant to X-LC (FIG. 2G).

[0218] Unexpectedly, Ykt6 was cleaved by X-LC (FIG. 2G). This finding was confirmed using purified GST-tagged Ykt6 fragment, which shifted to a lower molecular weight band after incubation with X-LC (FIG. 2H). The cleavage site was determined to be K173-S174 by mass spectrometry analysis of the intact Ykt6 versus the Ykt6 cleaved by X-LC (FIG. 13A). This is the homologous site to the cleavage site in VAMP2 (FIG. 2F), indicating that the location of the cleavage site is conserved across different VAMPs. Among VAMP members, VAMP4 contains the same pair of residues (K87-S88) at this site as Ykt6. It was found that GST-tagged cytoplasmic domain of VAMP4 was efficiently cleaved by X-LC (FIG. 2I). Consistently, X-LC cleaved native VAMP4 in BDE (FIG. 4J). As a control, Sec22b was not cleaved by X-LC in BDE. In addition, GST-tagged cytoplasmic domain of VAMP5 was also cleaved, although at a slower rate than VAMP2 and VAMP4 (FIG. 2I).The cleavage sites were confirmed to be K87-S88 in VAMP4 and R40-S41 in VAMP5 by mass spectrometry analysis (FIG. 14). Both are the homologous sites to the cleavage site in VAMP2 (FIG. 2F). The ability of X-LC to cleave VAMP4, VAMP5, and Ykt6 is highly unusual, as their sequences are substantially different from VAMP 1/2/3. BoNT/X is the first BoNT can cleave VAMPs beyond the canonical targets VAMP1/2/3[66]. X-LC also cleaved VAMP4 in BDE, and the cleavage was blocked by EDTA (FIG. 2J).

[0219] A remarkable feature of BoNT/X is its unique ability to cleave VAMP4 andYkt6. VAMP4 is widely expressed and is known to mediate vesicle fusion between trans-Golgi network (TGN) and endosomes, as well as homotypic fusion of endosomes [51,60]. Ykt6 is an atypical SNARE without a transmembrane domain [67-70]. It is anchored to membranes via lipidation, which allows dynamic regulation of its membrane association. Ykt6 is an essential protein in yeast, implicated in multiple membrane fusion events including ER-Golgi, intra-Golgi, endosome-Golgi-vacuolar, and autophagesome formation. Its function in mammalian cells remains to be established. BoNTs are traditionally known to be limited to target SNAREs that mediate vesicle exocytosis onto plasma membranes. BoNT/X is the first BoNT that is capable of cleaving SNAREs mediating various intracellular membrane trafficking events.

[0220] Interestingly, both VAMP4 and Ykt6 are enriched in neurons. Recent studies suggested that VAMP4 may also contribute to asynchronous synaptic vesicle exocytosis, enlargeosome exocytosis, and activity-dependent bulk endocytosis (ADBE) in neurons [61-63]. The role of Ykt6 in neurons remains to be established, but it has been shown to suppress the toxicity of a-synuclein in Parkinson's disease models[71-72] . The other substrate of BoNT/X, VAMP5, is mainly expressed in muscle cells and its function remains to be established[64]. BoNT/X will be a powerful tool for investigating VAMP4, Ykt6, and VAMP5 functions and related membrane trafficking events. In addition, VAMP4 has been implicated in granule release in immune cells [65], thus BoNT/X might have a unique potential among all BoNTs to modulate inflammatory secretion in immune cells.

Proteolytic activation of BoNT/X

[0221] BoNTs are initially produced as a single polypeptide. The linker region between LC and H_N needs to be cleaved by either bacterial or host proteases in a process known as "activation", which is essential for the activity of BoNTs. LC and H_N of BoNTs remain connected via an inter-chain disulfide bond prior to translocation of LC into the cytosol of cells, where the disulfide bond is reduced in order to release the LC into the cytosol. Sequence alignment revealed that BoNT/X contains the longest linker region between two conserved cysteines compared to all other BoNTs (C423-C467, FIG. 3A). In addition, the linker region of BoNT/X contains an additional cysteine (C461), which is unique to BoNT/X.

[0222] To examine whether the linker region between the LC and H_N of BoNT/X is susceptible to proteolytic cleavage, a recombinant X-LC-H_N fragment (residues 1-891) was produced in *E.coli* and subjected to limited proteolysis by endoproteinase Lys-C, which cuts at the C-terminal side of lysine residues. To identify the susceptible cleavage site under limited proteolysis conditions, X-LC-H_N was analyzed using Tandem Mass Tag (TMT) labeling and tandem mass spectrometry approach. TMT labels free N-terminus (and lysines). Limited proteolysis by Lys-C produces additional free N-termini, which would not exist in intact X-LC-H_N sample (see below for details). Briefly, intact X-LC-H_N samples were labeled with the light TMT and equal amount of X-LC-H_N samples were exposed to Lys-C and then labeled with the heavy TMT. Both samples were then digested with chymotrypsin, combined together, and subjected to quantitative mass spectrometry analysis. A list of identified peptides was shown in Table 2, below. The light TMT: heavy TMT ratios were usually within 2-fold of each other for each peptide, with the exception for 5 peptides starting with N439, which showed no signal for the light TMT labeling, indicating that this is a new N-terminal generated by Lys-C cutting (FIG. 3A, Table 2). Thus, Lys-C preferentially cuts K438-N439 under limited proteolytic conditions, demonstrating that the linker region is susceptible to proteases (FIG. 3A).

[0223] Whether this proteolytic activation is important for the function of BoNT/X was examined next. It has been previously shown that incubation of high concentrations of LC-H_N of BoNTs with cultured neurons resulted in entry of LC-H_N into neurons, likely through nonspecific uptake into neurons [46,47]. Using this approach, the potency of intact versus activated X-LC-H_N on cultured rat cortical neurons was compared. Neurons were exposed to X-LC-H_N in media for 12 hours. Cell lysates were harvested and immunoblot analysis was carried out to examine cleavage of SNARE proteins. As shown in FIG. 3B, X-LC-H_N entered neurons and cleaved VAMP2 in a concentration-dependent manner. X-LC-H_N activated by Lys-C showed a drastically increased potency than intact X-LC-H_N: 10 nM activated X-LC-H_N cleaved similar levels of VAMP2 as 150 nM intact X-LC-H_N (FIG. 3B). Note that the intact X-LC-H_N is likely susceptible to proteolytic cleavage by cell surface proteases, which is why it is still active on neurons at high concentrations. Interestingly, activated X-LC-H_N appears to be more potent than activated LC-H_N of BoNT/A (A-LC-H_N) and BoNT/B (B-LC-H_N), which did not show any detectable cleavage of their SNARE substrates in neurons under the same assay conditions (FIG. 3B).

Table 2. Peptide fragments of X-LC-H_N under limited proteolysis analyzed by TMT labeling and quantitative mass spectrometry.

His6-tagged recombinant X-LC-H_N was labeled with the light TMT. Equal amount of

X-LC-H_N samples were exposed to Lys-C and then labeled with the heavy TMT. Both samples

X-LC-H_N samples were exposed to Lys-C and then labeled with the heavy TMT. Both samples were then digested with chymotrypsin, combined together, and subjected to quantitative mass spectrometry analysis. A list of identified peptides was shown. The light TMT: heavy TMT ratios are within 2-fold of each other for all peptide, except five peptides (underlined) starting with N439. These five peptides showed no signal for the light TMT labeling, indicating that N439 is a new N-terminal generated by Lys-C cutting. The peptide sequences in Table 2 correspond, from top to bottom, to SEQ ID NOs: 94-226.

ScanF	z	Theo m/z	PPM	X Corr	Δ Corr	Ref.	Peptide	Start Pos	End Pos	Max Heavy	Max Light	H/L ratio
16633	2	611.384	1.46	1.751	0.651	xLcHN	K.L]EINK#F.N	3	8	4.03E+04	2.92E+04	1.38
16638	2	606.3736	0.95	1.71	0.654	xLcHN	K.LEINKF.N	3	8	4.03E+04	2.92E+04	1.38
15946	2	912.456	2.74	4.083	0.877	xLcHN	F.N]YNDPIDGINVITM*.R	9	22	4.34E+05	9.26E+05	0.47
15942	2	909.9508	2.28	4.546	0.661	xLcHN	F.NYNDPIDGINVITM*.R	9	22	4.34E+05	9.26E+05	0.47
17201	2	785.9092	1.26	2.455	0.273	xLcHN	F.NYNDPIDGINVI.T	9	20	1.14E+06	1.94E+06	0.59
11082	2	679.833	1.17	1.74	0.754	xLcHN	F.NYNDPIDGIN.V	9	18	8.01E+05	1.20E+06	0.67
11083	2	682.3382	0.96	2.6	0.742	xLcHN	F.N]YNDPIDGIN.V	9	18	8.01E+05	1.20E+06	0.67
19628	2	535.3264	0.97	1.391	0.701	xLcHN	D.P]IDGINVI.T	13	20	6.55E+04	1.23E+05	0.53
19626	2	532.8211	0.68	1.474	0.608	xLcHN	D.PIDGINVI.T	13	20	6.55E+04	1.23E+05	0.53
20815	4	802.2073	2.19	2.962	0.582	xLcHN	Y.N]PNYLNTPSEK#DEFLQGVIK#VL.E	78	99	4.57E+04	1.21E+05	0.38
20463	4	802.2073	1.96	2.726	0.385	xLcHN	Y.N]PNYLNTPSEK#DEFLQGVIK#VL.E	78	99	2.96E+04	8.18E+04	0.36
20799	4	798.4495	1.91	3.647	0.659	xLcHN	Y.NPNYLNTPSEKDEFLQGVIKVL.E	78	99	4.57E+04	1.21E+05	0.38
20568	4	798.4495	1.63	2.639	0.419	xLcHN	Y.NPNYLNTPSEKDEFLQGVI	78	99	2.96E+04	8.18E+04	0.36

							KVL.E					
22720	2	753.4631	1.96	2.339	0.222	xLcH N	L.LELISSS IPLPL.V	112	123	1.40E+04	2.80E+04	0.50
21170	2	696.9211	1.9	1.781	0.326	xLcH N	L.ELISSSI PLPL.V	113	123	2.75E+04	4.15E+04	0.66
21281	2	696.9211	1.86	2.099	0.282	xLcH N	L.ELISSSI PLPL.V	113	123	2.75E+04	4.15E+04	0.66
21378	2	696.9211	1.83	1.593	0.149	xLcH N	L.ELISSSI PLPL.V	113	123	2.75E+04	4.15E+04	0.66
19246	2	578.363	1.18	1.443	0.27	xLcH N	L.I]SSSIPL PL.V	115	123	1.61E+04	4.12E+04	0.39
19365	2	578.363	1.08	1.624	0.135	xLcH N	L.I]SSSIPL PL.V	115	123	1.61E+04	4.12E+04	0.39
19241	2	575.8577	1	1.484	0.209	xLcH N	L.ISSSIPL PL.V	115	123	1.61E+04	4.12E+04	0.39
19360	2	575.8577	0.91	1.673	0.298	xLcH N	L.ISSSIPL PL.V	115	123	1.61E+04	4.12E+04	0.39

13952	2	948.9912	2.47	2.562	0.54	xLcH N	L.V|SNGA LTLSDNE TIAY.Q	124	139	2.51E+05	3.67E+05	0.68
13949	2	946.486	2.42	1.729	0.599	xLcH N	L.VSNGA LTLSDNE TIAY.Q	124	139	2.51E+05	3.67E+05	0.68
6712	2	392.7318	0.19	1.523	0.206	xLcH N	L.VSNGA L.T	124	129	1.18E+05	2.03E+05	0.58
10243	2	678.3482	0.73	1.499	0.616	xLcH N	L.T]LSDN ETIAY.Q	130	139	2.14E+05	3.67E+05	0.58
10242	2	675.843	0.69	1.594	0.842	xLcH N	L.TLSDNE TIAY.Q	130	139	2.14E+05	3.67E+05	0.58
15890	2	1110.579	2.74	2.243	0.6	xLcH N	L.Q]ANLV IYGPGPDI ANNATY. G	150	168	5.20E+04	9.82E+04	0.53
15881	2	1108.073	1.86	2.121	0.673	xLcH N	L.QANLVI YGPGPDI ANNATY. G	150	168	5.20E+04	9.82E+04	0.53
11142	2	727.3879	1.39	1.945	0.635	xLcH N	L.VIYGPG PDIANN.A	154	165	1.91E+04	3.90E+04	0.49
12879	2	894.962	1.24	2.673	0.731	xLcH N	L.VIYGPG PDIANNA TY.G	154	168	6.62E+05	1.25E+06	0.53
10964	2	707.3541	2.8	1.39	0.466	xLcH N	Y.GPGPDI ANNATY. G	157	168	1.42E+04	2.24E+04	0.63
11091	2	456.2473	0.78	1.352	0.765	xLcH N	N.ATYGL Y.S	166	171	3.55E+04	8.08E+04	0.44
17435	2	1094.055	2.53	3.418	0.69	xLcH	Y.G]LYST	169	187	1.55E+05	2.27E+05	0.68

						N	PISNGEGT LSEVSF.S					
17410	2	1091.549	2.48	3.748	0.738	xLcH N	Y.GLYSTP ISNGEGT LSEVSF.S	169	187	1.55E+05	2.27E+05	0.68
19830	2	1259.631	2.15	2.885	0.676	xLcH N	Y.G]LYST PISNGEGT LSEVSFSP F.Y	169	190	1.98E+04	2.46E+04	0.81
20131	3	838.4197	2.08	2.781	0.742	xLcH N	Y.GLYSTP ISNGEGT LSEVSFSP F.Y	169	190	4.60E+05	5.32E+05	0.86
12546	2	862.9488	1.9	1.817	0.401	xLcH	Y.G]LYST	169	183	7.95E+04	7.83E+04	1.02

