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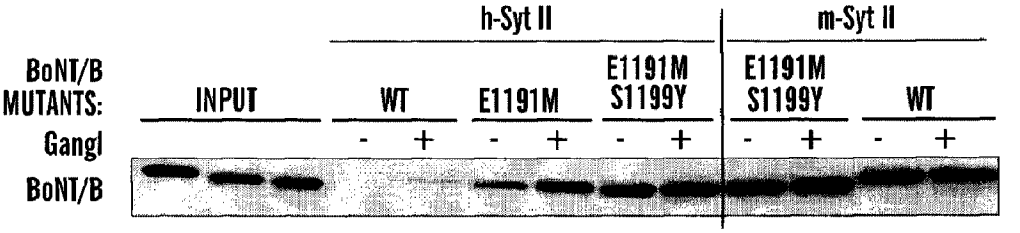

(57) Abrégé/Abstract:
Disclosed herein are botulinum neurotoxin (BoNT) polypeptides with a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c), comprising one or more substitution mutations corresponding to substitution mutations in serotype B, strain 1, V1118M; Y1183M; E1191M; E1191I; E1191Q; E1191T; S1199Y; S1199F; S1199L; SI 20 IV; or combinations thereof. Specific combination mutations include EI 191M and S1199L, E1191M and S1199Y, E1191M and S1199F, E1191Q and S1199L, E1191Q and S 1199Y, or E 1191 Q and S 1199F. Other substitution mutations are also disclosed. Isolated modified receptor binding domains, chimeric molecules, pharmaceutical compositions, and methods of using the same are also disclosed.

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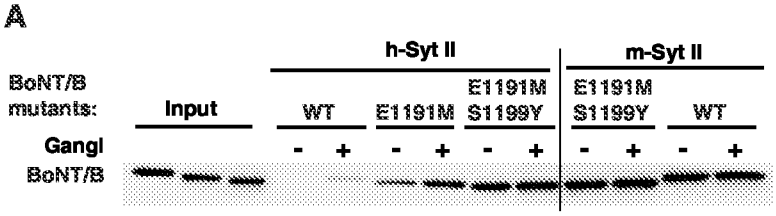

(57) Abstract: Disclosed herein are botulinum neurotoxin (BoNT) polypeptides with a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c), comprising one or more substitution mutations corresponding to substitution mutations in serotype B, strain 1, V1118M; Y1183M; E1191M; E1191I; E1191Q; E1191T; S1199Y; S1199F; S1199L; SI 20 IV; or combinations thereof. Specific combination mutations include El 191M and S1199L, E1191M and S1199Y, E1191M and S1199F, E1191Q and S1199L, E1191Q and S 1199Y, or E 1191 Q and S 1199F. Other substitution mutations are also disclosed. Isolated modified receptor binding domains, chimeric molecules, pharmaceutical compositions, and methods of using the same are also disclosed.

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ENGINEERED BOTULINUM NEUROTOXIN

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001]

GOVERNMENTAL SUPPORT

[0002] This invention was made with Government support under NCRR RR000168 awarded by the National Institute of Health. The Government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention relates to the field of therapeutics for neuromuscular disorders.

BACKGROUND OF THE INVENTION

[0004] Botulinum neurotoxins are a family of bacterial toxins, including seven major serotypes (BoNT/A-G) [1]. These toxins act by blocking neurotransmitter release from neurons, thus paralyzing animals and humans. 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 [2-4].

[0005] BoNT/A and BoNT/B are the only two BoNTs that are currently FDA-approved for use in humans [2-4]. These are toxins purified from bacteria without any sequence modifications (defined as wild type, WT). 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 [5]. Termination of BoNT usage often leaves patients with no other effective ways to treat/relieve their disorders. The possibility of antibody responses is directly related to both toxin doses and the frequency of injection [5]. Therefore, this limitation mainly occurs in treating muscle spasms, which involves relatively high doses of toxins. Consistently, antibody responses have not been observed in cosmetic applications, which use extremely low toxin doses [5].

[0006] The major adverse effects are also often associated with treating muscle spasms, but not cosmetic applications. This is because the adverse effects are largely due to diffusion of toxins to other regions of the body and the possibility of toxin diffusion is directly related to injected doses. The adverse effects ranges from transient non-serious events such as ptosis and diplopia to life-threatening events even death [6,7]. In a petition letter filed in 2008 by Dr. Sidney Wolfe to FDA, a total of 180 serious adverse events, including 16 deaths have been documented. As a result, FDA now requires the "Black box warning" on all BoNT products, highlighting the risk of the spread of toxins, following similar warnings issued by the European Union.

[0007] Because both the generation of neutralizing antibodies and toxin diffusion are directly related to injected doses, lowering toxin doses (while maintaining the same levels of toxin activity) is highly desired, which means the efficacy of individual toxin molecules has to be enhanced. Such modified BoNTs with improved specificity for neurons will also reduce any potential off-target effects due to non-specific entry into other cell types.

[0008] BoNTs target and enter neurons by binding to their specific receptors through their receptor binding domains, which are well-defined in the literature (BoNT-H_C, Fig. 1A, B) [1]. Receptor binding dictates the efficacy and specificity of BoNTs to recognize neurons. Improving the receptor binding ability of BoNTs will enhance their efficacy and specificity to target neurons. The receptors for most BoNTs have been identified (Fig. 1C). BoNT/B, D-C, and G share two homologous synaptic vesicle proteins synaptotagmin I and II (Syt I/II) as their receptors [8-13], while BoNT/A, E, D, and F use another synaptic vesicle protein SV2 [9,14-18]. In addition to protein receptors, all BoNTs require lipid co-receptor gangliosides (Fig. 1D), which are abundant on neuronal surfaces [19]. Among the two Syt isoforms in rodents and likely in most mammals, Syt II has ~10-fold higher binding affinity for BoNT/B than Syt I and is also the dominant isoform expressed in motor nerve terminals, which are the targeted neurons for BoNTs (Fig. 2A) [20,21]. Therefore, in rodents (on which most research has been conducted), Syt II is considered the major toxin receptor, while Syt I is a minor toxin receptor at motor nerve terminals.

[0009] One may argue that BoNTs already have high specificity to neurons, is it possible to further improve their binding to neurons? The answer is a "Yes" for humans, because it was recently discovered that the human Syt II has greatly diminished binding and function as the receptor for BoNT/B due to a unique amino acid change from rodent (rat/mouse) Syt II within the toxin binding site [13,22]. This is a change from phenylalanine (F) to leucine (L) at position 54 (mouse Syt II sequence) (Fig. 2B). Sequence alignments have revealed that

phenylalanine at this position is highly conserved in both Syt I and Syt II across vertebrates, including platypus, fish, rodents, and monkeys [23]. Only human and chimpanzee Syt II contains leucine at this position. As a result of this residue change, human and chimpanzee Syt II has greatly diminished binding to BoNT/B, D-C, and G (Fig. 2C) and is significantly less efficient in mediating the entry of BoNT/B (Fig. 2D), as compared to mouse Syt II. Since human and chimpanzee Syt I still contains phenylalanine at the same position and can bind BoNT/B, D-C, and G (Fig. 2E), the high affinity receptor for BoNT/B, D-C, and G in humans is restricted to the minor receptor Syt I. These findings provide an explanation for the clinical observations that a much higher dose of BoNT/B than BoNT/A (which binds a different receptor) is needed to achieve the same levels of therapeutic effects in patients [24,25]. Previously these observations were attributed to other reasons, such as the percentage of active neurontoxin in the preparations used. The recent observations of such binding differences of BoNT/B and human Syt II versus Syt II of other species suggests that different residues of BoNT/B may be involved in binding to human Syt II. As such, sequence modification to BoNT/B that is expected to affect binding to rodent SytII may have unpredictable affects on BoNT/B binding to human Syt II.

SUMMARY

[0010] One aspect of the invention relates to a botulinum neurotoxin (BoNT) polypeptide comprising a protease domain, a protease cleavage site, a translocation domain, and a modified receptor binding domain of *Clostridial botulinum* serotype B (B-H_c), comprising one or more substitution mutations corresponding to substitution mutations in serotype B, strain 1, selected from the group consisting of V1118M; Y1183M; E1191M; E1191I; E1191Q; E1191T; S1199Y; S1199F; S1199L; S1201V; and combinations thereof. In one embodiment, the modified (B-H_c) comprises two substitution mutations. In one embodiment, the two substitution mutations correspond to E1191M and S1199L, E1191M and S1199Y, E1191M and S1199F, E1191Q and S1199L, E1191Q and S1199Y, or E1191Q and S1199F. In one embodiment, the two substitution mutations correspond to E1191M and S1199L. In one embodiment, the two substitution mutations correspond to E1191M and S1199Y. In one embodiment, the two substitution mutations correspond to E1191M and S1199F. In one embodiment, the two substitution mutations correspond to E1191Q and S1199L. In one embodiment, the two substitution mutations correspond to E1191Q and S1199Y. In one embodiment, the two substitution mutations correspond to E1191Q and S1199F.

[0011] Another aspect of the invention relates to a botulinum neurotoxin (BoNT) polypeptide comprising a protease domain, a protease cleavage site, a translocation domain, and a modified receptor binding domain of Clostridial botulinum serotype B (B-Hc), comprising a substitution mutation at a position corresponding to S1199 or S1201 of serotype B, strain 1. In one embodiment, the substitution mutation produces enhanced binding of the modified B-Hc to human SytII and/or reduced binding of the modified B-Hc to human Syt I as compared to an identical molecule lacking the substitution mutation. In one embodiment, the substitution mutation produces enhanced binding of the modified B-Hc to human SytII and/or increased binding of the modified B-Hc to human Syt I as compared to an identical molecule lacking the substitution mutation. In one embodiment, the substitution mutation is selected from the group consisting of A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, T, W, Y and V substituted for S. In one embodiment, the substitution mutation is a non-naturally occurring amino acid substituted for S. In one embodiment, the modified B-Hc is of strain 1. In one embodiment, the protease domain, translocation domain, and protease cleavage site are from serotype selected from the group consisting of A, B, C, D, E, F, G, and combinations thereof. In one embodiment, the protease domain, translocation domain, and protease cleavage site are from serotype B, strain 1. In one embodiment, the protease domain, translocation domain, and protease cleavage site are from serotype A, strain 1.

[0012] Another aspect of the invention relates to a polypeptide comprising a modified receptor binding domain of Clostridial botulinum serotype B (B-Hc) comprising one or more substitution mutations corresponding to substitution mutations in serotype B, strain 1, selected from the group consisting of V1118M; Y1183M; E1191M; E1191I; E1191Q; E1191T; S1199Y; S1199F; S1199L; S1201V; and combinations thereof. In one embodiment, the modified (B-Hc) comprises two substitution mutations. In one embodiment, the two substitution mutations correspond to E1191M and S1199L, E1191M and S1199Y, E1191M and S1199F, E1191Q and S1199L, E1191Q and S1199Y, or E1191Q and S1199F. In one embodiment, the two substitution mutations correspond to E1191M and S1199L. In one embodiment, the two substitution mutations correspond to E1191M and S1199Y. In one embodiment, the two substitution mutations correspond to E1191M and S1199F. In one embodiment, the two substitution mutations correspond to E1191Q and S1199L. In one embodiment, the two substitution mutations correspond to E1191Q and S1199Y. In one embodiment, the two substitution mutations correspond to E1191Q and S1199F.

[0013] Another aspect of the invention relates to a polypeptide comprising a modified receptor binding domain of Clostridial botulinum serotype B (B-Hc) comprising a

substitution mutation at a position corresponding to S1199 or S1201 of serotype B, strain 1. In one embodiment, the substitution mutation produces enhanced binding of the modified B-Hc to human SytII and/or reduced binding of the modified B-Hc to human Syt I as compared to an identical molecule lacking the substitution mutation. In one embodiment, the substitution mutation produces enhanced binding of the modified B-Hc to human SytII and/or increased binding of the modified B-Hc to human Syt I as compared to an identical molecule lacking the substitution mutation. In one embodiment, the substitution mutation is selected from the group consisting of A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, T, W, Y and V substituted for S. In one embodiment, the substitution mutation is a non-naturally occurring amino acid substituted for S. In one embodiment, the modified B-Hc is of strain 1.