						N	PISNGEGT LS.E					
12571	2	860.4436	1.84	1.528	0.588	xLcH N	Y.GLYSTP ISNGEGT LS.E	169	183	7.95E+04	7.83E+04	1.02
19819	3	838.4197	1.61	2.655	0.662	xLcH N	Y.GLYSTP ISNGEGT LSEVSFSP F.Y	169	190	4.13E+04	4.59E+04	0.90
9948	2	851.4307	2.54	2.044	0.765	xLcH N	Y.STPISN GEGTLSE VS.F	172	186	1.92E+04	2.39E+04	0.81
18409	2	1090.541	2.45	4.434	0.844	xLcH N	Y.STPISN GEGTLSE VSFSPF.Y	172	190	8.21E+05	9.22E+05	0.89
18417	2	1090.541	2.39	3.307	0.82	xLcH N	Y.STPISN GEGTLSE VSFSPF.Y	172	190	8.21E+05	9.22E+05	0.89
18418	2	1093.047	2.39	2.983	0.845	xLcH N	Y.S]TPISN GEGTLSE VSFSPF.Y	172	190	8.21E+05	9.22E+05	0.89
18876	2	1093.047	1.97	1.841	0.679	xLcH N	Y.S]TPISN GEGTLSE VSFSPF.Y	172	190	8.21E+05	9.22E+05	0.89
14591	2	927.4701	1.96	3.362	0.646	xLcH N	Y.S]TPISN GEGTLSE VSF.S	172	187	2.09E+05	1.42E+05	1.48
14803	2	924.9649	1.88	1.934	0.462	xLcH N	Y.STPISN GEGTLSE VSF.S	172	187	2.96E+05	4.65E+05	0.64
14852	2	924.9649	1.87	1.538	0.369	xLcH N	Y.STPISN GEGTLSE VSF.S	172	187	2.96E+05	4.65E+05	0.64
18554	2	1093.047	1.72	3.044	0.86	xLcH N	Y.S]TPISN GEGTLSE VSFSPF.Y	172	190	8.21E+05	9.22E+05	0.89
18552	2	1090.541	1.68	3.643	0.813	xLcH N	Y.STPISN GEGTLSE VSFSPF.Y	172	190	8.21E+05	9.22E+05	0.89
14978	2	927.4701	1.68	1.401	0.31	xLcH N	Y.S]TPISN GEGTLSE VSF.S	172	187	4.96E+04	7.37E+04	0.67
18680	2	1093.047	1.66	1.656	0.717	xLcH N	Y.S]TPISN GEGTLSE VSFSPF.Y	172	190	8.21E+05	9.22E+05	0.89
20660	2	1093.047	1.63	2.182	0.831	xLcH	Y.S]TPISN	172	190	5.24E+05	5.44E+05	0.96

						xLcH N	GEGTLSE VSFSPF.Y					
17226	2	924.9649	1.62	2.459	0.698	xLcH	Y.STPISN GEGTLSE	172	187	3.25E+05	5.31E+05	0.61
						N	VSF.S					
17306	2	924.9649	1.62	2.126	0.588	xLcH N	Y.STPISN GEGTLSE VSF.S	172	187	3.25E+05	5.31E+05	0.61
10139	2	696.3644	1.61	1.942	0.628	xLcH N	Y.S]TPISN GEGTLS.E	172	183	6.39E+04	8.46E+04	0.76
18674	2	1090.541	1.59	2.423	0.765	xLcH N	Y.STPISN GEGTLSE VSFSPF.Y	172	190	8.21E+05	9.22E+05	0.89
20642	2	1090.541	1.55	2.608	0.823	xLcH N	Y.STPISN GEGTLSE VSFSPF.Y	172	190	5.24E+05	5.44E+05	0.96
8077	2	696.3644	1.54	1.763	0.707	xLcH N	Y.S]TPISN GEGTLS.E	172	183	1.09E+05	1.29E+05	0.84
18476	2	562.2872	1.17	1.812	0.784	xLcH N	L.SEVSFS PF.Y	183	190	4.49E+04	7.11E+04	0.63
18478	2	564.7924	0.95	1.57	0.965	xLcH N	L.S]EVSFS PF.Y	183	190	4.49E+04	7.11E+04	0.63
13190	3	652.0048	0.97	2.099	0.697	xLcH N	F.Y]LK#PF DESYGNY .R	191	202	1.98E+06	3.09E+06	0.64
12839	3	540.6289	0.38	2.441	0.423	xLcH N	F.Y]LK#PF DESY.G	191	199	2.43E+05	5.45E+05	0.44
7115	2	538.7403	0.69	1.476	0.615	xLcH N	F.D]ESYG NY.R	196	202	1.44E+05	3.14E+05	0.46
16124	3	661.3911	1.14	2.123	0.575	xLcH N	Y.G]NYRS LVNIVNK #F.V	200	212	2.81E+04	2.27E+04	1.24
11646	3	474.5894	0.49	2.077	0.602	xLcH N	H.NLYGIS NRNF.Y	235	244	2.80E+05	4.38E+05	0.64
10091	4	603.8103	1.23	2.98	0.756	xLcH N	F.YYNFD TGKIETSR QQN.S	245	260	3.05E+05	1.94E+05	1.57
9932	4	603.8103	1.08	2.5	0.638	xLcH N	F.YYNFD TGKIETSR QQN.S	245	260	3.05E+05	1.94E+05	1.57
10782	3	820.4359	0.65	2.156	0.438	xLcH N	Y.Y]NFDT GK#IETSR QQNSL.I	246	262	7.40E+04	1.33E+05	0.56
15039	3	819.1317	1.41	2.281	0.516	xLcH N	L.ISERLN TVTVEND LLKY.I	298	314	1.45E+05	2.76E+05	0.53
13824	3	619.6838	1.27	2.021	0.44	xLcH N	L.NTVTV ENDLLKY .I	303	314	3.10E+04	5.07E+04	0.61
10975	2	760.4251	1.45	2.089	0.663	xLcH	F.V]LNES	345	355	4.63E+05	6.60E+05	0.70

						N	NLAQRF.S					
17696	3	966.86	2.14	2.704	0.532	xLcH N	H.Y]LK#E RPIDPIYV NILDDNS Y.S	363	382	8.61E+04	1.42E+05	0.61
20823	2	827.9299	2.21	2.495	0.806	xLcH N	D.P]IYVNI LDDNSY. S	371	382	7.80E+04	8.16E+04	0.96
20825	2	825.4247	2.17	2.958	0.881	xLcH N	D.PIYVNI LDDNSY. S	371	382	7.80E+04	8.16E+04	0.96
12614	2	685.3561	1.34	1.843	0.609	xLcH N	N.I]LDDN SYSTL.E	376	385	1.01E+05	2.57E+05	0.39
11272	2	441.7421	1.48	1.795	0.903	xLcH N	Y.S]TLEG F.N	383	388	4.45E+04	1.36E+05	0.33
11261	2	439.2369	1.08	1.536	0.514	xLcH N	Y.STLEGF .N	383	388	4.45E+04	1.36E+05	0.33
14260	2	441.7421	0.49	1.462	0.848	xLcH N	Y.S]TLEG F.N	383	388	5.41E+04	1.32E+05	0.41
14246	2	439.2369	0.38	1.46	0.844	xLcH N	Y.STLEGF .N	383	388	5.41E+04	1.32E+05	0.41
13808	2	916.4629	2.48	2.036	0.498	xLcH N	F.NISSQG SNDFQGQ LL.E	389	403	2.86E+04	6.39E+04	0.45
17314	3	815.7238	2.05	2.149	0.621	xLcH N	F.NISSQG SNDFQGQ LLESSYF. E	389	408	1.26E+05	2.71E+05	0.47
6340	2	803.3788	0.75	2.554	0.721	xLcH N	F.NISSQG SNDFQGQ .L	389	401	1.03E+04	2.21E+04	0.47
11696	2	493.2814	0.67	1.62	0.518	xLcH N	L.L]YNAI Y.R	429	434	1.19E+05	1.87E+05	0.63
11692	2	490.7762	0.6	1.57	0.118	xLcH N	L.LYNAIY .R	429	434	1.19E+05	1.87E+05	0.63
14021	3	756.4332	2.14	2.305	0.363	xLcH N	K.N]YLNN IDLEDK# K#T.T	439	451	6.85E+05	5.12E+03	133. 95

12997	3	1009.909	1.93	3.044	0.553	xLcH N	K.N]YLNN IDLEDK# K#TTSK#T N.V	439	456	4.24E+05	0.00E+00	#DI V/0!
12932	3	1009.909	1.92	4.251	0.695	xLcH N	K.N]YLNN IDLEDK# K#TTSK#T N.V	439	456	4.24E+05	0.00E+00	#DI V/0!
14003	3	790.1157	1.59	2.894	0.542	xLcH N	K.N]YLNN IDLEDK# K#TT.S	439	452	4.40E+06	1.13E+04	389. 24
13105	4	729.173	1	3	0.599	xLcH N	K.N]YLNN IDLEDK# K#TTSK#T N	439	455	3.39E+06	3.14E+03	1078 .97

11567	3	747.091	1.33	2.711	0.518	xLcH N	N.YLNNID LEDKKTT .S	440	452	1.22E+04	9.52E+04	0.13
11515	3	747.091	1.33	2.729	0.496	xLcH N	N.YLNNID LEDKKTT .S	440	452	1.22E+04	9.52E+04	0.13
17001	2	857.9579	1.99	1.967	0.713	xLcH N	G.CIEVEN KDLF.L	467	476	3.29E+04	1.64E+05	0.20
16337	4	941.793	1.76	2.419	0.135	xLcH N	F.L]ISNK# DSLNDIN LSEEK#IK #PETTVF. F	477	501	1.27E+06	1.06E+06	1.19
16394	4	941.793	1.72	2.19	0.391	xLcH N	F.L]ISNK# DSLNDIN LSEEK#IK #PETTVF. F	477	501	1.27E+06	1.06E+06	1.19
17983	3	993.888	2.4	3.193	0.657	xLcH N	K.D]SLND INLSEEK# IK#PETTV F.F	482	501	3.27E+04	2.59E+03	12.6 4
18031	3	955.5456	2.06	3.607	0.764	xLcH N	D.S]LNDI NLSEEK#I K#PETTV F.F	483	501	4.90E+04	4.12E+04	1.19
11720	2	834.8912	2.14	1.689	0.726	xLcH N	L.SNYDFT EANSIPS.I	514	526	2.91E+04	2.50E+04	1.17
13409	2	1065.513	2.12	4.742	0.769	xLcH N	L.S]NYDF TEANSIPS ISQQ.N	514	530	7.05E+04	7.52E+04	0.94
8896	2	642.7908	1.42	1.851	0.708	xLcH N	L.SNYDFT EAN.S	514	522	2.12E+05	2.27E+05	0.94
8883	2	645.296	1.39	2.362	0.879	xLcH N	L.S]NYDF TEAN.S	514	522	2.12E+05	2.27E+05	0.94
17774	2	1051.044	2.7	1.967	0.9	xLcH N	Y.DFTEA NSIPSISQ QNIL.E	517	533	3.52E+04	3.28E+04	1.07
17438	2	1051.044	2.55	1.944	0.665	xLcH N	Y.DFTEA NSIPSISQ QNIL.E	517	533	9.04E+04	8.08E+04	1.12
12784	2	880.9387	1.87	2.537	0.778	xLcH N	Y.DFTEA NSIPSISQ Q.N	517	530	4.72E+05	5.06E+05	0.93
12779	2	883.4439	1.73	3.423	0.797	xLcH N	Y.D]FTEA NSIPSISQ Q.N	517	530	4.72E+05	5.06E+05	0.93
16126	4	911.7114	1.6	3.934	0.579	xLcH N	Y.DFTEA NSIPSISQ QNILERN EELYEPIR N.S	517	545	5.41E+05	2.02E+05	2.67
12449	2	937.9602	1.56	3.012	0.73	xLcH N	Y.DFTEA NSIPSISQ QN.I	517	531	5.16E+05	5.72E+05	0.90
12151	2	940.4654	1.47	1.476	0.458	xLcH	Y.D]FTEA NSIPSISQ	517	531	4.65E+05	4.96E+05	0.94

						N	QN.I				
20080	3	1106.923	2.52	3.592	0.677	xLcH N	N.S]IPSIS QQNILER NEELYEPI RNSLF.E	523	548	1.86E+04	1.52E+04	1.23
11314	3	700.7002	1.59	2.563	0.763	xLcH N	L.TDSVDE ALSNPNK VY.S	583	597	1.74E+05	1.84E+05	0.95
11315	3	704.0405	1.56	2.444	0.262	xLcH N	L.T]DSVD EALSNPN K#VY.S	583	597	1.74E+05	1.84E+05	0.95
11916	3	700.7002	1.52	2.049	0.508	xLcH N	L.TDSVDE ALSNPNK VY.S	583	597	2.27E+05	2.78E+05	0.82
7902	2	635.3637	0.25	1.478	0.567	xLcH N	L.SNPNK VY.S	591	597	6.79E+05	8.90E+05	0.76
7903	2	640.3742	0.25	1.485	0.282	xLcH N	L.S]NPNK #VY.S	591	597	6.79E+05	8.90E+05	0.76
14536	3	927.1457	1.59	2.027	0.553	xLcH N	Y.S]PFK# NM*SNTI NSIETGIT STY.I	598	618	1.47E+05	8.07E+04	1.83
14487	3	923.8054	1.54	2.35	0.708	xLcH N	Y.SPFKN M*SNTIN SIETGITS TY.I	598	618	1.47E+05	8.07E+04	1.83
13108	3	813.421	2.03	2.046	0.548	xLcH N	F.KNM*S NTINSIET GITSTY.I	601	618	2.41E+06	2.42E+06	1.00
12962	3	813.421	1.98	2.378	0.482	xLcH N	F.KNM*S NTINSIET GITSTY.I	601	618	2.41E+06	2.42E+06	1.00
13079	3	813.421	1.98	2.245	0.617	xLcH N	F.KNM*S NTINSIET GITSTY.I	601	618	2.41E+06	2.42E+06	1.00
13194	2	915.4701	2.12	1.504	0.654	xLcH N	M.S]NTIN SIETGITS TY.I	604	618	1.74E+05	3.22E+05	0.54
13170	2	912.9649	1.99	1.791	0.699	xLcH N	M.SNTINS IETGITST Y.I	604	618	1.74E+05	3.22E+05	0.54
12405	2	812.4274	2.38	1.349	0.781	xLcH N	N.TINSIET GITSTY.I	606	618	2.94E+04	3.41E+04	0.86
14318	3	837.465	1.53	2.439	0.647	xLcH N	F.S]DETG K#IDVIDK #SSDTL.A	632	648	2.23E+04	1.55E+04	1.44
19535	2	586.368	0.52	1.753	0.731	xLcH N	L.A]IVPYI GPL.L	649	657	7.93E+05	1.16E+06	0.69
19564	2	442.268	0.43	1.498	0.892	xLcH N	V.PYIGPL. L	652	657	1.30E+04	2.64E+04	0.49
9998	2	715.396	1.76	2.148	0.33	xLcH N	V.I]GGEL AREQVE. A	699	709	8.53E+04	1.32E+04	6.44
5372	3	494.9536	0.34	2.943	0.626	xLcH	L.SRQAN	754	762	2.50E+05	1.57E+04	15.9

						N	AIKM*.N				7
5363	3	494.9536	0.32	3.357	0.734	xLcH N	L.SRQAN AIKM*.N	754	762	2.50E+05	1.57E+04	15.9 7
17794	2	734.3537	1.89	2.212	0.79	xLcH N	F.SEFDDL INQY.K	879	888	3.00E+05	4.05E+05	0.74
14171	4	757.641	0.97	2.289	0.421	xLcH N	F.DDLINQ YKNEGSI LPETGGL EHH.H	882	904	1.02E+04	3.96E+04	0.26
7676	4	679.3532	1.49	2.624	0.624	xLcH N	Y.KNEGSI LPETGGL EHHHHH H.-	889	908	3.63E+04	3.74E+04	0.97
13193	3	588.3365	1.18	2.307	0.629	xLcH N	Y.KNEGSI LPETGGL. E	889	901	2.81E+05	3.08E+05	0.91
10362	3	677.037	1.15	2.582	0.615	xLcH N	Y.KNEGSI LPETGGL EH.H	889	903	8.01E+05	1.17E+06	0.68
9325	4	542.2943	1.06	2.617	0.58	xLcH N	Y.KNEGSI LPETGGL EHH.H	889	904	2.17E+05	2.96E+05	0.73

Unique feature of the disulfide bond in BoNT/X

[0224] The linker region of BoNT/X contains an additional cysteine (C461), which is unique to BoNT/X. To determine which cysteine forms the disulfide bond connecting the LC and HC, three X-LC-H_N mutants were generated, with each of the three cysteine residues mutated (C423S, C461S, and C467S). These three cysteine mutants, as well as wild type (WT) X-LC-H_N were subjected to limited proteolysis and then analyzed via SDS-PAGE and Coomassie Blue staining, with or without reducing agent DTT (FIG. 3C). It was found that mutating the cysteine on the LC (C423S) resulted in a protein that separated into two ~ 50 kDa bands, with or without DTT, indicating that C423S abolished the inter-chain disulfide bond. In contrast, mutants containing either C461S or C467S showed a single band at 100 kDa in the absence of DTT, which separated into two ~50 kDa bands in the presence of DTT, suggesting that both C461 and C467 on the H_N can form inter-chain disulfide bond with C423 on the LC. Also the X-LC-H_N (C423S) mutant appears to be more susceptible to Lys-C than both C461S and C467S mutants, resulting in further degradation of the protein (FIG. 3C). This result suggests that losing the inter-chain disulfide bond may increase the freedom of the LC and H_N, thus exposing more surface areas. Furthermore, a portion of WT X-LC-H_N formed aggregates at the top of the SDS-PAGE gel (FIG. 3C). These aggregates are due to formation of inter-molecular disulfide bond, as they disappeared in the presence of DTT (FIG. 3C, +DTT). C423, C461 and C467 are the only three cysteines in X-LC-H_N. Mutating any one of three cysteines abolished the X-LC-H_N aggregates (FIG. 3C, -DTT), indicating that formation of inter-molecular disulfide bond is due to existence of an extra cysteine in the linker region.

[0225] The majority of activated WT X-LC-H_N also separated to two ~50 kDa bands on SDS-PAGE gel without DTT (FIG. 3C). On the other hand, WT X-LC-H_N is similarly resistant to Lys-C as C461S and C467S mutants, and it showed no further degradation as C423S mutant did (FIG. 3C, +DTT), suggesting that WT X-LC-H_N is different from C423S mutant. One possible explanation is disulfide bond shuffling due to the existence of two cysteines close to each on the H_N (C461 and C467), which can rearrange the disulfide bond from inter-chain C423-C467 or C423-C467 to intra-chain C461-C467 under denatured conditions [48,49] . To test this hypothesis, an alkylating reagent, *N*-Ethylmaleimide (NEM), which reacts with sulfhydryls of free cysteine and permanently block any free cysteines, was used. As shown in FIG. 3D, WT X-LC-H_N pretreated with NEM showed largely as a single band at 100 kDa in the absence of DTT, and separated into two ~50 kDa bands in the presence of DTT. These results confirmed that native WT X-LC-H_N contains mainly inter-chain disulfide bond, which is susceptible to disulfide bond shuffling due to the existence of the third cysteine in the linker region.

[0226] Finally, the activity of the three X-LC-H_N cysteine mutants on cultured neurons was examined. As shown in FIG. 3E, mutating the cysteine on the LC (C423S) abolished the activity of X-LC-H_N, as evidenced by lack of VAMP2 cleavage in neurons. Mutating one of the two cysteines on the H_N (C461 or C467) did not significantly affect the potency of X-LC-H_N compared to wild type (WT) X-LC-H_N (FIG. 3E). These results confirmed that the interchain disulfide bond is essential for the activity of BoNT/X and demonstrated that functional inter-chain disulfide bond can be formed via either C423-C461 or C423-C467.

Generating full-length BoNT/X via sortase-mediated ligation

[0227] To evaluate whether BoNT/X is a functional toxin, it was necessary to generate and test full-length BoNT/X. However, BoNTs are one of the most dangerous potential bioterrorism agents. Therefore, the necessary precaution was taken, and the full-length active toxin gene was not generated. Instead, an approach to generate limited amounts of full-length BoNTs in test tubes under controlled conditions by the enzymatic ligation of two non-toxic fragments of BoNTs was developed. This method utilizes a transpeptidase known as sortase, which recognizes specific peptide motifs and covalently link two peptides together by forming a native peptide bond (FIG. 4A). This approach has been previously utilized to generate chimeric toxins and other fusion proteins [50,51].

[0228] An engineered sortase A, known as SrtA*, from *staphylococcus aureus* was generated[51]. SrtA* recognizes the peptide motif LPXTG (SEQ ID NO: 57), cleaves between T-G, and concurrently forms a new peptide bond between the protein containing LPXTG (SEQ ID

NO: 57) with other proteins/peptides containing one or more N-terminal glycine (FIG. 4A). Two non-toxic fragments of BoNT/X: (1) LC-H_N with LPETGG (SEQ ID NO: 58) motif and a His6-tag fused to the C-terminus; (2) the H_c of BoNT/X (X-H_c) with a GST tag and thrombin cleavage site at its N-terminus were produced. Cutting by thrombin releases X-H_c with a free glycine at the N-terminus. Incubation of these two fragments with SrtA* generated limited amount of ~ 150 kD full-length BoNT/X containing a short linker (LPETGS, SEQ ID NO: 59) between LC-H_N and H_C (FIGs. 4A-4B).

[0229] It was observed that X-H_c showed a strong tendency for aggregation in solution for unknown reasons once it is cut from GST tag, which might be the reason why the ligation efficiency is low for BoNT/X (FIG. 4B). In contrast, ligation of X-LC-H_N with the H_C of BoNT/A (A-H_C) using the same approach achieved a much higher efficiency, with majority of X-LC-H_N ligated into a full-length XA chimeric toxin (FIG. 8A).

BoNT/X is active on cultured neurons

[0230] To analyze the activity of full-length BoNT/X, cultured rat cortical neurons as a model system were used. Neurons were exposed to the sortase ligation mixture and various control mixtures in media. Cell lysates were harvested 12 hours later and immunoblot analysis was carried out to examine cleavage of SNARE proteins. As shown in FIG. 4C, X-LC-H_N alone cleaved some VAMP2 due to its high concentration in the reaction mixture. The control mixture containing X-LC-H_N and X-H_C but not sortase slightly enhanced cleavage of VAMP2 compared to X-LC-H_N alone. This result suggests that X-H_c might be associated with X-LC-H_N via non-covalent interactions. This interaction appears to be specific as the control mixture containing X-LC-H_N and A-Hc showed the same level of VAMP2 cleavage as X-LC-H_N alone (FIG. 8B). Ligating X-LC-H_N and X-H_C by sortase enhanced cleavage of VAMP2 over the mixture of X-LC-H_N and X-H_c without sortase (FIG. 4C), demonstrating that ligated full-length BoNT/X can enter neurons and cleave VAMP2. Similarly, ligated full-length XA chimeric toxin also entered neurons and cleaved VAMP2 (FIG. 8B).

[0231] Mixing X-H_C with X-LC-H_N increased the amounts of aggregates at the top of the SDS-PAGE gel compared to X-LC-H_N alone. These aggregates disappeared in the presence of DTT, suggesting that a portion of X-H_C formed inter-molecular disulfide bond with X-LC-H_N. The presence of DTT also increased the amount of ligated full-length BoNT/X, suggesting that a portion of BoNT/X aggregated via inter-molecular disulfide bond (FIG. 4B). The formation of these aggregates could significantly reduce the effective toxin monomer concentrations in solution. This could be an intrinsic weakness of BoNT/X sequence. X-H_C contains a single cysteine (C1240) and mutating this cysteine did not affect the activity of ligated BoNT/X (FIG. 9). Furthermore, C1240S mutant can be combined with C461S or C467S mutations in the X-LC-H_N to generate a modified BoNT/X with no free cysteines (FIG. 9). These mutant toxins maintained the same levels of activity as WT BoNT/X, but are more stable in solution as monomers than WT BoNT/X.

BoNT/X induced flaccid paralysis *in vivo* in mice

[0232] Whether BoNT/X is active *in vivo* was examined using a well-established non-lethal assay in mice, known as Digit Abduction Score (DAS). This assay measures local muscle paralysis following injection of BoNTs into mouse hind limb muscles [52,53]. BoNTs cause flaccid paralysis of limb muscles, which can be detected by the failure to spread the toes during the startle response. An activated sortase reaction mixture (FIG. 4B, lane 7) was injected into the gastrocnemius muscles of the right hind limb in mice. Within 12 hours, the right limb developed typical flaccid paralysis and the toes failed to spread (FIG. 4D). These results confirmed that BoNT/X is capable of causing flaccid paralysis *in vivo* as other BoNTs.

BoNT/X was not recognized by antisera raised against all known BoNTs

[0233] To further confirm that BoNT/X is a serologically unique BoNT, dot blot assays were carried out using antisera raised against known BoNTs, including all seven serotypes as well as one mosaic toxin (BoNT/DC). Four horse antisera were utilized (trivalent anti-BoNT/A, B, and E, anti-BoNT/C, anti-BoNT/DC, and anti-BoNT/F), as well as two goat antisera (anti-BoNT/G and anti-BoNT/D). These antisera were all capable of neutralizing their corresponding target BoNTs and prevented cleavage of SNARE proteins in neurons (FIG. 10), thus validating their specificity and potency. As shown in FIG. 4E, these antisera recognized their corresponding target toxins, yet none of them recognized BoNT/X. Note that the antisera raised against BoNT/DC and BoNT/C cross-react with each other, as they share high degree of similarity in their H_C. These result established BoNT/X as a new serological type of BoNTs.

Full-length inactive BoNT/X

[0234] Finally, whether full-length BoNT/X can be produced as a soluble protein was examined. To ensure the biosafety requirement, mutations in the LC of BoNT/X were introduced that inactivate its toxicity. Mutations at two residues R362A/Y365F in BoNT/A have been shown to inactivate the protease activity of the LC *in vitro* and abolishes the toxicity of full-length BoNT/A in mice *in vivo* [54-56]. These two residues are conserved in all BoNTs including BoNT/X. Therefore, the corresponding mutations were introduced at these two sites (R360A/Y363F in BoNT/X). As shown in FIG. 4F, this full-length inactivated form of BoNT/X ($BoNT/X_{RY}$) was produced and purified as a His6-tagged protein in *E.coli* recombinantly. It does not have any activity on neurons as VAMP2 was not cleaved in neurons (FIG. 11).

[0235] A substantial portion of BoNT/X_{RY} formed aggregates at the top of the SDS-PAGE gel (FIG. 4F). This is likely due to formation of inter-molecular disulfide bond from the extra cysteine in the linker region and the cysteine in the H_C, as adding DTT converted the aggregates to monomeric BoNT/X_{RY} (FIG. 4F). Mutating these cysteines does not affect the activity of BoNT/X (FIG. 9), and has the benefit of preventing formation of inter-molecular disulfide bond and aggregations of BoNT/X.

[0236] An inactive form of BoNT/X might be utilized as a vehicle to deliver therapeutics into neurons. Inactivation can be achieved by mutations at any one of the following residues or their combinations: R360, Y363, H227, E228, or H231, with the later three residues forming the conserved protease motif.

Purification of full-length inactive BoNT/X at industrial-scale

[0237] Whether full-length BoNT/X can be purified to a high degree of purity and with a good yield, which will be important for industrial production of BoNT/X (or its derivative) as a therapeutic toxin, was investigated. Several parameters of cell growth and expression were tested, such as temperature, time of induction and IPTG concentrations. The optimal parameters chosen for protein expression were culture of the cells at 37°C until they reached exponential growth, at which stage the temperature was reduced to 18°C and expression induced by addition of 1mM IPTG to the media. Cells were then cultured for 16 to 18 hours before harvesting. Presence of BoNT/X was verified by SDS-PAGE and showed a high level of over-expression in the soluble fraction (FIG. 11B).

[0238] Several small-scale purification trials were carried out to optimize the production process. Mechanical cell lysis using an Emulsiflex-C3 (Avestin, Mannheim, Germany) was the preferred method for intracellular protein extraction, and appeared more efficient that sonication. Various buffer conditions also had to be assessed for optimal recovery of BoNT/X. A reducing agent was included throughout the purification process and greatly decreased the propensity to unwanted aggregation. Additionally, glycerol was used as an additive during the early stage of the purification process and improved protein stability.

[0239] The BoNT/X construct was expressed with a HIS6-tag that could be used for affinity chromatography as a first purification step. For small-scale trials, a 5ml HIsTrapFF column (GE Healthcare, Danderyd, Sweden) was used. In order to achieve the highest purity from the initial chromatography, various concentrations of imidazole were tested. BoNT/X eluted from a concentration of 100mM imidazole; however, a major contaminant readily co-purified with the toxin. This contaminant appeared to non-specifically interact with BoNT/X and was identified by mass spectrometry as an *E. coli* host protein (bifunctional polymyxin resistance protein ArnA). The presence of this contaminant was dramatically reduced with the introduction of a high salt concentration (500 mM NaCl) and by carrying out an additional washing step at 100 mM imidazole during purification. This allowed for elution of a purer BoNT/X fraction at 250 mM imidazole. This later fraction could then be polished by size exclusion chromatography.

[0240] Once in place, this protocol was scaled up by expressing up to 12 L of media with the conditions described above. Additionally, a larger affinity chromatography matrix was prepared consisting of 15 ml of Protino® Ni-NTA agarose (Macherey-Nagel, Düren, Germany) to increase the yield of BoNT/X recovery. The final purification step was performed by size exclusion chromatography using a Superdex200-16/60 column (GE Healthcare, Danderyd, Sweden). Using this method, between 85 and 90% purity was obtained (FIG. 11C). The protein could be concentrated (using a Vivaspin concentrator with a 100kDa cut-off; GE Healthcare, Danderyd, Sweden) and appeared stable up to 10mg/ml. Complete details of the protein production process are described below. The yield of BoNT/X obtained was approximately 3 mg per liter of cell culture. Together these results demonstrated that BoNT/X can be purified at industrial scale to high purity.

[0241] Note that the purification was done in the presence of reducing agent, which would reduce the disulfide bond between the LC and the HC, so purified toxin would not be active. However, a designed BoNT/X derivative containing mutations at the cysteine sties (one mutation at C461 or C467, combined with mutating C1240) would be able to be purified without reducing agents. Note that an inactive form of BoNT/X (and its cysteine mutation derivative) might be utilized as a vehicle to deliver therapeutics into neurons. Inactivation can be achieved by mutations at any one of the following residues or their combinations: R360, Y363, H227, E228, or H231 (the later three residues form the conserved protease motif).

Identification of gangliosides as receptors for BoNT/X

[0242] Gangliosides are well-established lipid co-receptors for all BoNTs and a gangliosidebinding motif is well-conserved in BoNT/X (FIG. 1C). Highly purified full-length inactive BoNT/X was used to examine whether BoNT/X binds to neuronal cells via gangliosides/ An in vitro ELISA assay was developed to test for interaction with four major brain gangliosides: GD1a, GD1b, GT1b, and GM1. A-LC was use as a negative control to assess unspecific binding. Direct comparison with the receptor binding domain of BoNT/A (A-HC) was also performed. Binding of proteins were detected using an anti-His6-tag antibody. It was found that BoNT/X showed a dose-dependent binding to all four gangliosides over the non-specific binding level of A-LC (FIG. 12), suggesting that BoNT/X is capable of utilizing all four brain gangliosides as co-receptors. In accordance with previous reports, BoNT/A presented an equal preference for GDla and GT1b (FIG 12F) and their terminal NAcGal-Gal-NAcNeu moiety (with apparent EC50 values of 0.7 and 1.0μM, respectively, when fitted with a sigmoidal doseresponse model). In contrast, BoNT/X showed higher affinity for GDlb and GM1 over GDla and GT1b (FIG 12E). This would suggest BoNT/X has a preferred sialic acid recognition pattern, also seen in BoNT/B and TeNT. BoNT/X possesses the conserved SxWY motif at a homologous location to the one of the other toxins. The fact that it could recognize all four gangliosises, albeit with low affinity, may be an indication of multiple carbohydrate binding sites.

Discussion

[0243] The eighth serotype of BoNTs over 45 years after the identification of the last major BoNT serotype has been identified. BoNT/X has the lowest protein sequence identity to any other BoNTs and TeNT among this family of toxins, and this low level of identity is evenly distributed along the toxin sequence. As expected, BoNT/X was not recognized by any antisera raised against known BoNTs. It clearly represents a unique and distinct evolutionary branch of the toxin family.

[0244] BoNT/X was revealed by searching genomic sequences of *clostridium botulinum* strains and it represents the first major toxin type identified by genomic sequencing and bioinformatics approach. The strain 111 that contains BoNT/X gene was initially identified from an infant botulism patient in 1990s. Previous characterizations using classic neutralization assay have established BoNT/B2 as the major toxin of this strain. It is likely that BoNT/X is a silent toxin gene, or it was not expressed at detectable toxicity levels under the culture conditions in the lab. Therefore, it can only be identified by sequencing strain 111. This illustrates the importance of genomic sequencing and bioinformatics approaches for understanding microbial virulent factors.