[0014] Another aspect of the invention relates to a chimeric molecule comprising a first portion that is a modified receptor binding domain of Clostridial botulinum serotype B (B-Hc) linked to a second portion, wherein the modified B-Hc comprises one or more substitution mutations corresponding to substitution mutations in serotype B, strain 1, selected from the group consisting of V1118M; Y1183M; E1191M; E1191I; E1191Q; E1191T; S1199Y; S1199F; S1199L; S1201V and combinations thereof. In one embodiment, the modified B-Hc comprises two substitution mutations. In one embodiment, the two substitution mutations correspond to E1191M and S1199L, E1191M and S1199Y, E1191M and S1199F, E1191Q and S1199L, E1191Q and S1199Y, or E1191Q and S1199F. In one embodiment, the two substitution mutations correspond to E1191M and S1199L. In one embodiment, the two substitution mutations correspond to E1191M and S1199Y. In one embodiment, the two substitution mutations correspond to E1191M and S1199F. In one embodiment, the two substitution mutations correspond to E1191Q and S1199L. In one embodiment, the two substitution mutations correspond to E1191Q and S1199Y. In one embodiment, the two substitution mutations correspond to E1191Q and S1199F. In one embodiment, the modified B-Hc comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-Hc) comprising a substitution mutation at a position corresponding to S1199 or S1201 of serotype B, strain 1. In one embodiment, the substitution mutation produces enhanced binding of the modified B-Hc to human SytII and/or reduced binding of the modified B-Hc to human Syt I as compared to an identical molecule lacking the substitution mutation. In one embodiment, the substitution mutation produces enhanced binding of the modified B-Hc to human SytII and/or increased binding of the modified B-Hc to human Syt I as compared to an identical molecule lacking the substitution mutation. In one embodiment, the substitution mutation is selected from the group consisting of A, R, N,

D, C, Q, E, G, H, I, L, K, M, F, P, T, W, Y and V substituted for S. In one embodiment, the substitution mutation is a non-naturally occurring amino acid substituted for S. In one embodiment, the modified B-Hc is of strain 1. In one embodiment, the first portion and the second portion are linked covalently. In one embodiment, the first portion and the second portion are linked non-covalently. In one embodiment, the second portion is selected from the group consisting of a small molecule, a nucleic acid, a short polypeptide and a protein. In one embodiment, the second portion is a bioactive molecule. In one embodiment, the second portion is a therapeutic polypeptide or non-polypeptide drug.

[0015] Another aspect of the invention relates to a nucleic acid comprising a nucleotide sequence that encodes the polypeptide or chimeric molecule described herein.

[0016] Another aspect of the invention relates to a nucleic acid vector comprising the nucleic acid described herein.

[0017] Another aspect of the invention relates to a cell comprising the nucleic acid vector described herein or the nucleic acid described herein.

[0018] Another aspect of the invention relates to a cell expressing the polypeptide or chimeric molecule described herein.

[0019] Another aspect of the invention relates to a pharmaceutical composition comprising the botulinum neurotoxin (BoNT) polypeptide described herein, or the chimeric molecule described herein, or the nucleic acid vector described herein, or the nucleic acid described herein. In one embodiment, the pharmaceutical composition further comprises a pharmaceutically acceptable excipient.

[0020] Another aspect of the invention relates to a kit comprising a pharmaceutical composition described herein and directions for therapeutic administration of the pharmaceutical composition.

[0021] Another aspect of the invention relates to a method to produce a botulinum neurotoxin (BoNT) polypeptide, the method comprising the steps of culturing the host cell described herein under conditions wherein said BoNT polypeptide is produced. In oneembodiment, the method further comprises recovering the BoNT polypeptide from the culture.

[0022] Another aspect of the invention relates to a method for treating a condition associated with unwanted neuronal activity comprising administering a therapeutically effective amount of the BoNT polypeptide described herein to a subject to thereby contact one or more neurons exhibiting unwanted neuronal activity, to thereby treat the condition. In one embodiment, 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, and dermatological or aesthetic/cosmetic conditions.

[0023] Another aspect of the invention relates to a botulinum neurotoxin (BoNT) polypeptide described herein, the pharmaceutical composition of described herein, the chimeric molecule described herein, or the polypeptide described herein, any one of which for use in a medicament or medicine.

[0024] Another aspect of the invention relates to a botulinum neurotoxin (BoNT) polypeptide described herein, the pharmaceutical composition of described herein, the chimeric molecule described herein, or the polypeptide described herein, any one of which for use in treating a condition associated with unwanted neuronal activity.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Figure 1A- Figure 1D shows schematic models for how BoNTs target neurons (A), their overall protein structure (B), a list of identified receptors (C), and the structural model for BoNT/B binding to its receptors Syt and gangliosides (D). Figure 1A) A schematic view of BoNT actions: BoNTs recognize neurons by binding to their specific receptors (step 1), enter neurons via receptor-mediated endocytosis (step 2), the light chains of BoNTs then translocate across endosomal membranes into the cytosol (step 3), where these light chains act as proteases to cleave target host proteins (step 4). Panel A is adapted from Arnon, S. et al, JAMA, 285:1059, 2001 [33]. Figure 1B: BoNTs are composed of a light chain and a heavy chain, connected via a disulfide bond. The heavy chain can be further divided into two domains: the translocation domain (H_N) and the receptor binding domain (HC). These functional domains are well-defined and switchable between different BoNTs [1]. This suggests that the modified BoNT/B-H_C can be used to replace BoNT/A-H_C to generate chimeric toxins. Figure 1C) A list of identified toxin receptors. Figure 1D) A structural model showing binding of BoNT/B to its protein receptor, rodent Syt (I/II), as well as its lipid co-

receptor, gangliosides, on the cell surface. D is adapted from Chai et al, Nature, 444:1096, 2006[31].

[0026] Figure 2A – Figure 2G show prior data adapted from published literatures showing (1) human Syt II is not an effective receptor for BoNT/B, D-C, and G; (2) residue changes in the receptor binding domain of BoNT/B can significantly change the binding affinity to Syt II and the potency of toxins; (3) key residues within the receptor binding domain of BoNT/B that have been hypothesized to contribute to binding Syt II. Figure 2A) The comparison between rodent Syt I and Syt II indicates that Syt II is the major toxin receptor, while syt I is a minor toxin receptor in rodent motor neurons. Figure 2B) Human Syt II differs from mouse/rat Syt II by a single residue within the toxin binding site (residue 54 in mouse Syt II, 51 in human Syt II). Figure 2C) Glutathione *S*-transferase (GST) tagged recombinant mouse Syt II 1-87 (m-Syt II) and a mouse Syt II 1-87 mutant mimicking human Syt II (F54L, herein referred to as h-Syt II) were immobilized on glutathione-Sepharose beads, and were used to pull down BoNT/B, BoNT/D-C, or BoNT/G, with or without the presence of ganglioside (Gangl). All three toxins bind to m-Syt II 1-87, but not h-Syt II in the pull-down assays. Figure 2D) Cultured rat hippocampal neurons only express Syt I but not Syt II [8]. Therefore, knocking down (KD) Syt I generates neurons with no endogenous toxin receptors. Full-length m-Syt II and h-Syt II were then expressed in Syt I KD hippocampal neurons, and these neurons were exposed to BoNT/B (20 nM, 5 min exposure, 24 hrs incubation). It has been found that h-Syt II was significantly less efficient than m-Syt II in mediating the entry of BoNT/B, BoNT/D-C, and BoNT/G into Syt I KD neurons, as evidenced by the degrees of cleavage of toxin substrate synaptobrevin (Syb). Figure 2E) Rat Syt I 1-83 and human Syt I 1-80 were used to pull down BoNT/B, BoNT/D-C, and BoNT/G, as described in panel C. Human Syt I mediated similar levels of toxin binding as rat Syt I did for all three toxins. Figure 2A to E are adapted from the recent publication: Peng et al, J. Cell Science, 2012, 125:3233 [13]. Figure 2F) The binding affinity of BoNT/B (also defined as BoNT/B1) and one of its subtypes known as BoNT/B2 to rat Syt II are determined in an competition assay, by using the receptor binding domain of BoNT/B1 and B2 (right panel) to compete the binding of ^{125}I labeled BoNT/B1 on recombinant Syt II (left panel). The IC50 (which reflect the binding affinity) is 0.48 nM for BoNT/B1, and 2 nM for BoNT/B2, ~ 4-fold difference. This affinity difference is due to the C-terminal of the receptor binding domain (residue 1028-1291), because exchanging this region between BoNT/B1 and BoNT/B2 (right panel) virtually switches their binding affinity (right panel). Figure 2G) List of residues that are different between BoNT/B1 and BoNT/B2. These residues are thought to be the reason for

the binding affinity difference between these two toxins to rodent Syt II. Therefore, these may be key residues that can influence the binding affinity between BoNT/B and human Syt II. Panels F to G are adapted from Ihara et al, 2003, BBA, 1625:19 [29]. (H) Single residue mutations within the receptor binding domain of BoNT/A and BoNT/B, as indicated in the table, can significantly change the potency and toxicity of these toxins. This panel is adapted from Rummel et al, 2004, Mol. Microbiology, 51:631 [30]. (I) The co-crystal structure of BoNT/B (grey) binding to Syt II (red) reveals the key residues (listed in the right table) that form the binding pocket in BoNT/B. This panel is adapted from Jin et al, 2006, Nature, 444:1092 [32]and Chai et al, 2006, Nature, 444:1096 [31].

[0027] Figure 3A- Figure 3B shows targeted mutagenesis of BoNT/B-H_C and their effects on binding to m-Syt II and h-Syt II. Figure 3A) WT BoNT/B-H_C and indicated BoNT/B-H_C mutants were expressed as recombinant proteins in *E.Coli*. Bacterial lysates were harvested and incubated with immobilized m-Syt II (1-87) or h-Syt II (1-87). Bound pellets were analyzed by immunoblot assays, detecting BoNT/B-H_C using the HA antibody. "Input" represents bacterial lysates. Mutants that show strong binding to h-Syt II are indicated by arrows. Figure 3B) A table that categorizes BoNT/B-H_C mutations tested in Figure 3A.

[0028] Figure 4A- Figure 4B shows further characterization of selected BoNT/B-H_C mutants for their binding to Syt I and Syt II. Figure 4A) BoNT/B-H_C WT and indicated mutants were expressed in E.Coli. Harvested bacterial lysates were incubated with immobilized GST-tagged human Syt I (1-80), with or without the presence of gangliosides. Bound materials were analyzed by immunoblot assays detecting BoNT/B-H_C. E1191M significantly enhanced binding of BoNT/B-H_C to human Syt I, whereas V1118M has reduced binding to human Syt I than WT BoNT/B-H_C. Figure 4B) WT BoNT/B-H_C and E1191M mutant were purified as His6-tagged recombinant proteins and were incubated with immobilized GST-tagged m-Syt II (1-87) or h-Syt II (1-87), with or without the presence of lipid co-receptor gangliosides (Gangl). BoNT/B-H_C cannot bind to h-Syt II without gangliosides and only displays a weak binding in the presence of gangliosides. Purified E1191M mutant binds h-Syt II without gangliosides, and the binding is further enhanced in the presence of gangliosides.

[0029] Figure 5A- Figure 5B show that binding to human Syt I/II can be further enhanced by combining selected single residue substitutions. Figure 5A) Selected double mutants that combine two mutation sites as indicated were tested for their ability to bind m-Syt II and h-Syt II in pull-down assays as described in Fig. 3A. Combinations of two sites, E1191M or E1191Q with S1199L or S1199Y or S1199F (marked by arrows) displayed robust binding to h-Syt II. Figure 5B) Binding of selected double mutants to human Syt I was analyzed in pull-

down assays. All double mutants displayed significantly enhanced binding to human Syt I as compared to WT BoNT/B-H_C.