[0245] Silent BoNT genes have been frequently found previously in various *Clostridium botulinum* strains. It is not clear why these bacteria keep a silent toxin gene. It could be an evolutionarily degenerated gene. This is clearly the case when silent toxin genes contain premature stop code mutations. However, there are also cases that the silent gene encodes a full-length BoNT. Whether these silent full-length BoNTs might be expressed and exhibit toxicity under certain environmental conditions remains an intriguing question.

[0246] The general three-domain structures and functions of BoNTs are well conserved in BoNT/X, but it also has a few unique characteristics: (1) it shares VAMP as its target in neurons with BoNT/B, D, F, and G, but it cuts VAMP at a novel site (R66-A67 in VAMP2) that is unique to this toxin. This further expands the repertoire of toxins that can be used to ablate VAMP at different sites. (2) The inter-chain disulfide bond connecting LC and H_N is conserved in BoNT/X, but it also contains a unique additional cysteine in the linker region, which may lead to disulfide bond shuffling. The extra cysteine on H_N is not essential for the activity of LC-H_N (FIG. 3D), and mutating it has the benefit of preventing formation of inter-molecular disulfide bond (FIG. 3D, 4B).

[0247] His6-tagged X-LC-H_N fragment are stable in buffers as recombinant proteins. It showed a higher level of activity on neurons than both A-LC-H_N and B-LC-H_N (FIG. 3B), suggesting that its membrane translocation and/or protease activity might be more efficient than the corresponding fragments in BoNT/A and BoNT/B. X-LC-H_N could be a useful reagent for targeting VAMP1/2/3 in a broad range of cell types and tissues as its entry might not be restricted to neurons. For instance, it potentially can be utilized to reduce pain in a local region by targeting both sensory neurons and other cells that secrete inflammatory signals. It could also be used to generate chimeric toxins, such as XA (FIG. 8).

[0248] X-H_C is functional as its presence enhanced cleavage of VAMP2 in neurons than LC-H_N alone (FIG. 4C). However, X-H_C may have some unfavorable characterizes that remain to be further evaluated. For instance, sufficient levels of soluble X-H_C were only produced when it was fused with GST, which is known to facilitate protein folding/solubility, but not with His6 tag. Once released from GST tag, X-H_C is prone to aggregation. In addition, the cysteine in X-H_C may also form inter-molecular disulfide bond (FIG. 4B). Full-length inactive BoNT/X can be purified and exist as a soluble protein, suggesting that the solubility issue with X-H_C might at least partially due to separation of this domain from X-LC-H_N. For instance, X-LC-H_N might interact with X-H_C and covers its potential hydrophobic segments in the full-length context, which is not unusual for a multi-domain protein.

[0249] Gangliosides have long been established as neuronal receptors for all BoNT subtypes. It is demonstrated that BoNT/X can bind to all four of the most abundant gangliosides: GD1a, GDlb, GT1b, and GM1. Additionally it does so with remarkable difference in affinity and specificity when compared to BoNT/A. This is an intriguing property, as other BoNTs appear to have various degrees of preferences toward a subgroup of gangliosides. For instance, BoNT/A, E, F, and G prefer GDla and GT1b. BoNT/X might potentially recognize a broader range of neuron types compared to other BoNTs.

[0250] It is possible that BoNT/X has a low toxicity *in vivo,* which might explain why BoNT/X activity was not detected in the original study on strain 111. If this is the case, the reduced toxicity is likely due to its H_C domain, as X-LC-H_N appears to be more active than both A-LC-H_N and B-LC-H_N. The formation of inter-molecular disulfide bond might also reduce the effective toxin concentration. It will be necessary to produce full-length native BoNT/X in order to determine its potency *in vivo,* but it will be important to generate neutralizing antisera using non-toxic fragments of BoNT/X prior to producing full-length toxin.

[0251] Introducing full-length active toxin gene into any expression systems/organisms is always a significant biosafety concern and it has become a formidable hurdle for structure-function studies of biological toxins. This is particularly an important consideration for BoNTs as they are one of the six category A potential bioterrorism agents [4]. Here a method to assemble limited amount of full-length toxin biochemically from two complementary and non-toxic fragments was developed. Each fragment is expressed and purified individually, and then ligated together by sortase in test tubes. Other protein ligation methods such as split intein systems, which fuse two protein fragments through protein trans-splicing, can also be utilized [57]. By controlling the amount of precursor fragments in the reaction, the amount of ligated full-length toxin can be strictly controlled. This "semi-synthesis" approach can be used to produce multi-domain biological toxins and other toxic proteins under controlled conditions. It also provides a versatile platform for generating fusion and chimeric toxins, such as swapping the

H_C of two BoNTs, replacing H_C of BoNTs with other targeting proteins, or attaching additional cargo to toxins. As there is no full-length toxin cDNA ever generated and no expression of toxins in bacteria or any other living organisms, this approach significantly mitigates the biosafety concerns associated with producing wild type and mutant toxins and will greatly facilitate structure-function studies of biological toxins and toxic proteins.

Materials and Methods

[0252] Materials: Mouse monoclonal antibodies for syntaxin 1 (HPC-1), SNAP-25 (C171.2), and VAMP2 (C169.1) were generously provided by E. Chapman (Madison, WI) and are available from Synaptic Systems (Goettingen, Germany). Mouse monoclonal antibody for actin was purchased from Sigma (AC-15). Equine polyclonal antisera against BoNT/A/B/E, BoNT/C, BoNT/DC, BoNT/F, and goat polyclonal antisera against BoNT/G were obtained from the FDA. Goat polyclonal antibody against BoNT/D was purchased from Fisher Scientific (NB10062469). BoNT/A, BoNT/B, BoNT/C, BoNT/DC, BoNT/E, BoNT/F, and BoNT/G were purchased from Metabiologics (Madison, WI). BoNT/D was generously provided by E. Johnson (Madison, WI).

[0253] cDNA and constructs: The cDNAs encoding X-LC (residues 1-439) and X-H_C (residues 893-1306) was synthesized. The cDNA encoding X-H_N was generated in-house using Gibson assembly method. The cDNAs encoding A-LC (residues 1-425, M30196) and B-LC (residues 1-439, AB232927) were synthesized by GenScript (New Brunswick, NJ). These LCs were cloned into pET28 vectors for expression as His6-tagged proteins. X-H_C was cloned into pGEX4T to express as a GST-tagged protein. X-LC-H_N, A-LC-H_N, and B-LC-H_N were subcloned into pET28 vector, with a peptide sequence LPETGG (SEQ ID NO: 58) fused to their C-termini, and were purified as His6-tagged proteins. Full-length inactive form of BoNT/X was assembled in-house from mutated X-LC (R360A/Y363F), X-H_N, and X-H_C. It was cloned into pET28 vector with a His6-tagg fused to the C-terminus of BoNT/X. The cDNA encoding rat VAMP2 was generously provided by E. Chapman (Madison, WI). VAMP2 (1-96) was cloned into pET28 vector and expressed as a His6-tagged protein. VAMP2 (33-86) was cloned into a pGEX4T vector and expressed as a GST-tagged protein. The cDNA encoding mouse VAMP1, VAMP3, rat VAMP7, and VAMP8 was generously provided by C. Hu (Louisville, KY). They were cloned into a modified pcDNA3.1 vectors, with a HA tag fused to their C-termini. The construct encoding His6-tagged sortase (SrtA*) was generously provided by B. Pentelute (Boston, MA) and has been described previously [51].

[0254] Bioinformatics: The Uniprot database was searched with jackhmmer at the HMMER web server using a BoNT type A sequence (Uniprot accession number A5HZZ9) until convergence. Returned sequences were aligned with Clustal Omega and a NeighborNet phylogenetic network estimated with SplitsTree4.

[0255] Protein purification: *E.coli* BL21 (DE3) was utilized for protein expression. Induction of expression was carried out with 0.1 mM IPTG at 22 °C overnight. Bacterial pellets were disrupted in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl) by sonication and supernatants were collected after centrifugation at 20000g for 30 min at 4°C. Protein purification was carried out using AKTA Prime FPLC system (GE) and purified proteins were further desalted with PD-10 column (GE, 17-0851-01). Specifically, full-length inactive BoNT/X (BoNT/X_{RY}) was cloned into a pET22b vector. The corresponding plasmid was transformed into *E. coli* BL21 (DE3) competent cells. Resulting colonies were used to inoculate 100 ml overnight cultures of TB medium containing 100 µg/ml Carbenicillin in 250 ml shake-flask and grown at 37°C. Cultures for expression were first grown using a LEX Bioreactor (Epiphyte3, Ontario, Canada) at 37°C in 1.5 L of TB media until OD_{600} reached 0.8. The temperature was then reduced to 18°C for induction of expression with 1 mM IPTG, and grown for 16-17 hours. Cells were harvested and re-suspended on ice in 50 mM HEPES pH 7.2, 500 mM NaCl, 25 mM imidazole, 5% glycerol, 2 mM TCEP to allow for cell lysis with an Emulsiflex-C3 (Avestin, Mannheim, German) at 20,000 psi. Lysate was ultra-centrifuged at 200,000 g for 45 minutes at 4°C. Supernatnt was loaded onto a 15 ml Protino® Ni-NTA agarose (Macherey-Nagen, Düren, Germany) column that was then washed with 50 mM HEPES pH 7.2, 500 mM NaCl, 100 mM imidazole, 5% glycerol, 1 mM TCEP. Elution was carried out with 50 mM HEPES pH 7.2, 500 mM NaCl, 250 mM imidazole, 5% glycerol, 1 mM TCEP. The eluate was dialyzed overnight in 50 mM HEPES pH 7.2, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP at 4°C. Dialysate was concentrated using a Vivaspin concentrator (100kDa cut-off, GE Healthcare, Danderyd, Sweden) before being loaded on a Superdex200-16/60 column (GE Healthcare, Danderyd, Sweden) pre-equiliberated in the same buffer as was used for dialysis. The elution peak corresponding to BoNT/X was collected and concentrated so that the final sample was at a concentration of 10 mg/ml. The sample was aliquoted and flash-frozen in liquid nitrogen for storage at -80°C.

[0256] Ganglioside binding assay: Purified gangliosides GD1a, GDlb, GT1b, and GM1 (Carbosynth, Compton, UK) were dissolved in DMSO and diluted in methanol to reach a final concentration of 2.5 µg/ml; 100 µL was applied to each well of a 96-well PVC assay plate (catalog no. 2595, Corning; Corning, NY). After solvent evaporation at 21°C, the wells were washed with 200 µL PBS/0.1% (w/v) BSA. Nonspecific binding sites were blocked by incubation for 2.5 h at 4°C in 200 µL of PBS/2% (w/v) BSA. Binding assays were performed in 100 µL PBS/0.1% (w/v) BSA per well for 1 h at 4°C containing samples (triplicate) at concentrations ranging from 6 µM to 0.05 µM (in serial 2-fold dilution). Following incubation, wells were washed three times with PBS/0.1% (w/v) BSA and incubated with an HRP-conjugated anti-6xHis monoclonal antibody (1:2000, ThermoFisher) for 1 h at 4°C. After three washing steps with PBS/0.1% (w/v) BSA, bound samples were detected using Ultra-TMB (100 µL/well, ThermoFisher) as the substrate. The reaction was stopped after 15 minutes by addition of 100 µL 0.2M H_2SO_4, and the absorbance at 450 nm was measured using an Infinite M200PRO plate reader (Tecan, Männedorf, Switzerland). Data were analyzed with Prism7 (GraphPad Software).

[0257] Cleavage of SNARE proteins in rat brain detergent extracts (BDE): Rat BDE were prepared from fresh dissected adult rat brains as previously described [58]. Briefly, a rat brain was homogenized in 15 ml 320 mM sucrose buffer, followed by a centrifugation at 5000 rpm for 2 min at 4°C. Supernatants were collected and centrifuged at 11,000 rpm for 12 min. The pellet was collected and solubilized for 30 min in 15 ml Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl) plus 2% of Triton X-100 and a cocktail of protease inhibitors (Roche, CA). Samples were subsequently centrifuged at 17,000 rpm for 20 min to remove the insoluble materials. The final BDE concentration is ~ 2 mg/ml proteins. BDE (60 µl) were incubated with X-LC (0.5 µM), A-LC (1 µM), or B-LC (1 µM), respectively, for 1 hour at 37 °C, and were then analyzed by immunoblot using the enhanced chemiluminescence (ECL) method (Pierce). As controls, LCs were pre-incubated with 20 mM EDTA for 20 minutes at room temperature (RT) to de-active their activity prior to adding into BDE.

[0258] Cleavage of recombinant VAMP by X-LC: VAMP2 (1-96) was expressed and purified as a His6-tagged protein and VAMP2 (33-86) was expressed and purified as a GST-tagged protein. These proteins (0.6 mg/ml) were incubated with 0.1 µM X-LC in TBS buffer for 1 hour at 37 °C. Samples were either analyzed by SDS-PAGE gels and Coomassie Blue staining, or subjected to mass spectrometry analysis.

[0259] Cleavage of VAMPs in cell lysates: Full-length HA-tagged VAMP1, 3, 7, and 8 were transfected into HEK293 cells using PolyJet transfection reagents (SignaGen, MD) following the manufacturer's instruction. Cell lysates were harvested 48 hours later in RIPA buffer (50 mM Tris, 1% NP40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 400 µl per 10-cm dish) plus a protease inhibitor cocktail (Sigma-Aldrich). Cell lysates (250 µl) were incubated with X-LC (0.5 µM) for 1 hour at 37 °C. Samples were then analyzed by immunoblot.

[0260] Whole Protein Analysis by LC-MS/MS: Samples were analyzed at Taplin Biological Mass Spectrometry Core Facility at Harvard Medical School. Briefly, whole protein samples were loaded onto a 100 µm internal diameter C18 reverse phase HPLC column packed with 3cm of beads off-line using a pressure cell. The column was re-attached to an Accela 600 Pump (Thermo Fisher Scientific, Waltham, MA). A rapid gradient of increasing acetonitrile was used to elute the protein/peptide from the HPLC column. As peptides eluted, they were subjected to electrospray ionization and then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) to acquire a high resolution FTMS scan at 60000 resolution, a second scan at low resolution in the ion trap, and a final scan to perform data dependent MS/MS. The charge state envelopes were de-convoluted manually to obtain mono-isotopic masses when possible or average masses for the proteins. Peptide and protein identity were determined by matching protein databases with the acquired fragmentation pattern by the software program, Sequest (Thermo Fisher Scientific). All databases include a reversed version of all the sequences and the data was filtered to between a one and two percent peptide false discovery rate.

[0261] Identification of the protease cleavage site between LC and H_N: His6-tagged recombinant X-LC-H_N fragment (residues 1-891) was purified in *E.coli* and subjected to limited proteolysis by endoproteinase Lys-C (Sigma P2289, 100:1 (toxin:Lys-C) molar ratio, 25 minutes at room temperature). The cleavage site was determined by Tandem Mass Tag (TMT) labeling and tandem mass spectrometry approach. Briefly, intact X-LC-H_N samples were labeled with the light TMT and equal amount of X-LC-H_N samples were exposed to Lys-C and then labeled with the heavy TMT. Both samples were then digested with chymotrypsin, combined together, and subjected to quantitative mass spectrometry analysis.