[0030] Figure 6A-Figure 6D show further characterization of a representative double mutant, E1191M/S1199Y. Figure 6A) BoNT/B-H_C WT, E1191M, and E1191M/S1199Y mutants were expressed in E.Coli and purified as His6-tagged recombinant proteins. Equal amounts of these proteins (100 nM) were incubated with immobilized GST-tagged m-Syt II (1-87) or h-Syt II (1-87) as indicated, with or without the presence of gangliosides (Gangl). Bound materials were subjected to immunoblot analysis. "Input" represents the purified recombinant proteins in following orders: WT, E1191M, E1191M/S1199Y. WT BoNT/B-H_C cannot bind to h-Syt II without gangliosides and only displays a weak binding in the presence of gangliosides (lane 4, 5). E1191M mutant binds h-Syt II without gangliosides, and binding is further enhanced in the presence of gangliosides (lane 6,7). E1191M/S1199Y significantly enhanced binding to h-Syt II as compared to E1191M (lane 8, 9). Binding of E1191M/S1199Y to both h-syt II (lane 8,9) and m-Syt II (lane 10,11) are at similar levels as WT BoNT/B-HC binding to m-Syt II (lane 13,14). Figure 6B) Equal amounts of BoNT/B-H_C WT, E1191M, and E1191M/S1199Y mutants were incubated with GST tagged h-Syt I. Bound materials were subjected to immunoblot analysis. E1191M and E1191M/S1199Y both significantly enhanced binding to h-Syt I as compared to WT BoNT/B-H_C. Figure 6C) Titrations (nM) of purified WT BoNT/B-HC were incubated with m-Syt II, while titrations of purified E1191M/S1199Y were incubated with h-Syt II, as indicated. Bound materials were subjected to immunoblot analysis. Binding of E1191M/S1199Y to h-Syt II is at similar levels as binding of WT BoNT/B-H_C to m-Syt II. Figure 6D) Binding affinity between E1191M/S1199Y and h-Syt II was estimated based on quantifying the immunoblot results obtained in panel C. The Kd is estimated to be 19 +/- 3 nM for E1191M/S1199Y binding to h-Syt II, whereas the Kd for WT BoNT/B binding to m-Syt II is 68 +/- 12 nM. Therefore, binding of E1191M/S1199Y to h-Syt II is ~ 3.5 fold higher than WT BoNT/B binding to m-Syt II.

[0031] Figure 7 shows that BoNT/B-H_C E1191M/S1199Y mutant can bind to h-Syt II expressed on surface of neurons. Cultured rat hippocampal neurons express only Syt I, but not Syt II. Therefore, knocking-down (KD) Syt I expression via lentiviral infection created neurons without any endogenous Syt and that abolished the binding of WT and E1191M/S1199Y BoNT/B-H_C (the second frame from the left). M-Syt II, m-Syt II (F54L), and h-Syt II were then expressed in these neurons via lentiviral infection. WT BoNT/B-H_C can bind to m-Syt II, but not m-Syt II (F54L) or h-Syt II. E1191M/S1199Y mutant can bind

to both m-Syt II and h-Syt II on neuron surface. Synapsin was also labeled as a marker for synapses.

[0032] Figure 8 is the amino acid sequence of the BoNT/B-Hc (strain 1; BoNT/B1 Okra strain). Residues 857-1291 of BoNT/B, strain 1, GenBank: AB232927.1, (SEQ ID NO: 1).

[0033] Figure 9 is the nucleic acid sequence encoding BoNT/B-Hc (strain B1, Okra strain) residues 857-1291 of BoNT/B, strain 1, based on GenBank: AB232927.1), which has been optimized for expression in E. coli. The nucleic acid sequence is shown in SEQ ID NO: 2.

[0034] Figure 10 shows the amino acid sequence of *C. botulinum* serotype A (1296 a.a.) (SEQ ID NO: 3).

[0035] Figure 11 shows the amio acid sequence of *C. botulinum* serotype B (1291 a.a.) (SEQ ID NO: 4).

[0036] Figure 12 shows the amino acid sequence of *C.botulinum* serotype C1 (1291 a.a.) (SEQ ID NO: 5).

[0037] Figure 13 shows the amino acid sequence of *C.botulinum* serotype D (1276 a.a.) (SEQ ID NO: 6).

[0038] Figure 14 shows the amino acid sequence of *C.botulinum* serotype E (1252 a.a.)(SEQ ID NO: 7).

[0039] Figure 15 shows the amino acid sequence of *C.botulinum* serotype F (1274 a.a.) (SEQ ID NO: 8).

[0040] Figure 16 shows the amino acid sequence of *C.botulinum* serotype G (1297 a.a.) (SEQ ID NO: 9).

DETAILED DESCRIPTION OF THE INVENTION

[0041] Aspects of the invention relate to the generation of *C. botulinum* neurotoxin (BoNT) polypeptide which has improved binding to its human receptors through the incorporation of a modified receptor binding domain. From these findings, a new generation of therapeutic BoNTs can be created by utilizing the modified receptor binding domain identified herein, with improved efficacy and specificity to target human neurons than the currently utilized WT BoNTs.

Definitions

[0042] As used herein, the term "binding affinity" means how strong a molecule's binding activity is for a particular receptor system. In general, high binding affinity results from

greater intermolecular force between a binding domain and its receptor system while low binding affinity involves less intermolecular force between the ligand and its receptor. High binding affinity involves a longer residence time for the binding domain at its receptor binding site than is the case for low binding affinity. As such, a molecule with a high binding affinity means a lower concentration of that molecule is required to maximally occupy the binding sites of a receptor system and trigger a physiological response. Conversely, low binding affinity means a relatively high concentration of a molecule is required before the receptor binding sites of a receptor system is maximally occupied and the maximum physiological response is achieved. Thus, a botulinum neurotoxin of the present invention with increased binding activity due to high binding affinity will allow administration of reduced doses of the toxin, thereby reducing or preventing unwanted side-effects associated with toxin dispersal into non-targeted areas.

[0043] As the term is used herein, "significantly enhanced binding" when used to describe the binding affinity of a *C. botulinum* neurotoxin molecule of the present invention to a specific receptor, refers to an increase in binding affinity for a specific receptor that is substantially increased (e.g., by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the binding affinity of the wild type molecule) as compared to the non-substituted version of the molecule. In one embodiment, the enhanced binding is an order of magnitude or more higher than the Kd of the non-substituted neurotoxin (e.g., the neurotoxin with a naturally occurring BoNT H_C molecule). The term "significantly enhanced binding" when used to describe the binding affinity of a BoNT/B-H_C binding fragment produced by the point mutations described herein refers to an increase in binding affinity of the modified binding domain (expressed as an isolated fragment of the entire BoNT protein) to a specific receptor that is substantially increased (e.g., by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the binding affinity) as compared to the binding of the non-substituted version of the molecule. In one embodiment, the enhanced binding is significantly higher (e.g., 1.5X, 2.0X, 2.5X, 3.0X, etc.) than the Kd of the non-substituted fragment.

[0044] As used herein, the term "botulinum neurotoxin" means any polypeptide that can execute the overall cellular mechanism whereby a *C. botulinum* toxin enters a neuron and inhibits neurotransmitter release and encompasses the binding of a *C. botulinum* toxin to a low or high affinity receptor complex, the internalization of the toxin, the translocation of the toxin light chain into the cytoplasm and the enzymatic modification of a *C. botulinum* toxin substrate.

[0045] A "modified receptor binding domain" or "modified H_C", as the term is used herein, facilitates the binding of the *C. botulinum* neurotoxin molecule in which it is comprised, to a receptor for *C. botulinum* neurotoxin located on the surface of a target cell. Such a molecule is typically generated through genetic recombination technology. The modified H_C has a binding activity for the receptor for *C. botulinum* neurotoxin located on the surface of a target cell. As used herein, the term "binding activity" means that one molecule is directly or indirectly contacting another molecule via at least one intermolecular or intramolecular force, including, without limitation, a covalent bond, an ionic bond, a metallic bond, a hydrogen bond, a hydrophobic interaction, a van der Waals interaction, and the like, or any combination thereof. "Bound" and "bind" are considered terms for binding.

[0046] As used herein, the term "*C. botulinum* toxin protease domain" means a *C. botulinum* toxin domain that can execute the enzymatic target modification step of the intoxication process. Thus, a *C. botulinum* toxin 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 like a SNAP-25 substrate, a VAMP substrate and a Syntaxin substrate.

[0047] Non-limiting examples of *C. botulinum* toxin protease domains are provided in Table 1 and 2.

[0048] As used herein, the term "*C. botulinum* toxin translocation domain" or "H_N" means a *C. botulinum* toxin domain that can execute the translocation step of the intoxication process that mediates *C. botulinum* toxin light chain translocation. Thus, a H_N facilitates the movement of a *C. botulinum* toxin light chain across a membrane and encompasses the movement of a *C. botulinum* toxin light chain through the membrane an intracellular vesicle 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/C1 H_N, a BoNT/D H_N, a BoNT/E H_N, a BoNT/F H_N, and a BoNT/G H_N, the amino acid sequences of which are provided in Table 1 and Figures 10-16.

[0049] As used herein, the term "*C. botulinum* receptor-binding domain" is synonomous with "H_C domain" and means any naturally occurring *C. botulinum* receptor binding domain that can execute the cell binding step of the intoxication process, including, e.g., the binding of the *C. botulinum* toxin to a *C. botulinum* toxin-specific receptor system located on the plasma membrane surface of a target cell. It is envisioned that replacement of the binding activity can be achieved by, e.g., replacing the entire *C. botulinum* H_C domain with a modified (e.g., enhanced) H_C domain.

[0050] As used herein, the term "*C. botulinum* toxin target cell" means a cell that is a naturally occurring cell that a naturally occurring *C. botulinum* toxin is capable of

intoxicating, including, without limitation, motor neurons; sensory neurons; autonomic neurons; such as, e.g., sympathetic neurons and parasympathetic neurons; non-petidergic 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.

[0051] 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 flanking DNA or from the natural source of the DNA.

[0052] 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 polyepeptides). 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. Recast, 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).

[0053] The term “conservative” or “conservative substitution mutation” as used herein refers to a mutation where an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure, chemical properties, and/or hydropathic nature of the polypeptide to be substantially unchanged. The following groups of amino acids have been historically substituted for one another as conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, try, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. Other commonly accepted conservative substitutions are listed below:

Residue	Conservative Substitutions	Residue	Conservative Substitutions
Ala	Ser	Leu	Ile; Val
Arg	Lys	Lys	Arg; Gln
Asn	Gln; His	Met	Leu; Ile
Asp	Glu	Phe	Met; Leu; Tyr
Gln	Asn	Ser	Thr; Gly
Cys	Ser	Thr	Ser; Val
Glu	Asp	Trp	Tyr
Gly	Pro	Tyr	Trp; Phe
His	Asn; Gln	Val	Ile; Leu
Ile	Leu, Val		

[0054] 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 subsitutions 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 (hydrophyllic) 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.

[0055] 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 but are not limited to 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.

[0056] The term "therapeutically effective amount" refers to an amount that is sufficient to effect a therapeutically significant reduction in one or more symptoms of the condition when administered to a typical subject who has the condition. A therapeutically significant reduction in a symptom is, e.g. about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 100%, or more as compared to a control or non-treated subject.

[0057] The term "treat" or "treatment" refers to therapeutic treatment wherein the object is to eliminate or lessen symptoms. Beneficial or desired clinical results include, but are not limited to, elimination of symptoms, alleviation of symptoms, diminishment of extent of condition, stabilized (i.e., not worsening) state of condition, delay or slowing of progression of the condition.

[0058] 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. 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).

[0059] Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but are not limited to these examples. 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.

Embodiments

[0060] The observation that BoNT/B is less specific and potent in humans due to its inability to bind human Syt II, may explain why comparatively higher doses are required than BoNT/A. Higher BoNT/B doses correspond to increased chances for triggering antibody responses and for serious side-effects to occur. Therefore, improved binding of BoNT/B to

the human receptor Syt II, to increase its efficacy and specificity to target human neurons should allow a reduced amount of the toxin doses used in therapeutic applications.

[0061] Aspects of the invention arise from the finding that modifying the protein sequence of BoNT/B-H_C modifies binding of the fragment containing the receptor binding domain, to the human Syt II receptor. Specific modifications have been identified that enhance binding, thereby generating a domain that binds human Syt II with high-affinity. The modified BoNT/B-H_C, when in the context of a full length BoNT protein, retains these binding properties. Incorporation of a modified receptor binding domain with enhanced binding, into a molecule comprising the other BoNT domains, thereby generates a full length BoNT molecule with similarly enhanced receptor bindings. As such, new versions of BoNT with high-affinity binding to human Syt II are generated. BoNT with significantly enhanced binding can be used in similar therapies, albeit at lower doses than presently available BoNT molecules, thus providing safer methods of treatment.

[0062] The BoNT polypeptides, including full-length BoNT polypeptides and BoNT polypeptide fragments or domains described herein, and nucleic acid molecules which encode them, are explicitly encompassed in the invention. These polypeptides and nucleic acid molecules can be generated by recombinant DNA procedures known in the art. Such polypeptides are typically referred to as "recombinant polypeptides" or "recombinant nucleic acids".

[0063] BoNT has the overall structure shown in Figure 1B. BoNT is comprised of three domains, each domain having a specific and independent function: a protease domain (also referred to as the light chain), a translocation domain (H_N), and a receptor-binding domain (H_C). Domains of the various strains of *C. botulinum* neurotoxin have been shown to be largely interchangeable (as demonstrated by naturally occurred chimeric toxins such as BoNT/CD, which is composed of the light chain and H_N of BoNT/C, with the H_C of BoNT/D [34], in U.S. Patent 8,052,979). The protein can be in single chain form or di-chain form. The di-chain form results from the naturally occurring protease processing of a protease cleavage site located between the protease domain and the translocation domain. The protein is maintained in the Di-chain form following protease processing by the presence of a di-sulfide bond.

[0064] One aspect of the invention relates to a botulinum neurotoxin (BoNT) comprising a protease domain, a translocation domain, and a modified receptor binding domain of Clostridial botulinum serotype B, as described herein, and a protease cleavage site. Typically these are arranged in a linear amino-to-carboxyl single polypeptide order of the protease

domain, the protease cleavage site, the translocation domain and the modified receptor binding domain. However, different arrangements of the various domains are expected to function adequately. In one embodiment, the modified receptor binding domain comprises one or more substitution mutations which lead to significantly enhanced binding to the human Syt I receptor and/or the human Syt II receptor.

[0065] Strains of *Clostridia botulinum* produce seven antigenically-distinct types of Botulinum toxins (BoNTs), which have been identified by investigating botulism outbreaks in man (BoNT/A, /B, /E and /F), animals (BoNT/C1 and /D), or isolated from soil (BoNT/G). While all seven BoNT serotypes have similar structure and pharmacological properties, each also displays heterogeneous bacteriological characteristics. The genetic diversity of the *C. botulinum* strains is described in detail in Hill et al. (Journal of Bacteriology, Vol. 189, No. 3, p. 818-832 (2007))[35].

[0066] Toxins from the various *C. botulinum* strains share the same functional domain organization and overall structural architecture. *C. botulinum* toxins are each translated as a single chain polypeptide of approximately 150 kDa that is subsequently cleaved by proteolytic scission within a disulfide loop by a naturally-occurring protease, such as, e.g., an endogenous *C. botulinum* toxin protease or a naturally-occurring proteases produced in the environment. This posttranslational processing yields a di-chain molecule comprising an approximately 50 kDa light chain (LC) and an approximately 100 kDa heavy chain (HC) held together by a single disulfide bond and noncovalent interactions. Each mature di-chain molecule comprises three functionally distinct domains: 1) a proteolytic domain located in the LC that includes a metalloprotease region containing a zinc-dependent endopeptidase activity which specifically targets core components of the neurotransmitter release apparatus; 2) a translocation domain contained within the amino-terminal half of the HC (H_N) that facilitates release of the LC from intracellular vesicles into the cytoplasm of the target cell; and 3) a binding domain found within the carboxyl-terminal half of the HC that determines the binding activity and binding specificity of the toxin to the receptor complex located at the surface of the target cell. The locations of the specific domains within the toxin are provided in Table 1:

TABLE 1

C. botulinum toxin domains from various strains

Toxin	LC	H_N	H_C
BoNT/A	M1-K448	A449-K871	N872-L1296

BoNT/B	M1-K441	A442-S858	E859-E1291
BoNT/C1	M1-K449	T450-N866	N867-E1291
BoNT/D	M1-R445	D446-N862	S863-E1276
BoNT/E	M1-R422	K423-K845	R846-K1252
BoNT/F	M1-K439	A440-K864	K865-E1274
BoNT/G	M1-K446	S447-S863	N864-E1297

[0067] Complete amino acid sequences of the toxins are provided in Figures 10-16.

[0068] The binding, translocation and protease activity of these three functional domains are all necessary for toxicity. The overall cellular intoxication mechanism whereby *C. botulinum* toxins enter a neuron and inhibit neurotransmitter release is similar, regardless of serotype or subtype. Without wishing to be bound by theory, the intoxication mechanism involves at least four steps: 1) receptor binding, 2) complex internalization, 3) light chain translocation, and 4) protease target modification. The process is initiated when the H_C domain of a *C. botulinum* toxin binds to a toxin-specific receptor located on the plasma membrane surface of a target cell. The binding specificity of a receptor complex is thought to be achieved, in part, by specific combinations of gangliosides and protein receptors. Once bound, the toxin/receptor complexes are internalized by endocytosis and the internalized vesicles are sorted to specific intracellular routes. The translocation step is triggered by the acidification of the vesicle compartment. Once translocated, light chain endopeptidase of the toxin is released from the intracellular vesicle into the cytosol where it specifically targets one of three proteins known as the core components of the neurotransmitter release apparatus (vesicle-associated membrane protein (VAMP)/synaptobrevin, synaptosomal-associated protein of 25 kDa (SNAP-25) and Syntaxin). These core components are necessary for synaptic vesicle docking and fusion at the nerve terminal and constitute members of the soluble N-ethylmaleimide-sensitive factor-attachment protein-receptor (SNARE) family. BoNT/A and BoNT/E cleave SNAP-25 in the carboxyl-terminal region, releasing a nine or twenty-six amino acid segment, respectively, and BoNT/C1 also cleaves SNAP-25 near the carboxyl-terminus. The botulinum serotypes BoNT/B, BoNT/D, BoNT/F and BoNT/G, and tetanus toxin, act on the conserved central portion of VAMP, and release the amino-terminal portion of VAMP into the cytosol. BoNT/C1 cleaves syntaxin at a single site near the cytosolic plasma membrane surface. The selective proteolysis of synaptic SNAREs accounts for the block of neurotransmitter release caused by *C. botulinum* toxins in vivo. The SNARE protein targets of *C. botulinum* toxins are common to exocytosis in a variety of non-neuronal types; in these cells, as in neurons, light

chain peptidase activity inhibits exocytosis, see, e.g., Yann Humeau et al., How Botulinum and Tetanus Neurotoxins Block Neurotransmitter Release, 82(5) Biochimie. 427-446 (2000); Kathryn Turton et al., Botulinum and Tetanus Neurotoxins: Structure, Function and Therapeutic Utility, 27(11) Trends Biochem. Sci. 552-558. (2002); Giovanna Lalli et al., The Journey of Tetanus and Botulinum Neurotoxins in Neurons, 11(9) Trends Microbiol. 431-437, (2003).

[0069] The botulinum neurotoxin of the present invention comprises a modified receptor binding domain. The modified receptor binding domain exhibits significantly enhanced binding to one or more human receptors typically bound and utilized by one or more *C. botulinum* toxin strains. Examples of specific modified receptor binding domains are provided herein. The isolated modified receptor binding domain polypeptide described herein is also encompassed by the present invention, as is the isolated nucleic acid molecule by which it is encoded.

[0070] The botulinum neurotoxin of the present invention also comprises a protease domain, also referred to in the art as a light chain variant. The light chain variant may be a naturally occurring light chain variant, such as, e.g., *C. botulinum* toxin light chain isoforms and *C. botulinum* toxin light chain subtypes; or a non-naturally occurring *C. botulinum* toxin light chain variant, such as, e.g., conservative substitution *C. botulinum* toxin light chain variants.

[0071] The botulinum neurotoxin of the present invention also comprises a toxin translocation domain (H_N).

[0072] The various domains described herein (e.g., H_N, H_c, or protease domain) include, without limitation, naturally occurring variants, such as, e.g., isoforms and subtypes; non-naturally occurring variants, such as, e.g., conservative substitution mutations. Non-naturally-occurring variants, refers to a domain that has at least one amino acid change from the corresponding region of the reference sequences (e.g., from Table 1 or Figures 10-16) and can be described in percent identity to the corresponding region of that reference sequence.

[0073] It is recognized by those of skill in the art that within each serotype of *C. botulinum* toxin there can be naturally occurring *C. botulinum* domain variants that differ somewhat in their amino acid sequence, and also in the nucleic acids encoding these proteins. A naturally occurring *C. botulinum* toxin domain (e.g., light chain, H_N or H_C) variant envisioned for use in the generation of the BoNT of the present invention can function in substantially the same manner as the reference *C. botulinum* toxin domain on which the naturally occurring *C. botulinum* domain variant is based, and can be substituted for the reference *C. botulinum* toxin domain in any aspect of the present invention.

[0074] A non-limiting example of a naturally occurring *C. botulinum* toxin domain variant is a *C. botulinum* toxin domain isoform such as, e.g., a BoNT/A domain isoform, a BoNT/B domain isoform, a BoNT/C1 domain isoform, a BoNT/D domain isoform, a BoNT/E domain isoform, a BoNT/F domain isoform, and a BoNT/G domain isoform. A *C. botulinum* toxin domain isoform can function in substantially the same manner as the reference *C. botulinum* toxin domain on which the *C. botulinum* toxin domain isoform is based, and can be substituted for the reference *C. botulinum* toxin domain in any aspect of the present invention.

[0075] Another non-limiting example of a naturally occurring *C. botulinum* toxin domain variant is a *C. botulinum* toxin domain subtype such as, e.g., a domain from subtype BoNT/A1, BoNT/A2,BoNT/A3, BoNT/A4, BoNT/A5; a domain from subtype BoNT/B1, BoNT/B2, BoNT/B3, BoNT/B4, BoNT/B5, BoNT/B6, BoNT/B7; a domain from subtype BoNT/C1-1, BoNT/C1-2, BoNT/D-C; a domain from subtype BoNT/E1, BoNT/E2, BoNT/E3, BoNT/E4, BoNT/E5, BoNT/E6, BoNT/E7, BoNT/E8; and a domain from subtype BoNT/F1, BoNT/F2, BoNT/F3, BoNT/F4, BoNT/F5, BoNT/F6, BoNT/F7. A *C. botulinum* toxin domain subtype can function in substantially the same manner as the reference *C. botulinum* toxin domain on which the *C. botulinum* toxin domain subtype is based, and can be substituted for the reference *C. botulinum* toxin domain in any aspect of the present invention.

[0076] As used herein, the term "non-naturally occurring variant" (e.g., *C. botulinum* toxin light chain variant, H_C and H_N) means a *C. botulinum* domain produced with the aid of human manipulation, including, without limitation, domains produced by genetic engineering using random mutagenesis or rational design and *C. botulinum* domains produced by chemical synthesis. Non-limiting examples of non-naturally occurring *C. botulinum* domain variants include, e.g., conservative *C. botulinum* domain variants. As used herein, the term "conservative *C. botulinum* domain variant" means a *C. botulinum* domain that has at least one amino acid substituted by another amino acid or an amino acid analog that has at least one property similar to that of the original amino acid from the reference *C. botulinum* domain sequence (e.g., Table 1 and Figures 10-16). The variant may have one, two, three, four, five or more conservative amino acid substitutions compared to the reference domain sequence. Examples of properties include, without limitation, similar size, topography, charge, hydrophobicity, hydrophilicity, lipophilicity, covalent-bonding capacity, hydrogen-bonding capacity, a physicochemical property, of the like, or any combination thereof. A conservative *C. botulinum* domain variant can function in substantially the same manner as

the reference *C. botulinum* toxin domain on which the conservative *C. botulinum* toxin domain variant is based, and can be substituted for the reference *C. botulinum* domain in any aspect of the present invention.

[0077] A non-naturally occurring *C. botulinum* toxin domain variant may substitute one or more amino acids (e.g., one, two, three, four, five or more) from the reference *C. botulinum* toxin domain on which the naturally occurring *C. botulinum* toxin domain is based. A non-naturally occurring *C. botulinum* toxin domain variant can also possess 95% or more (e.g., 96%, 97%, 98% or 99%) amino acid identity to the reference *C. botulinum* toxin domain on which the naturally occurring *C. botulinum* domain variant is based.