[0262] Cysteine alkylation by NEM: Lys-C activated X-LC-H_N fragment was diluted into sodium phosphate buffer (10 mM, pH 6.5) at the final concentration of 0.3 mg/ml, with or without NEM at indicated concentrations (20, 10, and 5 mM) and incubated for 10 minutes at RT. NEM was freshly prepared in sodium phosphate buffer. Samples were mixed with 3 × neutral loading dyes (200 mM Tris pH 6.8, 30% glycerol, 6% Lithium Dodecyl sulfate, 10mM NEM and 0.06% BPB). For samples without NEM, the same 3 × SDS loading dye without NEM was used. Samples were further incubated with the loading dye at RT for 10 minutes, heated for 10 min at 55 °C, and then analyzed by SDS-PAGE and Coomassie Blue staining.

[0263] Neuron culture and immunoblot analysis: Primary rat cortical neurons were prepared from E18-19 embryos using a papain dissociation kit (Worthington Biochemical, NJ), as we described previously [58]. Experiments were carried out on DIV 14-16. Neurons were exposed to BoNT/X fragments or sortase ligation mixture in media for 12 hrs. Cells were then washed and lysed with RIPA buffer (50 mM Tris, 1% NP40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS) plus a protease inhibitor cocktail (Sigma-Aldrich). Lysates were centrifuged for 10 min at maximum speed using a microcentrifuge at 4°C. Supernatants were subjected to SDS-PAG and immunoblot analysis.

[0264] Dot blot: BoNTs (0.2 µg in 1 µl) were spotted onto nitrocellulose membranes and dried (10 minutes at room temperature). The membranes were blocked with 5% milk in TBST (TBS plus 0.05% Tween20) for 30 min and then incubated with indicated antisera (1:500 dilution) for 30 min. The membranes were then washed three times with TBST and incubated with HRP (horseradish peroxidase) conjugated secondary antibodies for 30 min, washed three more times with TBST, and analyzed with the ECL method (Pierce). We note that the BoNT/X sample was composed of purified X-LC-H_N and X-Hc at 1:1 ratio.

[0265] Sortase-mediated ligation: GST-X-H_C was cleaved overnight at 4 °C by thrombin before adding into the mixture of proteins. Ligation reaction was set up in 50 µl TBS buffer with addition of X-LC-H_N (8 µM), thrombin-cleaved GST-X-H_C (25 µM), Ca^{2+} (10 mM), and sortase (10 µM), for 40 min at RT. In FIG. 4C, neurons were exposed to 5 µl of the mixture in media for 12 hrs. In DAS assay described in FIG. 4D, 25 µl of the mixture was injected into the hind leg of mice.

[0266] DAS assay: Sortase ligation mixture was first activated with limited proteolysis using trypsin (60:1 molar ratio (the total amount of the proteins : trypsin), 30 min at RT). We chose trypsin instead of Lys-C here as we can stop the proteolysis by adding trypsin inhibitor (Soybean trypsin inhibitor, 1:10 ratio (trypsin : trypsin inhibitor). Mice (CD-1 strain, 21-25g, n = 6) were anesthetized with isoflurane (3-4%) and were injected with sortase ligation mixture using a 30-gauge needle attached to the sterile Hamilton Syringes, into the gastrocnemius muscles of the right hind limb. BoNTs result in paralysis of the hind paw in the startle response. Muscle paralysis was observed within 12 hours after the injection as previously described [52,53].

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[0267]

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- **KRZEWSKI, K.GIL-KRZEWSKA, A.WATTS, J.STERN, J.N.STROMINGER, J.L.**VAMP4- and VAMP7-expressing vesicles are both required for cytotoxic granule exocytosis in NK cellsEur J Immunol, 2011, vol. 41, 3323-3329 [0267]
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- **HASEGAWA, H. et al.**Mammalian ykt6 is a neuronal SNARE targeted to a specialized compartment by its profilin-like amino terminal domainMol Biol Cell, 2003, vol. 14, 698-720 [0267]
- **DASTE, F.GALLI, T.TARESTE, D.**Structure and function of longin SNAREsJ Cell Sci, 2015, vol. 128, 4263-4272 [0267]
- **COOPER, A.A. et al.**Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's modelsScience, 2006, vol. 313, 324-328 [0267]
- **THAYANIDHI, N. et al.**Alpha-synuclein delays endoplasmic reticulum (ER)-to-Golgi transport in mammalian cells by antagonizing ER/Golgi SNAREsMol Biol Cell, 2010, vol. 21, 1850-1863 [0267]

Patentkrav

1. Isoleret Clostridial botulinum-neurotoksin (BoNT)-polypeptid, som omfatter en aminosyresekvens, der har mindst 85 %, mindst 86 %, mindst 87 %, mindst 88 %, mindst 89 %, mindst 90 %, mindst 91 %, mindst 92 %, mindst 93 %, mindst 94 %, mindst 95 %, mindst 96 %, mindst 97 %, mindst 98 %, mindst 99 % eller mindst 99,5 % identitet med SEQ ID NO: 3, eventuelt hvor det isolerede BoNT-polypeptid omfatter aminosyresekvensen ifølge SEQ ID NO: 3, hvor BoNT-polypeptidet ikke krydsreagerer med et antistof mod BoNT-serotype A, B, C, D, E, F eller G.

2. Isoleret BoNT-polypeptid ifølge krav 1, som omfatter en aminosyresekvens, der har mindst 85 %, mindst 86 %, mindst 87 %, mindst 88 %, mindst 89 %, mindst 90 %, mindst 91 %, mindst 92 %, mindst 93 %, mindst 94 %, mindst 95 %, mindst 96 %, mindst 97 %, mindst 98 %, mindst 99 % eller mindst 99,5 % identitet med SEQ ID NO: 2, eventuelt hvor det isolerede BoNT-polypeptid omfatter aminosyresekvensen ifølge SEQ ID NO: 2.

3. Isoleret BoNT-polypeptid ifølge krav 2, som omfatter en aminosyresekvens, der har mindst 85 %, mindst 86 %, mindst 87 %, mindst 88 %, mindst 89 %, mindst 90 %, mindst 91 %, mindst 92 %, mindst 93 %, mindst 94 %, mindst 95 %, mindst 96 %, mindst 97 %, mindst 98 %, mindst 99 % eller mindst 99,5 % identitet med SEQ ID NO: 1, eventuelt hvor det isolerede BoNT-polypeptid omfatter aminosyresekvensen ifølge SEQ ID NO: 1.

4. Modificeret BoNT/X-polypeptid ifølge krav 3, som omfatter en eller flere substitutionsmutation(er) i en position svarende til C461, C467 eller C1240 i SEQ ID NO: 1, eventuelt hvor
(i) substitutionsmutationen svarer til C461S, C461A, C467S, C467A, C1240S, C1240A, C461S/C1240S, C416S/C1240A, C461A/C1240S, C461A/C1240A, C467S/C1240S, C461S/C1240A, C467A/C1240S eller C467A/C1240A i SEQ ID NO: 1, eller
(ii) det modificerede BoNT/X-polypeptid omfatter aminosyresekvensen ifølge et hvilket som helst af SEQ ID NO: 4-17.

5. Modificeret BoNT/X-polypeptid ifølge krav 2, som omfatter en enkelt substitutionsmutation i en position svarende til C461 eller C467 i SEQ ID NO: 2, eventuelt hvor
(i) substitutionsmutationen svarer til C461S, C461A, C467S eller C467A i SEQ ID NO: 2, eller
(ii) det modificerede BoNT/X-polypeptid omfatter aminosyresekvensen ifølge et hvilket som helst af SEQ ID NO: 18-21.

6. Modificeret BoNT/X-polypeptid ifølge krav 3, som omfatter en eller flere substitutionsmutation(er) i en position svarende til R360, Y363, H227, E228 eller H231 i SEQ ID NO: 1, eventuelt hvor
(i) den ene eller de flere substitutionsmutation(er) svarer til R360A/Y363F, H227Y, E228Q eller H231Y i SEQ ID NO: 1, eller
(ii) det modificerede BoNT/X-polypeptid omfatter aminosyresekvensen ifølge et hvilket som helst af SEQ ID NO: 31-38.

7. Modificeret BoNT/X-polypeptid ifølge krav 6, som yderligere omfatter én enkelt substitutionsmutation i en position svarende til C461 eller C467 i SEQ ID NO: 1, eventuelt hvor den enkelte substitutionsmutation svarer til C461A, C461S, C467A eller C467S i SEQ ID NO: 1.

8. Modificeret BoNT/X ifølge krav 6 eller krav 7, som yderligere omfatter en substitutionsmutation i en position svarende til C1240 i SEQ ID NO: 1.

9. Nukleinsyremolekyle, som omfatter et polynukleotid, der koder for et polypeptid, der omfatter en aminosyresekvens, der har mindst 85 %, mindst 86 %, mindst 87 %, mindst 88 %, mindst 89 %, mindst 90 %, mindst 91 %, mindst 92 %, mindst 93 %, mindst 94 %, mindst 95 %, mindst 96 %, mindst 97 %, mindst 98 %, mindst 99 % eller mindst 99,5 % eller 100 % identitet med BoNT-polypeptidet ifølge et hvilket som helst af kravene 1-8.

10. Nukleinsyrevektor, som omfatter nukleinsyremolekylet ifølge krav 9.

11. Celle, som omfatter nukleinsyremolekylet ifølge krav 9 eller nukleinsyrevektoren ifølge krav 10.

12. Farmaceutisk sammensætning, som omfatter BoNT-polypeptidet ifølge et hvilket som helst af kravene 1-8, eventuelt hvor den farmaceutiske sammensætning yderligere omfatter et farmaceutisk acceptabelt hjælpestof.

13. Modificeret BoNT-polypeptid ifølge et hvilket som helst af kravene 1-8 eller farmaceutisk sammensætning ifølge krav 12 til anvendelse til terapi.

14. Modificeret BoNT-polypeptid eller farmaceutisk sammensætning til anvendelse ifølge krav 13 til behandling af en tilstand forbundet med uønsket neuronaktivitet, eventuelt hvor tilstanden er forbundet med overaktive neuroner eller kirtler, eventuelt hvor tilstanden er valgt fra gruppen, der består af: spasmodisk dysfoni, spasmodisk torticollis, laryngeal dystoni, oromandibulær dysfoni, lingual dystoni, cervikal dystoni, fokal dystoni i hånden, blefarospasme, strabismus, hemifacial spasme, øjenlågsforstyrrelse, cerebral parese, fokal spasticitet og andre stemmeforstyrrelser, spasmodisk colitis, neurogen blære, anismus, lemmespasticitet, tics, tremor, bruksisme, analfissur, akalasi, dysfagi og andre muskeltonusforstyrrelser og andre forstyrrelser, der er kendetegnet ved ufrivillige bevægelser af muskelgrupper, tåreflåd, hyperhydrose, kraftig spytsekretion, kraftige gastrointestinale sekretioner, sekretionsforstyrrelser, smerter fra muskelspasmer, hovedpine, dermatologiske tilstande, obesitas/reduceret appetit.

15. Modificeret BoNT-polypeptid eller farmaceutisk sammensætning til anvendelse ifølge krav 13, hvor tilstanden ikke er forbundet med uønsket neuronaktivitet, eventuelt hvor tilstanden er valgt fra gruppen, der består af: psoriasis,

allergi, hæmofagocytisk lymfohistiocytose og alkoholisk pankreassygdom.

16. BoNT-polypeptid ifølge et hvilket som helst af kravene 6-8, som er koblet til et terapeutisk middel, hvor BoNT-polypeptidet er egnet til fremføring af det terapeutiske middel til målceller, eventuelt hvor målcellerne er neuroner.

17. Ikke-terapeutisk fremgangsmåde til behandling af en kosmetisk eller æstetisk tilstand ved anvendelse af BoNT-polypeptidet ifølge et hvilket som helst af kravene 1-8, eventuelt hvor tilstanden er pandefurer eller hudrynker.

DRAWINGS

FIG. 1A

FIG. 1B

FIG. 1C

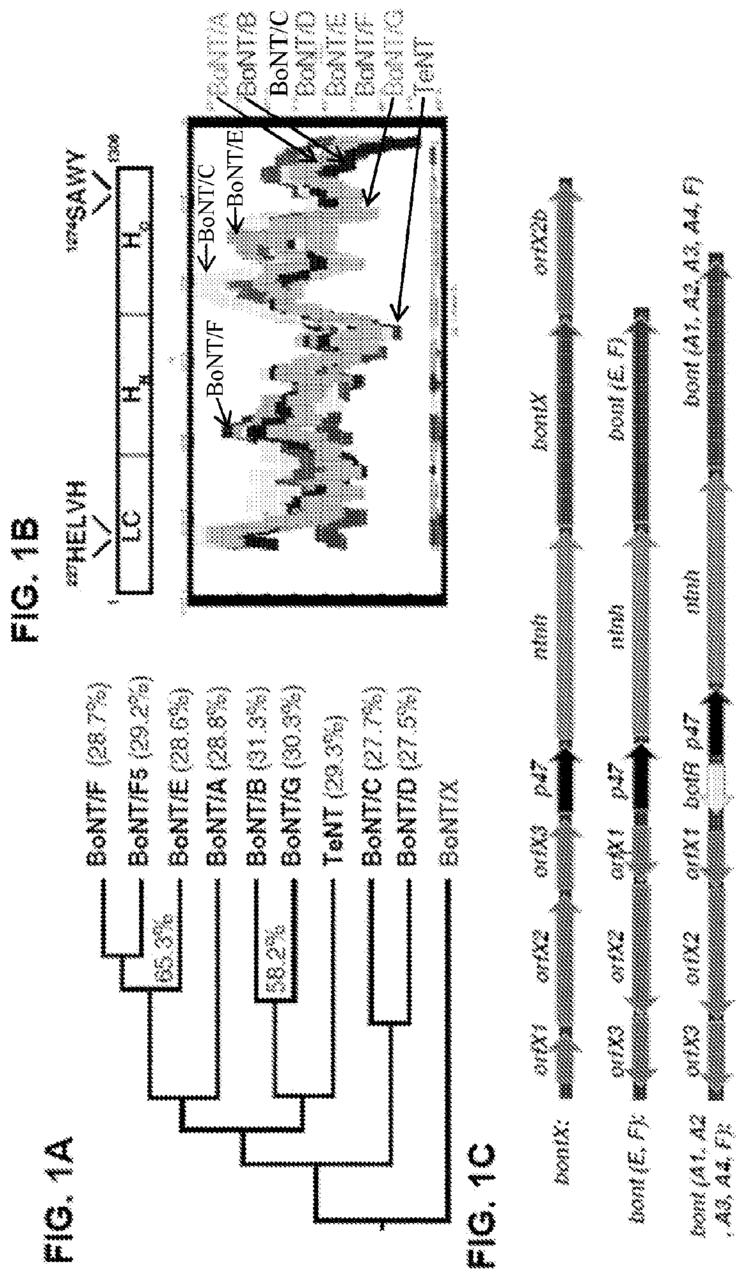


FIG. 1D

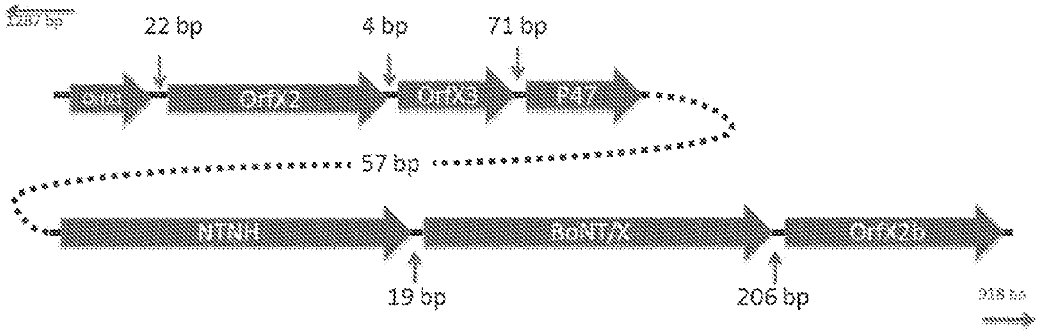

1287 bp
22 bp
4 bp
71 bp
OrfX2
OrfX3
P47
57 bp
NTNH
BoNT/X
OrfX2b
19 bp
206 bp
918 bp