[0078] Various non-naturally occurring *C. botulinum* neurotoxins or specific domains thereof, are described in International Patent Publications WO95/32738, WO96/33273, WO98/07864 and WO99/17806.

[0079] The C. botulinum neurotoxin or specific domain thereof described herein will typically contain naturally occurring amino acid residues, but in some cases non-naturally occurring amino acid residues may also be present. Therefore, so-called "peptide mimetics" and "peptide analogues", which may include non-amino acid chemical structures that mimic the structure of a particular amino acid or peptide, may also be used within the context of the invention. Such mimetics or analogues are characterised generally as exhibiting similar physical characteristics such as size, charge or hydrophobicity, and the appropriate spatial orientation that is found in their natural peptide counterparts. A specific example of a peptide mimetic compound is a compound in which the amide bond between one or more of the amino acids is replaced by, for example, a carbon-carbon bond or other non-amide bond, as is well known in the art (see, for example Sawyer, in Peptide Based Drug Design, pp. 378-422, ACS, Washington D.C. 1995).

[0080] In one aspect of the invention, the botulinum neurotoxin (BoNT) of the present invention comprises a modified receptor binding domain of *C. botulinum* serotype B (BoNT/B-H_C). The modified BoNT/B-H_C comprises one or more substitution mutations which lead to significantly enhanced binding to the human Syt I receptor and/or the human Syt II receptor. In one embodiment, the BoNT/B-H_c is from BoNT/B1 (GenBank access No.: AB232927.1). The amino acid sequence of BoNT/B1-H_C Okra strain, used as the reference template in the present invention is shown in Figure 8. The generation of B-H_c from other strains by substitution of the amino acids that correspond to the specified position(s) in B1 described herein is also envisioned. Also encompassed in the invention is an isolated, purified modified receptor binding domain polypeptide described herein. The present

invention also encompasses a polypeptide comprising a modified receptor binding domain described herein. The invention also encompasses a nucleic acid molecule which encodes such a polypeptide. In one embodiment, the modified receptor binding domain is BoNT/B-H_C (e.g., from BoNT/B1).

[0081] Modification of the BoNT/B-H_C protein sequence can be performed by either targeted mutagenesis (site-directed mutagenesis) or random mutagenesis of each amino acid residue within the region known for binding Syt I/II. These Syt binding regions are well defined by previous studies relatingto mouse or rate Syt receptors [1,29,3631,32] but have not been clearly determined for interactions between BoNT/B-H_c and human Syt receptors. Different subtypes of BoNT/B can be used as the template to create the same or similar mutations by generating corresponding mutations described herein for B1-H_C. The corresponding position for selected residues to be mutated can be readily identified by sequence alignment with the B1 subtype. The resulting polypeptide products are encompasses by the instant invention, as are polypeptides comprising said products and nucleic acid molecules encoding said polypeptides and products.

[0082] Amino acid sequence modifications to produce the modified receptor binding domain can be mutation of a single residue to a different amino acid (single site substitution), mutation of multiple residues at the same time (multiple sites substitution), deletion of one or more residues (deletion), and insertion of one or more residues (insertion), as well as combinations thereof. Methods for mutating proteins are well-known in the art (e.g., targeted single site and multiple sites substitutions on the DNA encoding the BoNT/B-H_C sequence).

[0083] In one embodiment, one or more residues in BoNT/B-H_C that either contact rodent Syt II or the surrounding regions, based on previous literatures on BoNT/B receptor binding domain [29] and reported BoNT/B-Syt II structure (PDB ID: 2NM1) [31,32], are modified. These include, without limitation those positions that correspond to position Y1181, P1197, A1196, F1204, F1194, P1117, W1178, Y1183, V1118, S1116, K1113, K1192, S1199, S1201, E1191, E1245, Y1256 of BoNT/B-B1. In one embodiment, one or more of these residues is modified to a hydrophobic amino acid (e.g., V, I, L, M, F, W, C). In one embodiment, one or more of these residues is modified to a less hydrophobic amino acid (e.g., A, Y, H, T, S, P, Q, N and G). Combinations of various modifications are also envisioned, including, without limitation, mutations of two or more recited positions, to any variety of the herein recited various amino acids.

[0084] In one embodiment, the BoNT/B-H_C has one or more substitution mutation (e.g., at positions which correspond to positions E1191, S1199, S1201, V1118, P1117, Y1183,

A1196, and Y1181 of B1) that enhances binding to human Syt II as compared to WT BoNT/B-H_C. In one embodiment, the mutation comprises one or more mutations that correspond to E1191M/I/T/L/Q (E1191M, E1191I, E1191T, E1191L, or E1191Q), V1118M, S1199Y/L/F (S1199Y, S1199L, or S1199F), S1201V, P1117S/M/Y (P1117S, P1117M, or P1117Y), Y1183M, Y1181M, A1196Y of B1, or combinations thereof (Fig. 3A, B). Suitably the mutations are selected form the above mutations at positions 1118, 1191 and 1199 or combinations thereof. In particular, mutations selected from one or more of V1118M, E1191M/Q/I and S1199Y may be beneficial. More particularly, the mutation that corresponds to position E1191M or E1191Q of B1 is envisioned, since they display the strongest enhancement for binding h-Syt II. The mutations corresponding to E1191M or E1191Q of B1 also significantly enhanced binding of BoNT/B-H_C to human Syt I as compared to WT BoNT/B-H_C (Fig. 4A). In one embodiment, the BoNT/B-H_C has two substitution mutations.

[0085] Multiple site substitutions can also be generated by combining mutations in these identified key residues. Such multiple site substitution mutants have further enhanced binding to human Syt I and h-Syt II (Fig. 5). As a non-limiting example, mutations that combine two single site substitutions such as those corresponding to E1191M or E1191Q with S1199L, S1199Y or S1199F of B1 displayed significantly enhanced binding to both human Syt I and h-Syt II (Fig. 5). The enhancement in binding strength was surprising given the relatively modest enhancement in bidning activity achieved by mutations at the 1199 position alone.

[0086] In one embodiment substitution of a residue corresponding to position E1191, S1199, S1201, V1118, P1117, A1196, Y1181, and Y1183 of BoNT/B- B1 is envisioned, since it will yield a BoNT/B-H_C mutant with enhanced binding to human Syt II. Additional combination substitutions at positions including, but not limited to those that correspond to E1191, S1199, S1201, V1118, P1117, Y1181, Y1183, and A1196 of B1 yield BoNT/B-H_C mutants with enhanced binding to human Syt II.

[0087] Accordingly, the invention encompasses polypeptides comprising BoNT/B-H_C with modified amino acid sequence relative to the sequence of WT BoNT/B-H_C, wherein the modified BoNT/B-H_C has significantly enhanced binding to human Syt I and II as compared to WT BoNT/B-H_C. The invention further encompasses nucleic acid molecules encoding such polypeptides. In a preferred embodiment, the modified BoNT/B-H_C mutants contain amino acids substitutions at one or combinations of the amino acid residues corresponding to V1118, E1191, S1199, S1201, P1117, Y1181, Y1183, and A1196 of B1. In one

embodiment, these modifications include mutations corresponding to E1191M or E1191Q in combination with S1199L, S1199Y or S1199F of B1.

[0088] The present invention also encompass mutant full-length BoNT/B that contain the same amino acid substitutions in B-H_c as described above for therapeutic applications in humans. In a preferred embodiment, the full-length BoNT/B mutants contain amino acids substitutions at one or combinations of the amino acid residues corresponding to position E1191, V1118, S1199, S1201, P1117, Y1181, Y1183, and A1196 of B1. In one embodiment, the modifications include combinations of E1191M or E1191Q with S1199L, S1199Y or S1199F. The mutations can be made in the same manner as disclosed above for BoNT/B-H_C, using any one of BoNT/B subtypes as templates. These mutant BoNT/B toxins have significantly enhanced binding to both human Syt II and human Syt I, therefore will achieve higher efficacy and specificity to target human neurons than WT BoNT/B.

[0089] Toxin diffusion and generation of neutralization antibodies are not limited to BoNT/B, but also observed for BoNT/A, indicating that the binding affinity of BoNT/A to its receptor SV2 also needs to be improved. Because BoNT/B binding to Syt I/II has much higher affinity than BoNT/A binding to SV2 [14,20,26,27], a modified BoNT/B receptor binding domain (BoNT/B-H_C) with the ability to bind human Syt II can also be used to replace BoNT/A-H_C to generate a modified chimeric BoNT/A with greater efficacy and specificity for human neurons than WT BoNT/A. [28 29 30]

[0090] It is further envisioned that the modified BoNT/B-H_C described above can be utilized to replace the H_C of all other BoNTs. The H_C regions of each BoNTs are well defined and their replacement can be performed via standard PCR fusion of DNA encoding BoNT/B-H_C with the H_N-LC of other BoNTs, which has been well-established in the art. In addition, these replacements may also be performed using the C-terminal part of BoNT/B-H_C (designated as H_{CC}), which is the region containing the binding site for protein receptors and gangliosides in each BoNT. The resulting chimeric toxins will have the ability to target human neurons via binding to human Syt I/II. As a non-limiting example, modified BoNT/B-H_C can be used to replace the H_C of BoNT/A. The resulting polypeptides are encompassed by the instant invention. These chimeric toxin will have a higher efficacy and specificity targeting human neurons than WT BoNT/A. Such a chimeric BoNT/A toxin can be used for therapeutic applications in humans and offers significant improvements over WT BoNT/A.

[0091] Another aspect of the invention relates to an isolated nucleic acid molecule comprising a nucleotide sequence that encodes the polypeptides described herein (e.g., modified receptor binding domain or the botulinum neurotoxin comprising the modified

receptor binding domain, described herein). In one embodiment, the nucleic acid molecule comprises the nucleic acid sequence shown in in Figure 9. Such nucleic acid molecules can be produced by recombinant DNA techniques.

[0092] Another aspect of the invention relates to a nucleic acid vector comprising the nucleic acid molecule described herein. In one embodiment the vector is an expression vector. Such an expression vector is referred to herein as an expression construct, and comprises a nucleic acid molecule disclosed herein operably-linked to the expression vector useful for expressing the nucleic acid molecule in a cell or cell-free extract. A wide variety of expression vectors can be employed for expressing a nucleic acid molecule encoding a *C. botulinum* neurotoxin of the present invention including, without limitation, a viral expression vector; a prokaryotic expression vector; eukaryotic expression vectors, such as, e.g., a yeast expression vector, an insect expression vector and a mammalian expression vector; and a cell-free extract expression vector. It is further understood that expression vectors useful to practice aspects of these methods may include those which express the *C. botulinum* neurotoxin under control of a constitutive, tissue-specific, cell-specific or inducible promoter element, enhancer element or both. Non-limiting examples of expression vectors, along with well-established reagents and conditions for making and using an expression construct from such expression vectors are readily available from commercial vendors that include, without limitation, BD Biosciences-Clontech, Palo Alto, Calif.; BD Biosciences Pharmingen, San Diego, Calif.; Invitrogen, Inc, Carlsbad, Calif.; EMD Biosciences-Novagen, Madison, Wis.; QIAGEN, Inc., Valencia, Calif.; and Stratagene, La Jolla, Calif. The selection, making and use of an appropriate expression vector are routine procedures well within the scope of one skilled in the art and from the teachings herein.

[0093] Another aspect of the invention relates to a cell comprising the nucleic acid molecule or expression construct 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). These protocols are routine procedures within the scope of one skilled in the art and from the teaching herein.

[0094] It is also envisioned that the modified BoNT/B-H_C described here can be utilized as a delivery tool to target neurons in humans. For example, the modified BoNT/B-H_C can be linked to other therapeutic agents, covalently or non-covalently, and acts as the targeting vehicle to deliver the therapeutic agents to neurons in humans by binding to human Syt I/II. As such, another aspect of the invention relates to a chimeric polypeptide molecule comprising a first portion that is a modified receptor binding domain of *C.botulinum* serotype B, comprising one or more substitution mutations which leads to significantly enhanced binding to the human Syt I receptor and/or the human Syt II receptor, 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 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.

[0095] Another aspect of the present invention relates to a pharmaceutical composition comprising the *C. botulinum* neurotoxin, or chimeric molecule described herein. In one

embodiment, the polypeptide described herein is an active ingredient in a composition comprising a pharmaceutically acceptable carrier (referred to herein as a pharmaceutical composition). A "pharmaceutically acceptable carrier" means any pharmaceutically acceptable means to mix and/or deliver the targeted delivery composition to a subject. 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, solvent or encapsulating material, involved in carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the composition and is compatible with administration to a subject, for example a human. Such compositions can be specifically formulated for administration via one or more of a number of routes, such as the routes of administration described herein. Supplementary active ingredients also can be incorporated into the compositions. When an agent, formulation or pharmaceutical composition described herein, is administered to a subject, preferably, a therapeutically effective amount is administered. As used herein, the term "therapeutically effective amount" refers to an amount that results in an improvement or remediation of the condition. In one embodiment, the pharmaceutical composition is formulated for administration by injection. In one embodiment, the pharmaceutical composition involves the botulinum neurotoxin encapsulated in microspheres. In one embodiment, the pharmaceutical composition involves the botulinum neurotoxin formulated for slow release.
[0096] In one embodiment, the botulinum neurotoxin, polypeptide, or chimeric molecule of the present invention is in the form of a controlled release formula. Such compositions and methods for adminstration are provides in U.S. Patent publication No. 2007/0020295.

[0097] 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. 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_1, D and E are synthesized by nonproteolytic strains and are therefore typically unactivated when recovered from culture. Serotypes B and F are produced by both proteolytic and nonproteolytic 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 exact proportion of nicked to unnicked 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 invention 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.

[0098] Also encompassed in the present invention is a kit comprising the pharmaceutical composition described herein. The kit may further comprise a delivery tool or device for the therapeutic administration of the composition, and/or instructions for therapeutic administration.

[0099] Another aspect of the invention relates to a delivery tool or device for administration of the pharmaceutical compositions described herein, pre-loaded with the pharmaceutical composition (e.g., for single use). Such devices may be a syringe or a microneedle ***device*** for delivery of the compositions. The syringe may be a single use syringe pre-loaded with an effective amount of the composition. The microneedle device may comprise one or more microneedles coated with the composition described herein, such as is described in U.S. Patent Publication 2010/0196445.

Methods of Treatment

[00100] The present invention also includes methods for treating a condition 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). Such conditions are associated with unwanted neuronal activity, as determined by the skilled practitioner. The method comprises the step of administering a therapeutically effective amount of a pharmaceutical composition described herein (e.g., containing a botulinum neurotoxin (BoNT) or a chimeric molecule) to the appropriate location in the mammal to reduce the unwanted neuronal activity, to thereby treat the condition. Adminstration is by a route that contacts an effective amount of the composition to neurons exhibiting the unwanted activity.

[00101] Specific conditions envisioned for 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 invention can be used to treat dermatological or aesthetic/cosmetic conditions, for example, reduction of brow furrows, reduction of skin wrinkles. The present invention can also be used in the treatment of sports injuries.

[00102] 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, using substantially similar methods as disclosed by Borodic, a modified neurotoxin can be administered to a mammal, preferably a human, to treat spinal curvature. In a suitable embodiment, a modified neurotoxin comprising botulinum type E fused with a leucine-based motif is administered. Even more preferably, a modified neurotoxin 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.

[00103] In addition, the modified neurotoxin can be administered to treat other neuromuscular disorders using well known techniques that are commonly performed with botulinum type A. For example, the present invention 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.

[00104] 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.

In one embodiment, substantially similar methods to that of

Sanders et al. can be employed, but using a modified neurotoxin, to treat autonomic nervous system disorders such as the ones discussed above. For example, a modified neurotoxin 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.

[00105] 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.

In one embodiment, substantially similar methods to that of Binder can be employed, but using a modified neurotoxin, 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 modified neurotoxin. 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.

[00106] Furthermore, a modified neurotoxin can be administered to a mammal to treat pain that is not associated with a muscular disorder, such as spasm. In one broad embodiment, methods of the present invention to treat non-spasm related pain include central administration or peripheral administration of the modified neurotoxin.

[00107] 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.

In one embodiment, substantially similar methods to that of Foster et al. can be employed, but using the compositions described herein to treat pain. The pain to be treated can be an acute pain or chronic pain.

[00108] 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. In one embodiment, the modified neurotoxin is administered subcutaneously at or near the location of pain, for example, at or near a cut. In some embodiments, the modified neurotoxin 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 modified neurotoxin 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 invention

[00109] Routes of administration for such methods are known in the art and easily adapted to the methods described herei 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). 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.

[00110] The embodiments described here and in the following examples are for illustrative purposes only, and various modifications or changes apparent to those skilled in the art are included within the scope of the invention.

[00111] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[00112] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[00113] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used to describe the present invention, in connection with percentages means $\pm 1\%$.

[00114] In one respect, the present invention relates to the herein described compositions, methods, and respective component(s) thereof, as essential to the invention, yet open to the inclusion of unspecified elements, essential or not ("comprising"). In some embodiments, other elements to be included in the description of the composition, method or respective component thereof are limited to those that do not materially affect the basic and

novel characteristic(s) of the invention ("consisting essentially of"). This applies equally to steps within a described method as well as compositions and components therein. In other embodiments, the inventions, compositions, methods, and respective components thereof, described herein are intended to be exclusive of any element not deemed an essential element to the component, composition or method ("consisting of").

[00115] All patents, patent applications, and publications identified

are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00116] The present invention may be as defined in any one of the following numbered paragraphs.

1. A botulinum neurotoxin (BoNT) polypeptide comprising:
   a) a protease domain;
   b) a protease cleavage site;
   c) a translocation domain; and
   d) a modified receptor binding domain of *Clostridial botulinum* serotype B ($B\text{-}H_c$), comprising one or more substitution mutations corresponding to substitution mutations in serotype B, strain 1, selected from the group consisting of:

      V1118M; Y1183M; E1191M; E1191I; E1191Q; E1191T; S1199Y; S1199F; S1199L; S1201V; and combinations thereof.

2. The BoNT polypeptide of paragraph 1, wherein the modified ($B\text{-}H_c$) comprises two substitution mutations.

3. The BoNT polypeptide of paragraph 2, wherein the two substitution mutations correspond to E1191M and S1199L, E1191M and S1199Y, E1191M and S1199F, E1191Q and S1199L, E1191Q and S1199Y, or E1191Q and S1199F.

4. The BoNT polypeptide of one of paragraphs 2-3, wherein the two substitution mutations correspond to E1191M and S1199L.

5. The BoNT polypeptide of one of paragraphs 2-3, wherein the two substitution mutations correspond to E1191M and S1199Y.

6. The BoNT polypeptide of one of paragraphs 2-3, wherein the two substitution mutations correspond to E1191M and S1199F.

7. The BoNT polypeptide of one of paragraphs 2-3, wherein the two substitution mutations correspond to E1191Q and S1199L.

8. The BoNT polypeptide of one of paragraphs 2-3, wherein the two substitution mutations correspond to E1191Q and S1199Y.

9. The BoNT polypeptide of one of paragraphs 2-3, wherein the two substitution mutations correspond to E1191Q and S1199F.

10. A botulinum neurotoxin (BoNT) polypeptide comprising:
    a) a protease domain;
    b) a protease cleavage site;
    c) a translocation domain; and
    d) a modified receptor binding domain of *Clostridial botulinum* serotype B ($B\text{-}H_c$), comprising a substitution mutation at a position corresponding to S1199 or S1201 of serotype B, strain 1.

11. The BoNT polypeptide of paragraph 10 wherein the substitution mutation produces enhanced binding of the modified $B\text{-}H_c$ to human SytII and/or reduced binding of the modified $B\text{-}H_c$ to human Syt I as compared to an identical molecule lacking the substitution mutation.

12. The BoNT polypeptide of paragraph 10 wherein the substitution mutation produces enhanced binding of the modified $B\text{-}H_c$ to human SytII and/or increased binding of

the modified B-H_c to human Syt I as compared to an identical molecule lacking the substitution mutation.

13. The BoNt polypeptide of any one of paragraphs 11-12 wherein the substitution mutation is selected from the group consisting of A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, T, W, Y and V substituted for S.

14. The BoNt polypeptide of any one of paragraphs 11-13 wherein the substitution mutation is a non-naturally occurring amino acid substituted for S.

15. The BoNT polypeptide of any one of paragraphs 1 -14, wherein the modified B-H_c is of strain 1.

16. The BoNT polypeptide of any one of paragraphs 1-15 wherein the protease domain, translocation domain, and protease cleavage site are from serotype selected from the group consisting of A, B, C, D, E, F, G, and combinations thereof.

17. The BoNT polypeptide of paragraph 16, wherein the protease domain, translocation domain, and protease cleavage site are from serotype B, strain 1.

18. The BoNT polypeptide of paragraph 16, wherein the protease domain, translocation domain, and protease cleavage site are from serotype A, strain 1.

19. A polypeptide comprising a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c) comprising one or more substitution mutations corresponding to substitution mutations in serotype B, strain 1, selected from the group consisting of V1118M; Y1183M; E1191M; E1191I; E1191Q; E1191T; S1199Y; S1199F; S1199L; S1201V; and combinations thereof.

20. The polypeptide of paragraph 19, wherein the modified (B-H_c) comprises two substitution mutations.

21. The polypeptide of paragraph 20, wherein the two substitution mutations correspond to E1191M and S1199L, E1191M and S1199Y, E1191M and S1199F, E1191Q and S1199L, E1191Q and S1199Y, or E1191Q and S1199F.

22. The polypeptide of one of paragraphs 20-21, wherein the two substitution mutations correspond to E1191M and S1199L.

23. The polypeptide of one of paragraphs 20-21, wherein the two substitution mutations correspond to E1191M and S1199Y.

24. The polypeptide of one of paragraphs 20-21, wherein the two substitution mutations correspond to E1191M and S1199F.

25. The polypeptide of one of paragraphs 20-21, wherein the two substitution mutations correspond to E1191Q and S1199L.

26. The polypeptide of one of paragraphs 20-21, wherein the two substitution mutations correspond to E1191Q and S1199Y.

27. The polypeptide of one of paragraphs 20-21, wherein the two substitution mutations correspond to E1191Q and S1199F.

28. A polypeptide comprising a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c) comprising a substitution mutation at a position corresponding to S1199 or S1201 of serotype B, strain 1.

29. The polypeptide of paragraph 28, wherein the substitution mutation produces enhanced binding of the modified B-H_c to human SytII and/or reduced binding of the modified B-H_c to human Syt I as compared to an identical molecule lacking the substitution mutation.

30. The polypeptide of paragraph 28, wherein the substitution mutation produces enhanced binding of the modified B-H_c to human SytII and/or increased binding of

the modified B-H_c to human Syt I as compared to an identical molecule lacking the substitution mutation.

31. The polypeptide of any one of paragraphs 29-30 wherein the substitution mutation is selected from the group consisting of A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, T, W, Y and V substituted for S.

32. The polypeptide of any one of paragraphs 29-31 wherein the substitution mutation is a non-naturally occurring amino acid substituted for S.

33. The polypeptide of any one of paragraphs 19 - 32, wherein the modified B-H_c is of strain 1.

34. A chimeric molecule comprising a first portion that is a modified receptor binding domain of *Clostridial botulinum* serotype B (B-H_c) linked to a second portion, wherein the modified B-H_c comprises one or more substitution mutations corresponding to substitution mutations in serotype B, strain 1, selected from the group consisting of:

V1118M; Y1183M; E1191M; E1191I; E1191Q; E1191T; S1199Y; S1199F; S1199L; S1201V and combinations thereof.

35. The chimeric molecule of paragraph 33, wherein the modified B-H_c comprises two substitution mutations.

36. The chimeric molecule of paragraph 35, wherein the two substitution mutations correspond to E1191M and S1199L, E1191M and S1199Y, E1191M and S1199F, E1191Q and S1199L, E1191Q and S1199Y, or E1191Q and S1199F.

37. The chimeric molecule of one of paragraphs 35-36, wherein the two substitution mutations correspond to E1191M and S1199L.

38. The chimeric molecule of one of paragraphs 35-36, wherein the two substitution mutations correspond to E1191M and S1199Y.