FIG. 1E

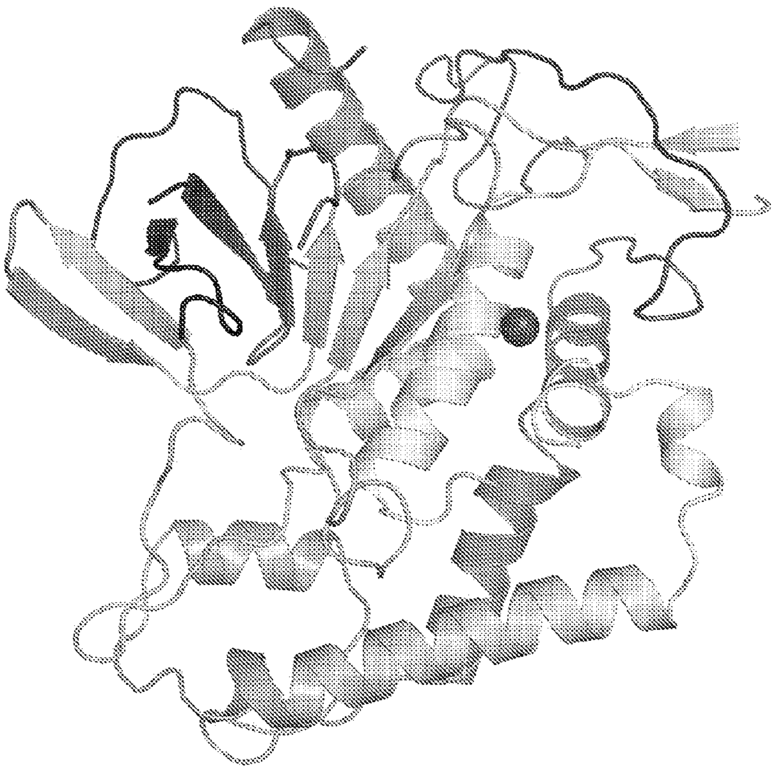


FIG. 2A

FIG. 2B

FIG. 2C

FIG. 2D

FIG. 2E

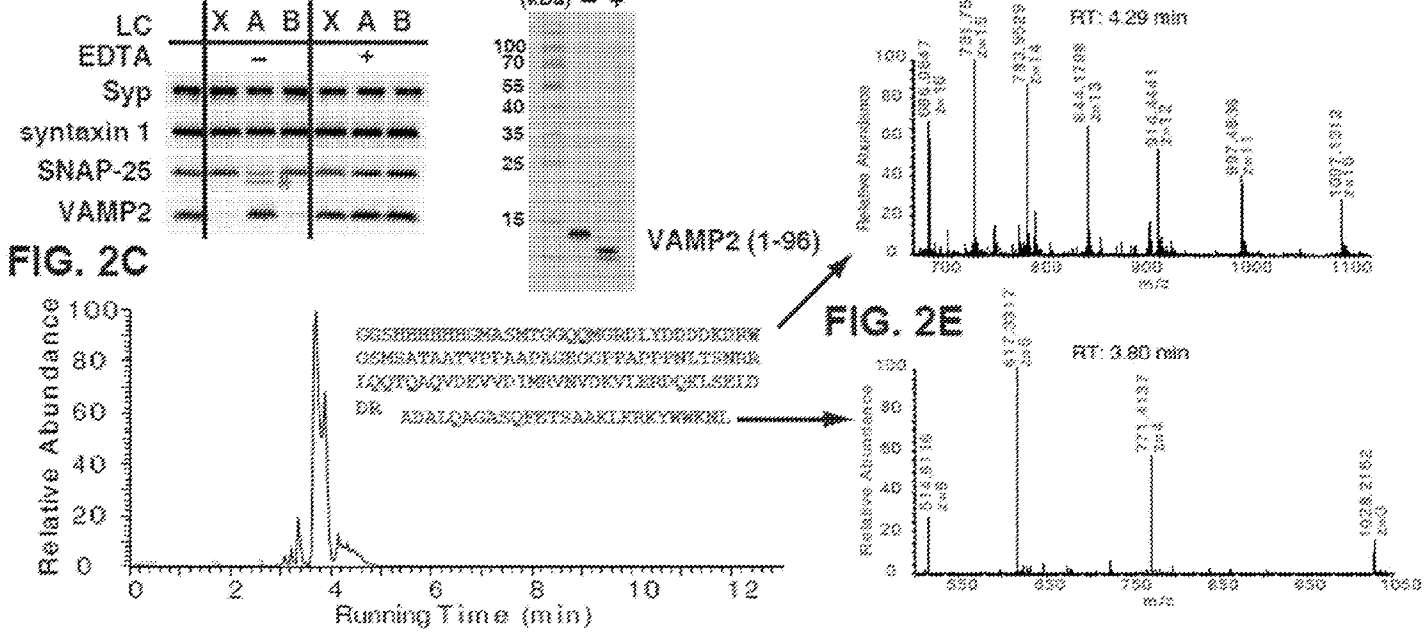


FIG. 2F

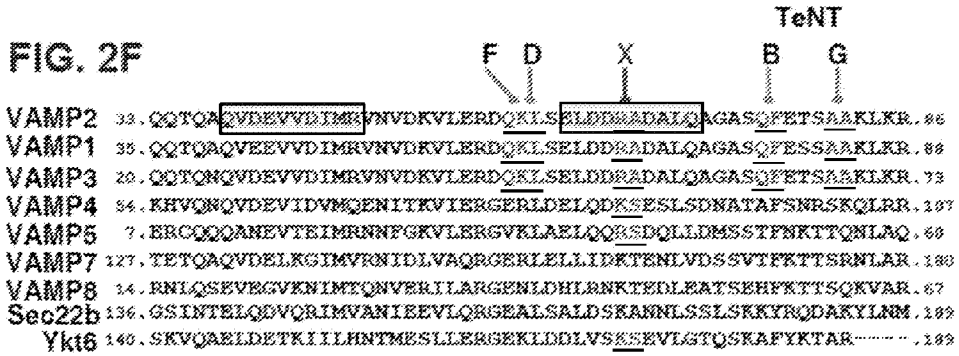


FIG. 2G

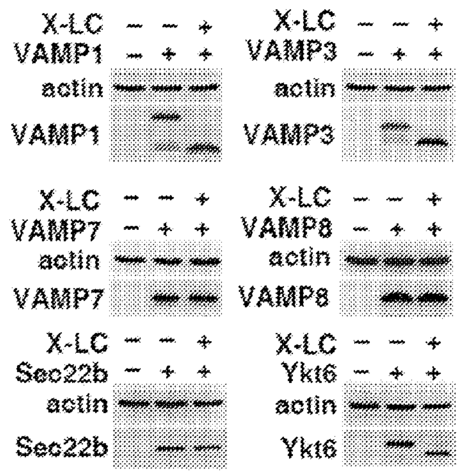


FIG. 2H

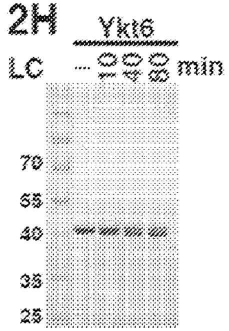


FIG. 2I

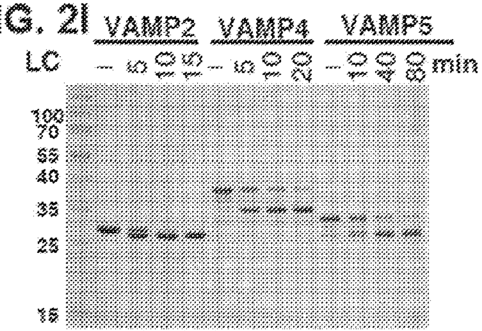
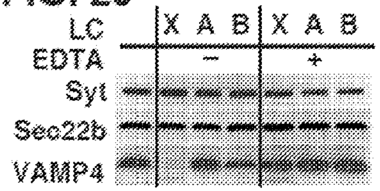


FIG. 2J



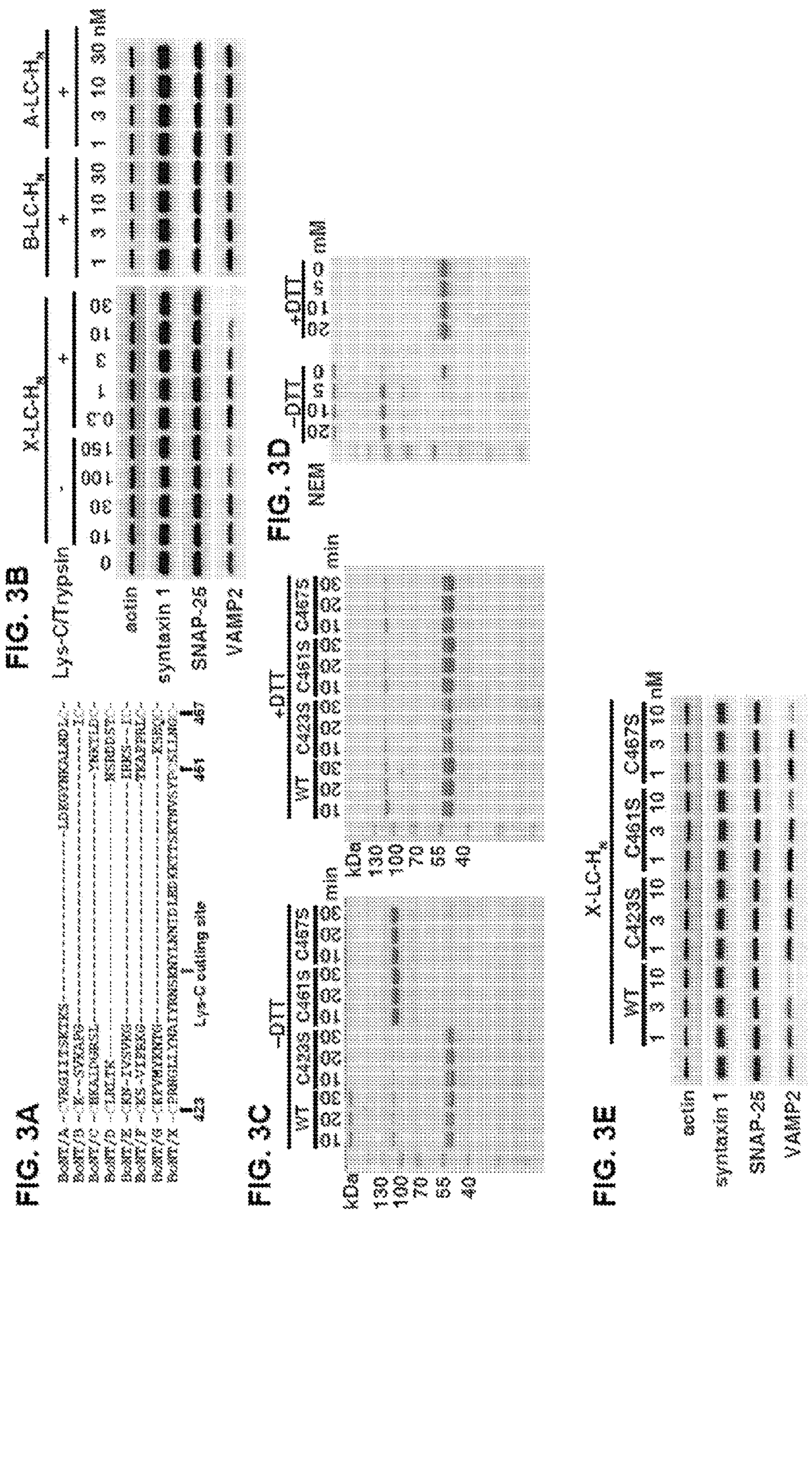


FIG. 3A
Lys-C cutting site
FIG. 3B
Lys-C/Trypsin
X-LC-H_N
B-LC-H_N
A-LC-H_N
actin
syntaxin 1
SNAP-25
VAMP2
FIG. 3C
-DTT
+DTT
WT
C423S
C461S
C467S
min
kDa
FIG. 3D
NEM
FIG. 3E
X-LC-H_N
nM

FIG. 4A

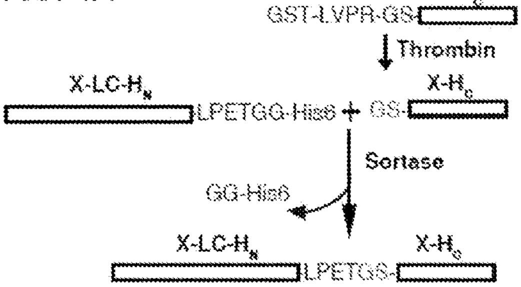


FIG. 4B

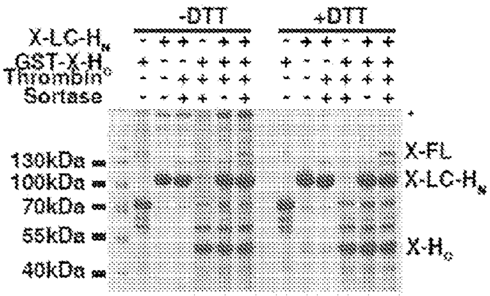


FIG. 4C

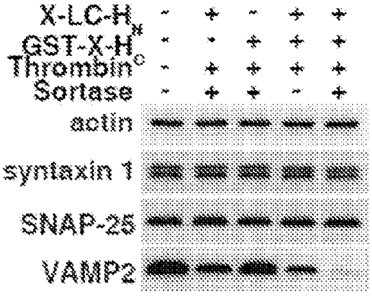


FIG. 4D

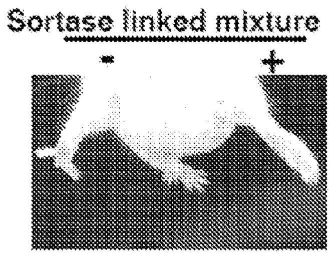


FIG. 4E

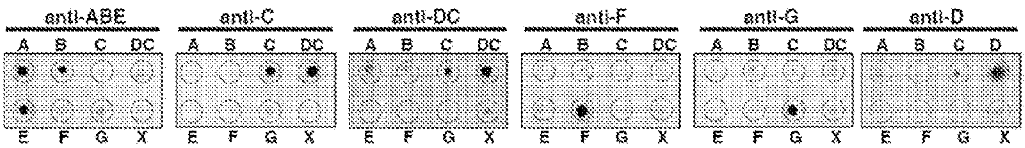


FIG. 4F

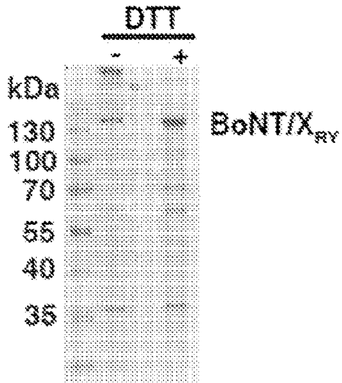
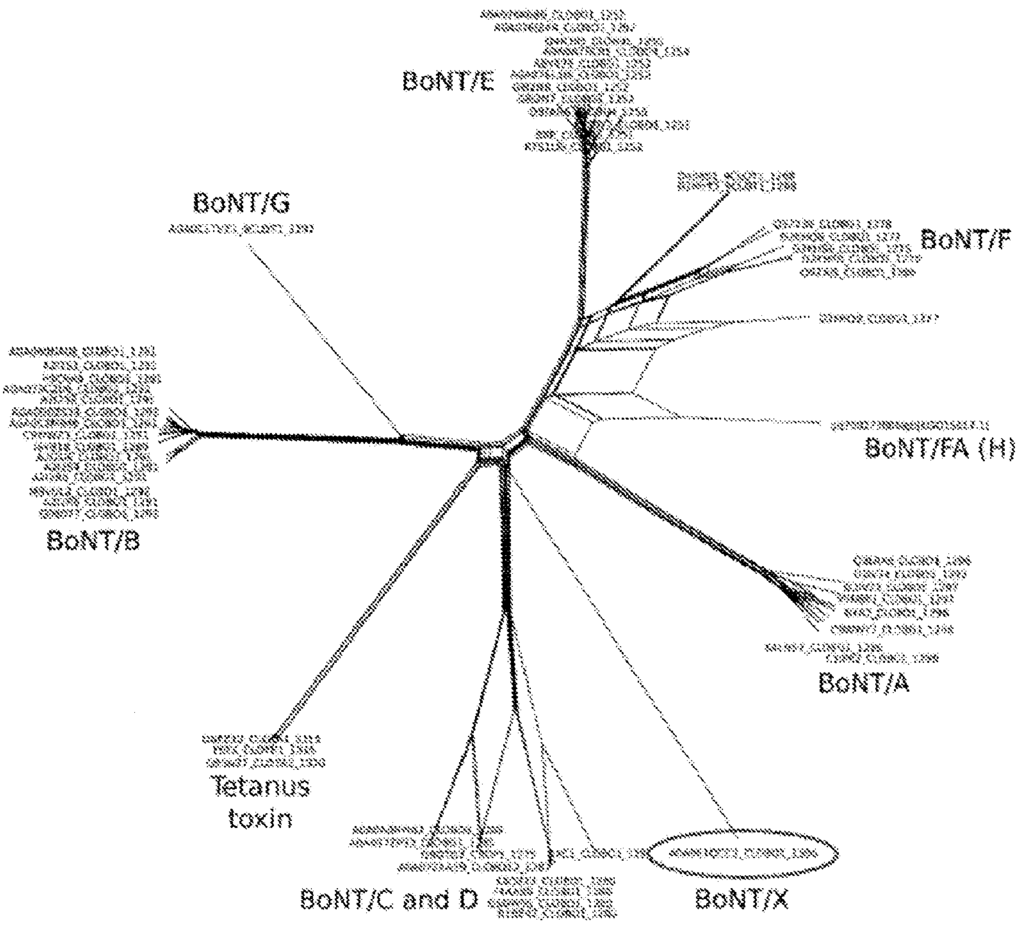


FIG. 5


BoNT/E
BoNT/G
BoNT/F
BoNT/FA (H)
BoNT/B
BoNT/A
Tetanus toxin
BoNT/C and D
BoNT/X

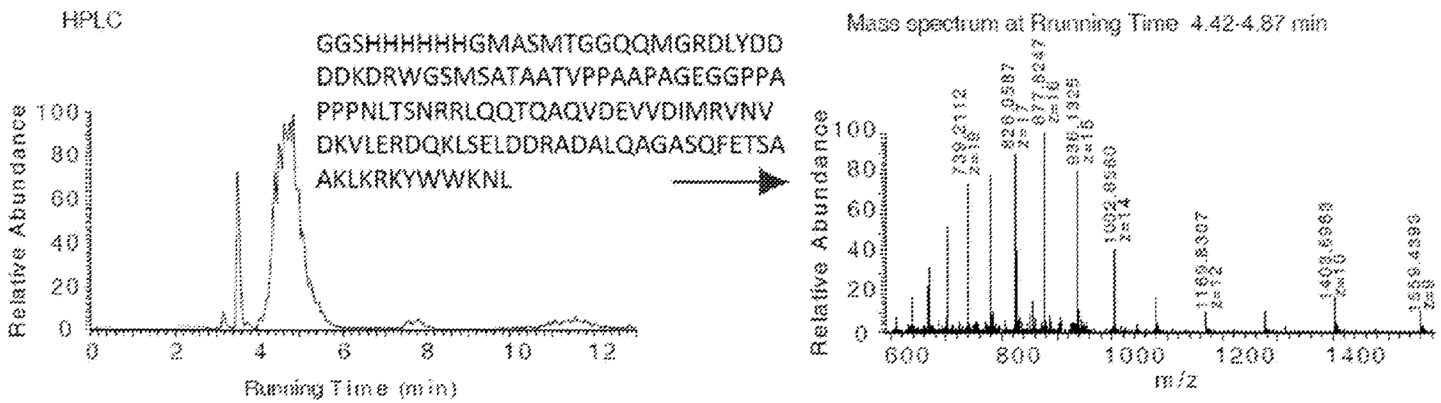

HPLC
Relative Abundance
Running Time (min)
GGSHHHHHHGMASMTGGQQMGRDLYDD
DDKDRWGSMSATAATVPPAAPAGEGGPPA
PPPNLTSNRRLQQTQAQVDEVVDIMRVNV
DKVLERDQKLSELDDRADALQAGASQFETSA
AKLKRKYWWKNL
Mass spectrum at Running Time 4.42-4.87 min
Relative Abundance
739.2112
z=18
826.0587
z=17
877.8247
z=16
936.1325
z=15
z=14
1169.8307
z=12
1403.6968
z=10
1559.4396
z=9
m/z

FIG. 6

FIG. 7A

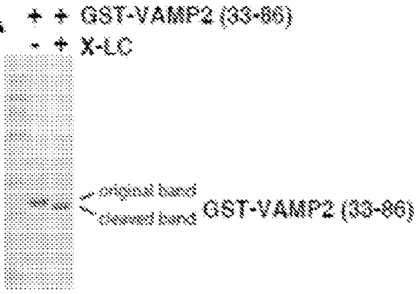


FIG. 7B

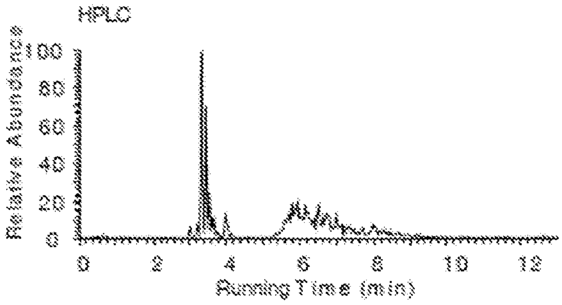
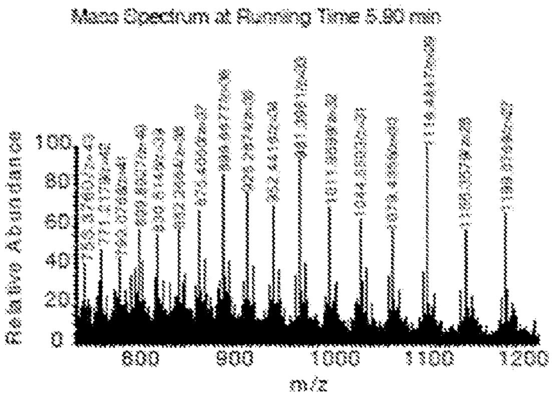


FIG. 7C



MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDK
WRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHN
MLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKV
DFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDAL
DVVLYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIA
WPLQGWQATFGGGDHPPKSDLVPRGSQQTQAQVDEV
VDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETSA
AKLKR

VAMP2 (33-66) VAMP2 (67-86)

FIG. 7D HPLC

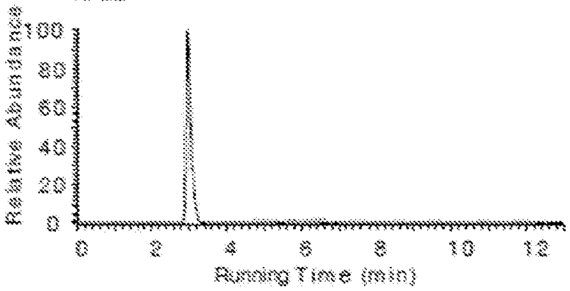


FIG. 7E

Mass Spectrum at Running Time 3.02 min

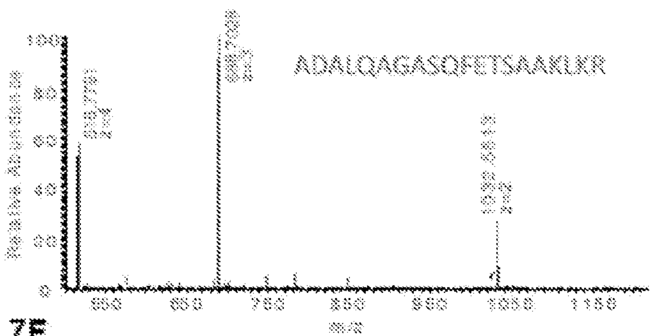
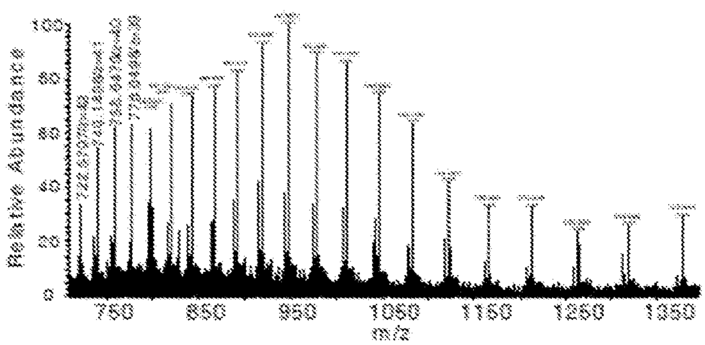


FIG. 7F

Mass Spectrum at Running Time 5.90 min



MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDK
WRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHN
MLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKV
DFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDAL
DVVLYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIA
WPLQGWQATFGGGDHPPKSDLVPRGSQQTQAQVDEV
VDIMRVNVDKVLERDQKLSELDDR

FIG. 8A

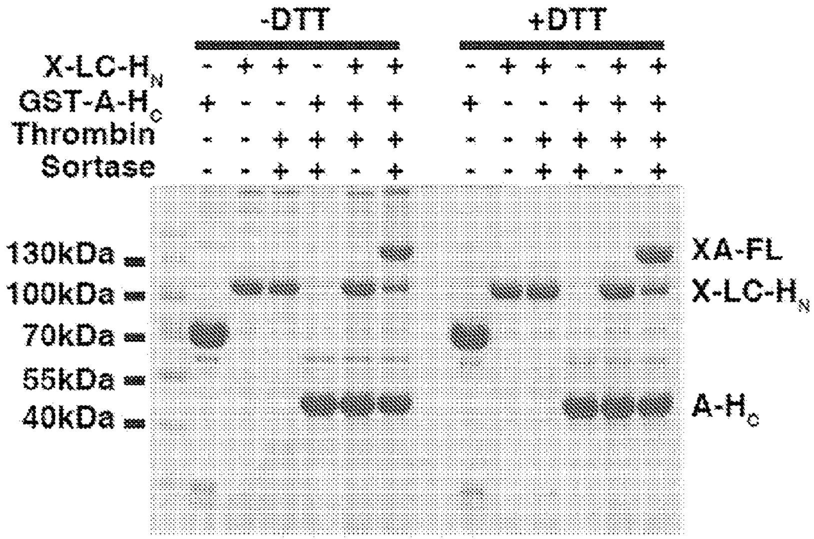

-DTT
+DTT
X-LC-HN
GST-A-HC
Thrombin
Sortase
130kDa
100kDa
70kDa
55kDa
40kDa
XA-FL
X-LC-HN
A-HC

FIG. 8B

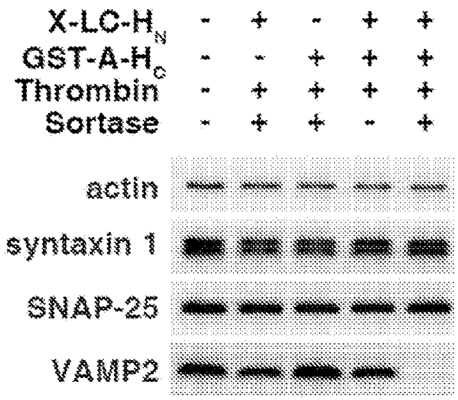
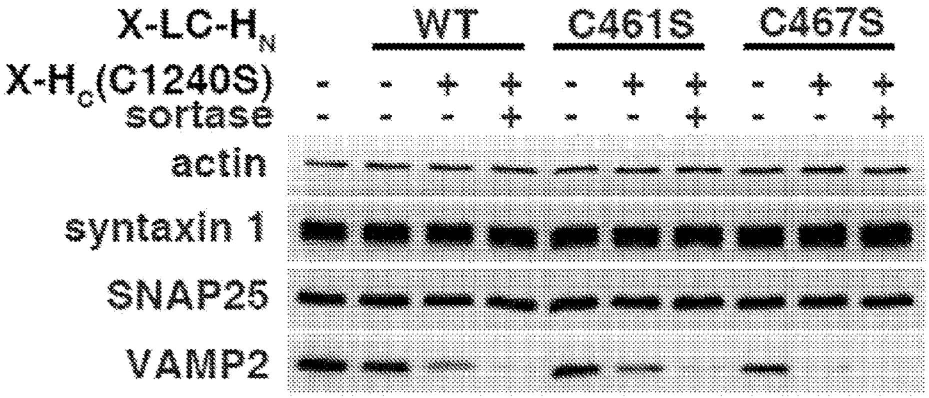

X-LC-HN
GST-A-HC
Thrombin
Sortase
actin
syntaxin 1
SNAP-25
VAMP2

FIG. 9


X-LC-H_N
WT
C461S
C467S
X-H_C(C1240S)
sortase
actin
syntaxin 1
SNAP25
VAMP2

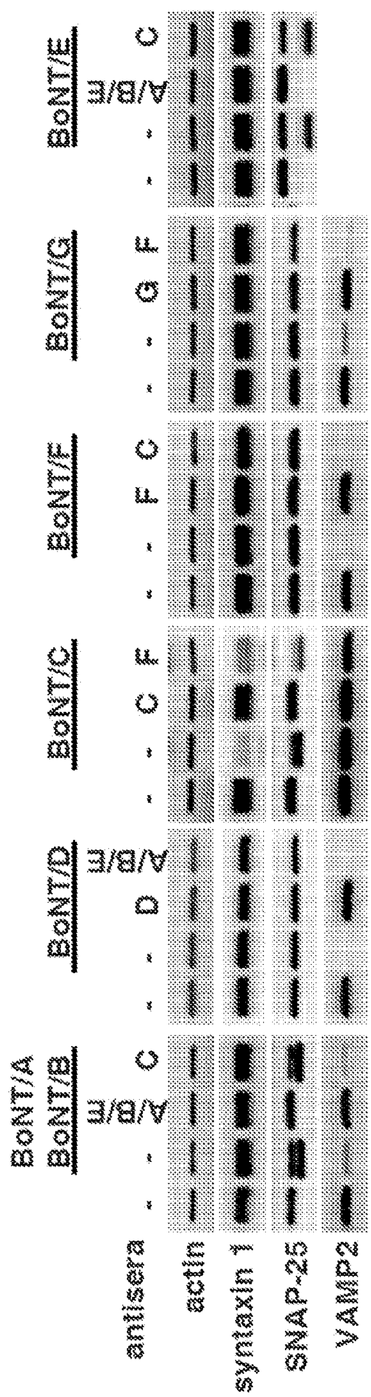

BoNT/A
BoNT/B
BoNT/D
BoNT/C
BoNT/F
BoNT/G
BoNT/E
antisera
actin
syntaxin 1
SNAP-25
VAMP2
A/B/E
C
D
F
G