39. The chimeric molecule of one of paragraphs 35-36, wherein the two substitution mutations correspond to E1191M and S1199F.

40. The chimeric molecule of one of paragraphs 35-36, wherein the two substitution mutations correspond to E1191Q and S1199L.

41. The chimeric molecule of one of paragraphs 35-36, wherein the two substitution mutations correspond to E1191Q and S1199Y.

42. The chimeric molecule of one of paragraphs 35-36, wherein the two substitution mutations correspond to E1191Q and S1199F.

43. The chimeric molecule of paragraph 34, wherein the modified B-H_c comprises a modified receptor binding domain of Clostridial botulinum serotype B (B-H_c) comprising a substitution mutation at a position corresponding to S1199 or S1201 of serotype B, strain 1.

44. The chimeric molecule of paragraph 43, wherein the substitution mutation produces enhanced binding of the modified B-H_c to human SytII and/or reduced binding of the modified B-H_c to human Syt I as compared to an identical molecule lacking the substitution mutation.

45. The chimeric molecule of paragraph 43, wherein the substitution mutation produces enhanced binding of the modified B-H_c to human SytII and/or increased binding of the modified B-H_c to human Syt I as compared to an identical molecule lacking the substitution mutation.

46. The chimeric molecule of any one of paragraphs 44-45 wherein the substitution mutation is selected from the group consisting of A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, T, W, Y and V substituted for S.

47. The chimeric molecule of any one of paragraphs 44-46 wherein the substitution mutation is a non-naturally occurring amino acid substituted for S.

48. The chimeric molecule of any one of paragraphs 43 – 47, wherein the modified B-H_c is of strain 1.

49. The chimeric molecule of any one of paragraphs 32-48, wherein the first portion and the second portion are linked covalently.

50. The chimeric molecule of any one of paragraphs 32-48, wherein the first portion and the second portion are linked non-covalently.

51. The chimeric molecule of any one of paragraphs 32-50 wherein the second portion is selected from the group consisting of a small molecule, a nucleic acid, a short polypeptide and a protein.

52. The chimeric molecule of paragraph 51, wherein the second portion is a bioactive molecule.

53. The chimeric molecule of paragraph 51 or 52, wherein the second portion is a therapeutic polypeptide or non-polypeptide drug.

54. A nucleic acid comprising a nucleotide sequence that encodes the polypeptide or chimeric molecule of any one of paragraphs 1- 53.

55. A nucleic acid vector comprising the nucleic acid of paragraph 54.

56. A cell comprising the nucleic acid vector of paragraph 55 or the nucleic acid of paragraph 54.

57. A cell expressing the polypeptide or chimeric molecule of any one of paragraphs 1-53.

58. A pharmaceutical composition comprising the botulinum neurotoxin (BoNT) polypeptide of any one of paragraphs 1-18, or the chimeric molecue of any one of paragraphs 34-53, or the nucleic acid vector of paragraph 55 or the nucleic acid of paragraph 54.

59. The pharmaceutical composition of paragraph 58, further comprising a pharmaceutically acceptable excipient.

60. A kit comprising a pharmaceutical composition of paragraph 58 or 59 and directions for therapeutic administration of the pharmaceutical composition.

61. A method to produce a botulinum neurotoxin (BoNT) polypeptide, the method comprising the steps of culturing the host cell of paragraph 57 under conditions wherein said BoNT polypeptide is produced.

62. The method of paragraph 61 further comprising recovering the BoNT polypeptide from the culture.

63. A method for treating a condition associated with unwanted neuronal activity comprising administering a therapeutically effective amount of the BoNT polypeptide of any one of paragraphs 1-18 to a subject to thereby contact one or more neurons exhibiting unwanted neuronal activity, to thereby treat the condition.

64. The method of paragraph 63, wherein 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, and dermatological or aesthetic/cosmetic conditions.

65. The botulinum neurotoxin (BoNT) polypeptide of any one of paragraphs 1-18, the pharmaceutical composition of paragraph 58 or 59, or the chimeric molecule of any one of paragraphs 34-53, or the polypeptide of any one of paragraphs 19-33, for use in medicine.

66. The botulinum neurotoxin (BoNT) polypeptide of any one of paragraphs 1-18, the pharmaceutical composition of paragraph 58 or 59, or the chimeric molecule of any one of paragraphs 34-53, or the polypeptide of any one of paragraphs 19-33, for use in treating a condition associated with unwanted neuronal activity.

[00117] The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

[00118] The following experiments were performed to determine if it is possible to change the binding affinity of BoNT/B to human Syt II by modifying the BoNT/B receptor binding domain. The hypothesis is based on a series of previous studies: (1) It has been shown in 1998 that a naturally occurring BoNT/B subtype toxin, BoNT/B2, exhibits ~4 fold lower binding affinity to Syt II than BoNT/B [28](also defined as BoNT/B1, Fig. 2F). This affinity difference was demonstrated to be due to a few amino acid differences within their receptor binding domains in 2003 [29](Fig. 2F, G), demonstrating for the first time that changing residues within the receptor binding domain of BoNT/B can change the binding affinity to Syt II. These studies also identified key residues that influence binding affinity to Syt II (Fig. 2G). (2) It has been reported in 2004 that single residue mutations within the receptor binding domain of BoNT/A and BoNT/B can dramatically change the toxicity and potency of these toxins (Fig. 2H), demonstrating that changes in receptor-binding affinity can translate into changes of toxicity and potency of toxins [30]. (3) The co-crystal structure of BoNT/B bound to rat Syt II has been solved [31,32], and key residues that form the binding site for Syt II have been resolved[31,32]. These previous studies all utilized the rodent Syt II, but not human Syt II.

[00119] Target residues for engineering BoNT/B receptor binding domain to change its binding affinity to human Syt II were identified from all these previous studies with rodent Syt II binding.

[00120] The receptor binding domain of BoNT/B is well defined [1]. Previous studies established that changing residues within the receptor binding domain of BoNT/B can modulate the binding affinity of BoNT/B to rat or mouse Syt II [29,30]. Co-crystal structure of BoNT/B bound to rat Syt II has also been solved by two studies in 2006 [31,32]. The residue

change in human Syt II is a relatively conservative change from F to L, both are hydrophobic residues. However, the difference in the binding affinity of BoNT/B for rodent Syt II is significantly higher than for human Syt II. Furthermore, it is not obvious how the binding interaction between BoNT/B and human Syt II might be modified to compensate for the lack of this phenylalanine residue in the middle of the binding site. Whereas positive binding interactions can be envisaged (and visualized in published crystal structures) between WT BoNT/B-H_C and rat or mouse Syt II, e.g. involving stacking or packing of hydrophobic rings, or between a WT BoNT/B-H_C and a modified human Syt II in which the phenylalanine is substituted into the sequence; such interactions may not be reproducible between a modified BoNT/B-HC and a WT human Syt II protein. This suggests that changing a few or even one residue in BoNT/B might not be able to restore/improve binding to human Syt II without major changes in the global structure of BoNT/B-Syt II complexes.

[00121] The conserved phenylalanine at position 54 forms multiple hydrophobic contacts with BoNT/B. Because leucine (in humans) is also hydrophobic, disruption of BoNT/B binding is likely due to size/shape differences between phenylalanine and leucine. The key to the invention was therefore to identify possible changes in BoNT/B-H_C region that may accommodate and compensate for the change from phenylalanine to leucine. The approach was two-fold: to focus on residues directly contacting phenylalanine 54 in rodent Syt II: or to focus on residues within the surrounding region of BoNT.B-Hc, which might compensate for the lack of a positive binding interaction with phenylalanine at position 54. These residues that are potentially within the corresponding binding region between BoNT/B and human Syt II were judged by reference to the BoNT/ B-rat Syt II co-crystal structure (Fig. 2I), to possibly include Y1181, P1197, A1196, F1204, F1194, P1117, W1178, Y1183, V1118, S1116, K1113, K1192, S1199, S1201, E1191, E1245, and Y1256. Residues 1117, 1191, and 1199 have also been shown to be among the list of residues that influence binding of BoNT/B2 to rodent Syt II in an earlier study (Fig. 2G)[29]. Because the precise effect from residue substitutions is impossible to predict, a "trial-and-error" approach was employed. At first, single residue substitutions were carried out, followed by selected combinations. Specifically, each of the listed key residues were systematically substituted with hydrophobic residues with different sizes – with the screen limited to hydrophobic residues in order to ensure that important hydrophobic contacts were maintained. These hydrophobic substitution residues include: V, I, L, M, F, W, C, and other less hydrophobic amino acids including A, Y, H, T, S, P, Q, N, and G.

[00122] A key to the success of the invention was to develop a feasible and economical way for screening mutants. The basic approach was to detect binding of soluble recombinant BoNT/B-H_C to immobilized mouseSyt II (F54L) in pull-down assays as described in Fig. 2C. However, it was not feasible to purify all mutants for pull-down assays. Therefore, whether it was possible to pull down BoNT/B-H_C from a small amount of bacterial lysates directly with Syt II, without the need for purification, was tested. The rationale was that the binding affinity of BoNT/B-Syt II might be high enough for this approach (Kd ~ 0.23 nM) [20]. Indeed, it was found that immobilized rat Syt II could "affinity-purify" enough WT BoNT/B-H_C directly from merely 6 ml of bacterial lysates (Fig. 3A). This newly developed method greatly simplified the effort to screen a fairly large number of BoNT/B-H_C mutants. Using this method, screening of BoNT/B-H_C mutants for their binding to both a mouse Syt II 1-87 (m-Syt II) and a mutated mouse Syt II that mimicking human Syt II sequence (F54L, h-Syt II) was tested. Bound materials were subjected to immunoblot analysis detecting BoNT/B-H_C using the anti-HA antibody (Fig. 3A).

[00123] The majority of mutants were found to fall into two categories: (1) fail to bind m-Syt II and h-Syt II, such as F1204L and V1118W (Fig. 3B); (2) still bind m-Syt II, but fail to bind h-Syt II, such as F1204W and E1191W (Fig. 3B). These binding results are largely omitted here except a few examples illustrated in Fig. 3A.

[00124] Among mutants screened, a few that bound both m-Syt II and h-Syt II, including V1118M, S1199Y/L/F, Y1183M, S1201V, E1191M/I/Q/T (Fig. 3B) were identified. Thus, these residues were determined to be at key positions for accommodating the L residues in human Syt II or for compensating for the lack of phenylalanine residue at this position in human Syt II. Although human Syt I is expressed at significantly lower levels in motor neurons than human Syt II, it is nevertheless an important and capable toxin receptor, as demonstrated by the effectiveness of BoNT/B in patients. In order to achieve the highest possible binding to human neurons, in some aspects the modified BoNT/B mutants should desireably not adversely affect binding to human Syt I. Ideally, they may even increase binding to Syt I. Therefore, the binding of selected BoNT/B mutants to immobilized human Syt I, using the same small-scale pull-down assay (Fig. 4A) was further examined. Because Syt I binding to BoNT/B has a lower affinity as compared to Syt II, it requires the presence of lipid co-receptor gangliosides [10,20]. This need was addressed by adding purified brain gangliosides into bacterial lysates in the pull-down assays. As indicated in Fig. 4A, human Syt I fragment (1-80) containing the toxin binding site was purified as GST-tagged proteins and immobilized on beads to pull down WT and mutant BoNT/B-HC, with and

without the presence of gangliosides (Gangl). As expected, WT BoNT/B-HC binds Syt I only in the presence of gangliosides. It was found that the mutants E1191M and E1191Q significantly increased binding to Syt I: these mutants can even bind to human Syt I without gangliosides (Fig.4A). Other mutants either reduced binding to Syt I (e.g. V118M) or maintained the similar levels of binding as compared to WT BoNT/B-H_C (e.g. S1201V). This indicates that E1191M and E1191Q are mutants that both enable binding to human Syt II and enhance binding to human Syt I.

[00125] Mutation V1118M was also of interest as it binds to human Syt II, but not human Syt I. Therefore, it has the potential to be used to create therapeutic toxins that are more specific for neurons that express Syt II than the WT BoNT/B in humans, thus reducing non-specific entry into Syt-I expressing cells in humans.