FIG. 10

FIG. 11A

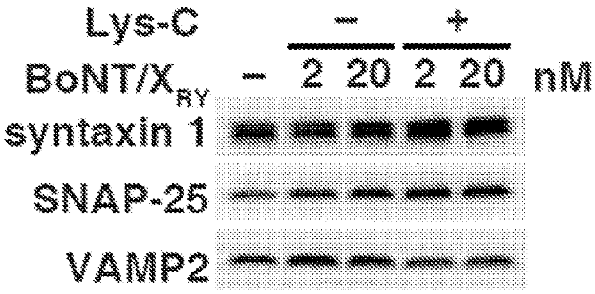


FIG. 11B

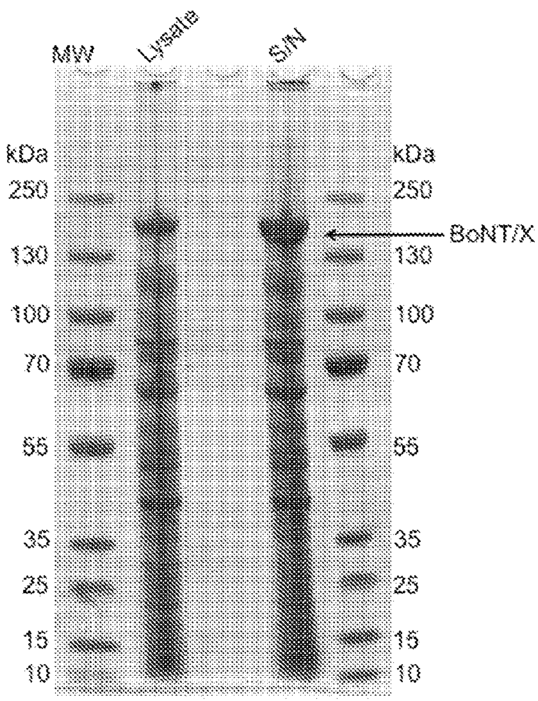


FIG. 11C

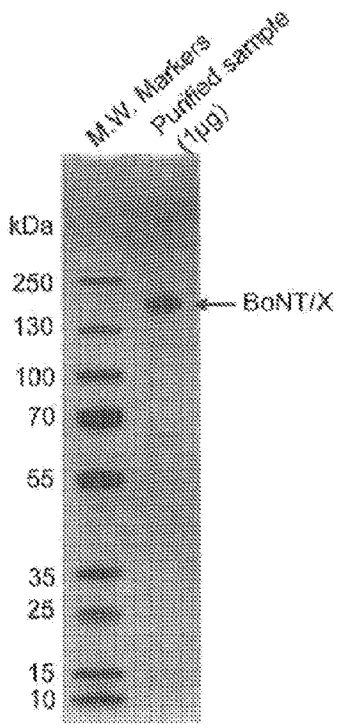


FIG. 12A

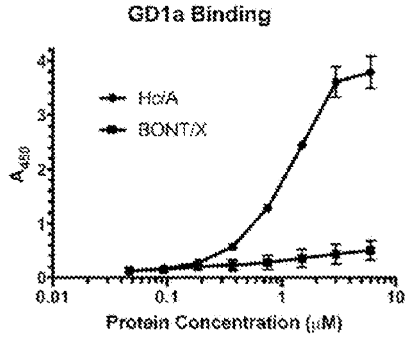


FIG. 12B

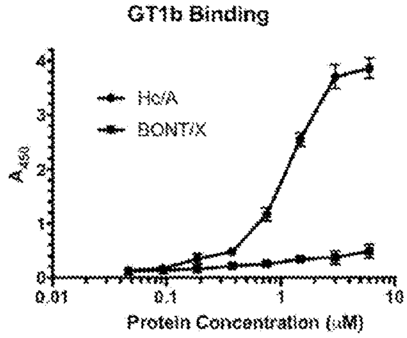


FIG. 12C

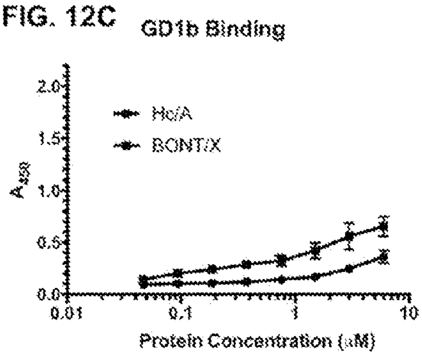


FIG. 12D

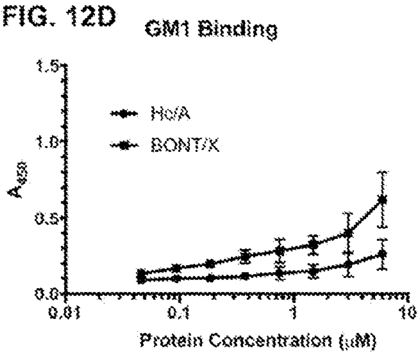


FIG. 12E

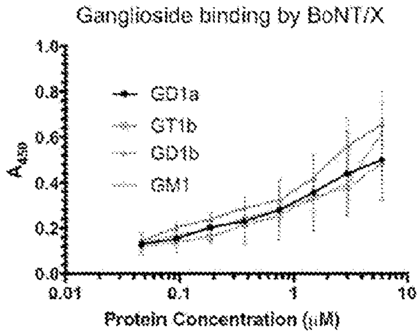


FIG. 12F

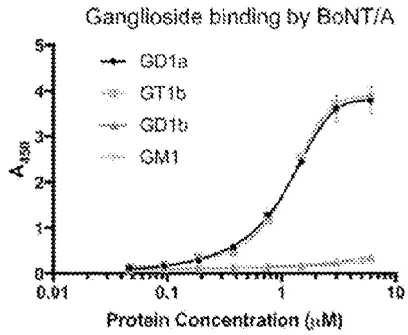


FIG. 13A

FIG. 13B

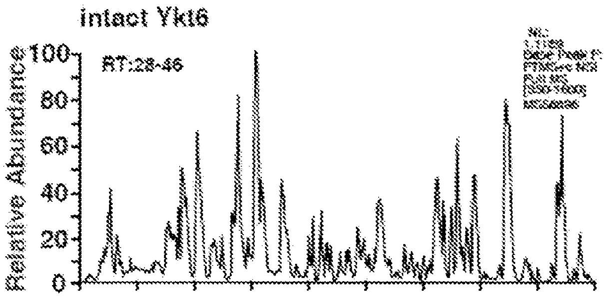


FIG. 13C

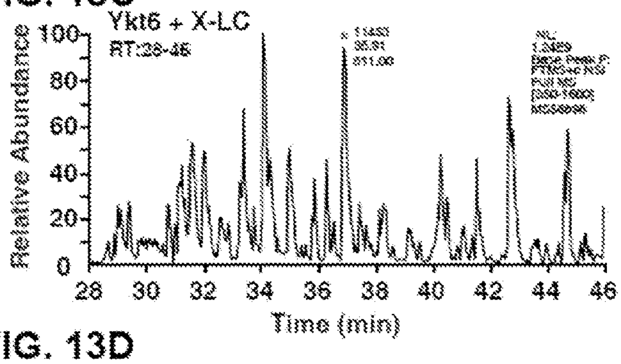


FIG. 13D

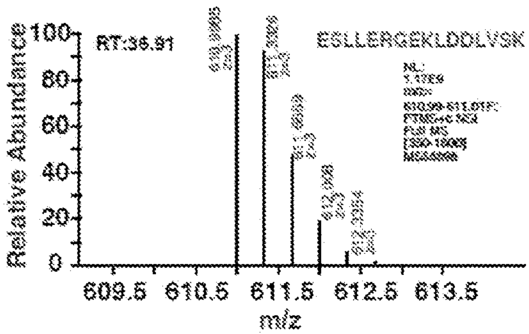


FIG. 14A

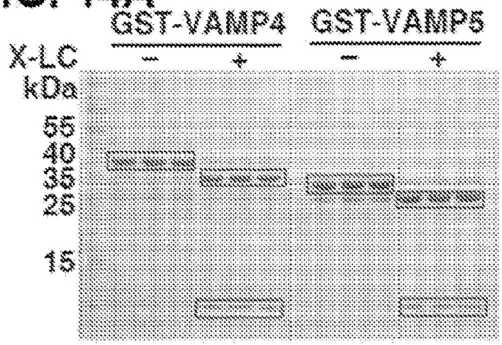
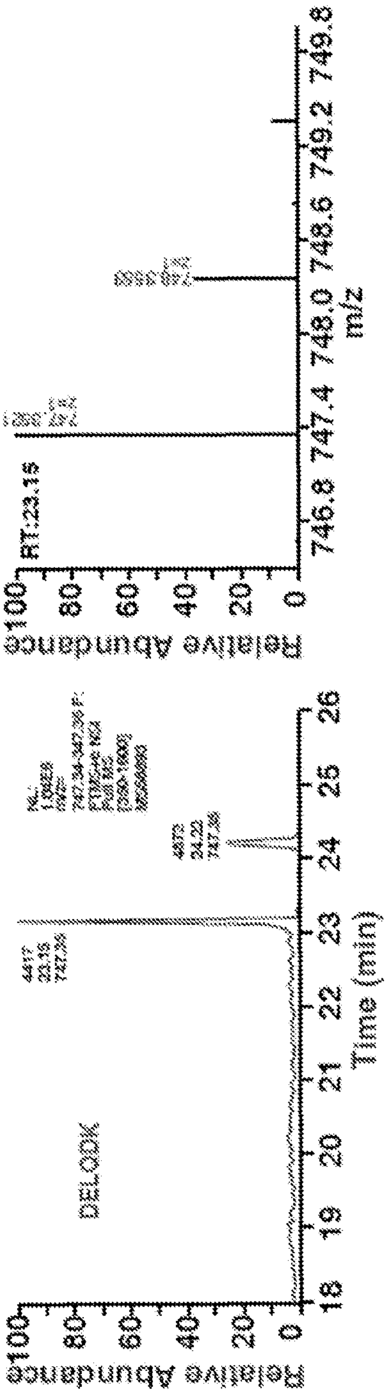


FIG. 14B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Relative Abundance
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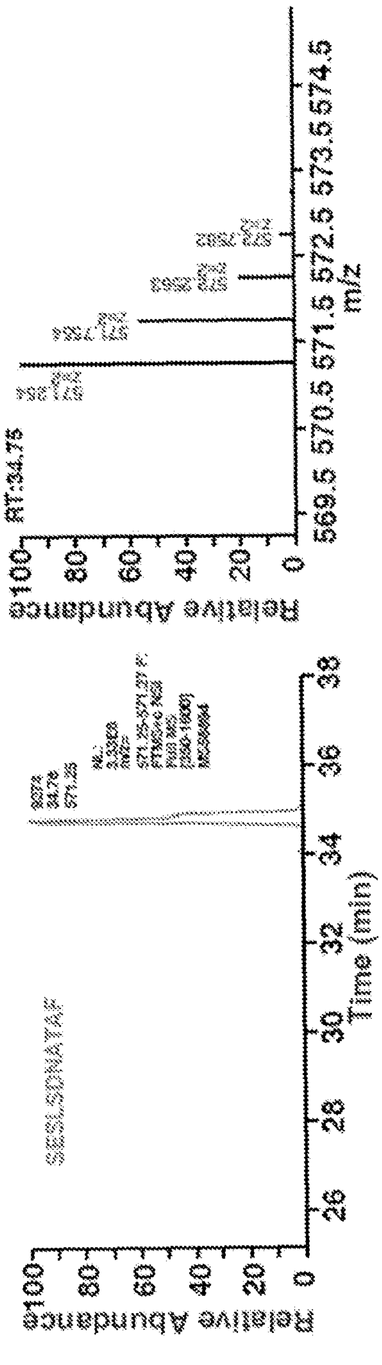
40
20
0
18
19
20
21
22
23
24
25
26
Time (min)
RT:23.15
746.8
747.4
748.0
748.6
749.2
749.8
m/z

FIG. 14C


Relative Abundance
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60
40
20
0
SESLSDNATAF
26
28
30
32
34
36
38
Time (min)
Relative Abundance
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60
40
20
0
RT:34.75
569.5 570.5 571 571.5 572 572.5 573.5 574.5
m/z

FIG. 14D

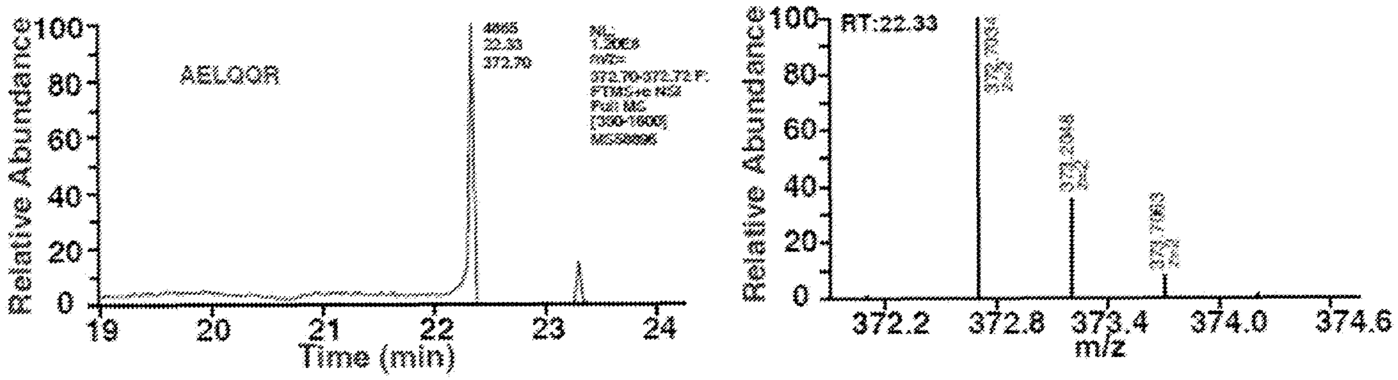
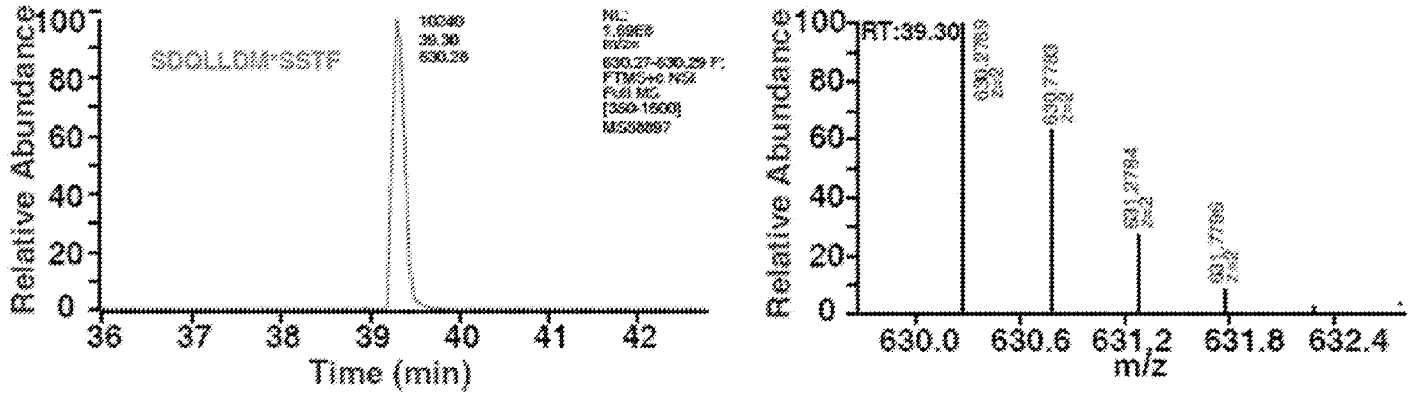


FIG. 14E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20
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SDQLLDM*SSTF
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37
38
39
40
41
42
Time (min)
Relative Abundance
RT:39.30
630.0
630.6
631.2
631.8
632.4
m/z

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.