[00126] Using E1191M as an example, its interactions with human Syt II were further validated using purified recombinant proteins, which allows us to compare binding of equal amounts of WT BoNT/B-H_C and the E1191M mutant to m-Syt II and h-Syt II (Fig. 4B). E1191M was found to bind to both m-Syt II and h-Syt II without gangliosides, and adding gangliosides further elevated the binding (Fig. 4B). These results confirmed that E1191M gains the ability to bind human Syt II in the absence of gangliosides and can form high-affinity complexes with human Syt II in the presence of the lipid co-receptor gangliosides.

[00127] Using E1191M/Q as the backbone, experiments were performed to analyze whether combining it with other residue substitutions may further enhance binding to human Syt I/II. Combining S1199L/Y/ or /F with E1191M/or Q generated double mutants that display significantly higher binding to human Syt II (Fig. 5A). For instance, E1191M/S1199Y achieved similar levels of binding to both m-Syt II and h-Syt II (Fig. 5A, lane 5 and 6). This was a significant enhancement as compared to E1191M alone, which mediated less binding to h-Syt II than its binding to m-Syt II (Fig. 4B). Furthermore, all selected double mutants displayed significantly higher binding to human Syt I than WT BoNT/B-H_C (Fig. 5B).

[00128] Using E1191M/S1199Y as an example, binding of WT, E1191M, and E1191M/S1199Y to h-Syt II were further compared using equal amounts of purified recombinant proteins. As shown in Fig. 6A, WT BoNT/B-H_C could not bind to h-Syt II in the absence of gangliosides under the current assay conditions. E1191M showed a modest binding to h-Syt II without gangliosides, while binding of E1191M/S1199Y to h-Syt II was significantly enhanced as compared to E1191M alone, especially without gangliosides

(comparing lanes 6 versus 8). Furthermore, both E1191M and E1191M/S1199Y significantly enhanced binding to human-Syt I as compared to WT BoNT/B-H_C (Fig. 6B).

[00129] Binding of WT BoNT/B-H_C to m-Syt II is known to have a high affinity [20,21]. Thus the binding between E1191M/S1199Y to h-Syt II versus the "golden standard": WT BoNT/B-HC binding to m-Syt II was compared. As shown in Fig. 6C, titration of BoNT/B-H_C concentrations revealed that E1191M/S1199Y has similar levels of binding at all concentrations as WT binding to m-Syt II. The Kd was estimated to be ~19 nM between E1191M/S1199Y and h-Syt II, and ~ 68 nM for WT BoNT/B-H_C binding to m-Syt II under this assay condition (Fig. 6D) This is a gigantic improvement for binding h-syt II as compared to WT BoNT/B-H_C, which failed to bind h-Syt II in these assay conditions (Fig. 6A). In conclusion, combining E1191M with S1199Y provided a synergistic improvement in binding affinity, outweighing an additive improvement over the E1191M mutant and yielded new BoNT/B-H_C mutants with high affinity binding to both human Syt I and Syt II. By contrast, combinations of some other beneficial individual mutations did not result in further improved double-mutant BoNT/B-H_C domains.

[00130] Finally, whether E1191M/S1199Y mutant can recover the binding to h-Syt II on the neuron surface was examined. Cultured rat hippocampal neurons only express Syt I, but not Syt II. Syt I was knocked down (KD) in these neurosn and then replaced with exogenous m-Syt II, m-Syt II (F54L), and h-Syt II via lentiviral transduction. Binding of WT BoNT/B-H_C and E1191M/S1199Y to these neurons was then tested (Fig. 7). WT BoNT/B-H_C only bound to m-Syt II, whereas E1191M/S1199Y bound to both m-Syt II (F54L) and h-Syt II on the neuron surface, demonstrating that E1191M/S1199Y mutant can use h-Syt II as a functional receptor in neurons.

Materials and Methods

[00131] ***Antibodies and materials***: The mouse monoclonal anti-HA antibody was purchased from Covance (16B12). Bovine mixed brain gangliosides were purchased from Matreya LLC (Pleasant Gap, PA) and were reconstituted in Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl) as previously described [9]. BoNT/B (Okra) was purified in E. Johnson's lab (Madison, WI) from indicated strains.

[00132] ***cDNA and constructs:*** DNA encoding BoNT/B-H_C (residue 856-1291, based on GenBank access No:AB232927.1) was synthesized by Geneart Inc. and its codon has been optimized for expression in *E.Coli*. DNA encoding BoNT/B-H_C was subcloned into pET28a

vector, with both a His6 tag and a HA tag (YPYDVPDYA) fused to its N-terminus. Mutations in BoNT/B-H_C were generated via PCR using Quickchange Site-directed Mutagenesis Kit (Agilent Technologies, CA), following the manufacturer's manual. The following DNA were generously provided by indicated groups: rat Syt I (T.C. Sudhof, Palo Alto, CA), mouse Syt II (M. Fukuda, Ibaraki, Japan), human Syt I (R.B. Sutton, Lubbock, TX). GST tagged Syt I/II fragments and Syt II mutations were described previously [10,13,14]. All constructs were verified by sequencing.

[00133] ***Protein expression and purification:*** WT and mutants of BoNT/B-H_C were expressed as His6 tagged recombinant proteins in *E.Coli*. Syt I/II fragments and mutants were expressed as GST tagged recombinant proteins in *E.Coli*. Both GST-fusion and His_6-fusion proteins were purified as previously described [9], with the induction temperature at 20°C overnight with 0.25 mM IPTG.

[00134] ***GST pull-down assays:*** Two types of pull-down assays were carried out. The first series were used to screen binding of mutant BoNT/B-H_C to GST-tagged mouse Syt II (m-Syt II) and a mutant mouse Syt II (F54L) that mimicking human Syt II sequence (designated as h-Syt II in Examples 1 to 6). Briefly, 6 ml of *E.Coli* expressing BoNT/B-H_C were spin down, re-suspended in 800 µl TBS, sonicated, and then incubated with 2% Triton X™-100 for 1 hr at 4°C. Samples were then spun down at maximal speed for 15 min in a microcentrifuge at 4°C. The supernatants were collected and were used for pull-down assays by incubating with 10 µg of Syt proteins immobilized on glutathione-Sepharose beads (GE bioscience, Piscataway, NJ) at 4°C for 1 hr. Samples were washed three times in washing buffer (TBS + 0.5% Triton), and analyzed by immunoblot assaysdetecting BoNT/B-H_C using the anti-HA antibody. For mutants with enhanced binding to h-Syt II, further pull-down assays were carried out by purifying these mutant BoNT/B-H_C as His6 tagged proteins as described previously [9]. Pull-down assays were then carried out using immobilized Syt fragments in 100 µl TBS buffer plus 0.5% Triton X™-100, with or without gangliosides (60 µg/ml), for 1 hr at 4 °C. Beads were washed three times using TBS buffer plus 0.5% Triton X™-100. Ten percent of bound materials were subjected to SDS-PAGE followed by immunoblot analysis.

[00135] ***Immunostaining:***Culture neurons were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X™-100, and subjected to immunostaining analysis detecting both BoNT/B-H_C (with an HA antibody) and synapsin. Images were collected using a confocal microscope (Leica TCS SP5; 40× oil objective).

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Claims

1. A polypeptide comprising a modified receptor binding domain of *Clostridial botulinum* serotype B (B-H_c), the modified receptor binding domain comprising a sequence with at least 95% identity to a sequence corresponding to residues 859-1291 of SEQ ID NO:4, and one or more substitution mutations at positions with reference to SEQ ID NO: 4, wherein one of the substitution mutations is selected from the group consisting of : E1191M; E1191I; E1191Q; and E1191T,

wherein the substitution mutation produces enhanced binding of the modified B-H_c to human synaptotagmin II (Syt II) as compared to an identical molecule lacking the substitution mutation.

2. A botulinum neurotoxin (BoNT) polypeptide comprising:

a) a protease domain;

b) a protease cleavage site;

c) a translocation domain; and

d) a modified receptor binding domain of *Clostridial botulinum* serotype B (B-H_c), the modified receptor binding domain comprising a sequence with at least 95% identity to a sequence corresponding to residues 859-1291 of SEQ ID NO:4, and one or more substitution mutations at positions with reference to SEQ ID NO: 4, wherein one of the substitution mutations is selected from the group consisting of :

E1191M; E1191I; E1191Q; and E1191T,

wherein the substitution mutation produces enhanced binding of the modified B-H_c to human synaptotagmin II (Syt II) as compared to an identical molecule lacking the substitution mutation.

3. The polypeptide or BoNT polypeptide of claim 1 or 2 , wherein the modified B-H_c is of strain 1.

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4. The BoNT polypeptide of claim 2 or 3 , wherein the protease domain, translocation domain, and protease cleavage site are from serotype A, B, C, D, E, F, G, or any combination thereof.

5. The BoNT polypeptide of claim 4, wherein the protease domain, translocation domain, and protease cleavage site are from serotype B, strain 1.

6. The BoNT polypeptide of claim 4, wherein the protease domain, translocation domain, and protease cleavage site are from serotype A, strain 1.

7. The polypeptide or BoNT polypeptide of any one of claims 1 to 6, wherein the modified (B-H_c) comprises two substitution mutations.

8. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to

E1191M and S1199Y;
E1191M and S1199L;
E1191M and S1199F;
E1191Q and S1199L;
E1191Q and S1199Y;
E1191Q and S1199F;
E1191M and S1201V;
E1191M and Y1183M; or
E1191M and V1118M.

9. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to E1191M and S1199Y.

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10. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to E1191M and S1199L.

11. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to E1191M and S1199F.

12. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to E1191Q and S1199L.

13. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to E1191Q and S1199Y.

14. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to E1191Q and S1199F.

15. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to E1191M and S1201V.

16. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to E1191M and Y1183M.

17. The polypeptide or BoNT polypeptide of claim 7, wherein the two substitution mutations correspond to E1191M and V1118M.

18. The polypeptide or BoNT polypeptide of any one of claims 1 to 17 that is a chimeric molecule comprising a first portion that is the modified receptor binding domain of *Clostridial*

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botulinum serotype B (B-H_c) linked to a second portion, wherein the second portion is a small molecule, a nucleic acid, a short polypeptide or a protein.

19. The polypeptide or BoNT polypeptide of claim 18, wherein the first portion and the second portion are linked covalently.

20. The polypeptide or BoNT polypeptide of claim 18, wherein the first portion and the second portion are linked non-covalently.

21. The polypeptide or BoNT polypeptide of any one of claims 18-20, wherein the second portion is a small molecule.

22. The polypeptide or BoNT polypeptide of any one of claims 18-21, wherein the second portion is a bioactive molecule.

23. The polypeptide or BoNT polypeptide of any one of claims 18-22, wherein the second portion is a therapeutic polypeptide or non-polypeptide drug.

24. A nucleic acid comprising a nucleotide sequence that encodes the polypeptide or BoNT polypeptide of any one of claims 1 to 23.

25. A nucleic acid vector comprising the nucleic acid of claim 24.

26. A cell comprising the nucleic acid vector of claim 25 or the nucleic acid of claim 24 or expressing the polypeptide or BoNT polypeptide of any one of claims 1-23.

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27. A pharmaceutical composition comprising the polypeptide or botulinum neurotoxin (BoNT) polypeptide of any one of claims 1-23, or the nucleic acid vector of claim 25 or the nucleic acid of claim 24 and a pharmaceutically acceptable excipient.

28. A kit comprising the pharmaceutical composition of claim 27, and directions for therapeutic administration of the pharmaceutical composition.

29. A method to produce a polypeptide or botulinum neurotoxin (BoNT) polypeptide, the method comprising the steps of culturing the cell of claim 26 under conditions wherein said polypeptide or BoNT polypeptide is produced.

30. The method of claim 29 further comprising recovering the polypeptide or BoNT polypeptide from the culture.

31. The polypeptide or botulinum neurotoxin (BoNT) polypeptide of any one of claims 1-23, or the pharmaceutical composition of claim 27, for use in treating a condition associated with unwanted neuronal activity, wherein the condition is 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, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions, secretory disorders, pain from muscle spasms, headache pain, or dermatological or aesthetic/cosmetic conditions.

32. Use of the polypeptide or BoNT polypeptide of any one of claims 1-23 in the manufacture of a medicament for the treatment of a condition associated with unwanted neuronal activity, wherein the condition is spasmodic dysphonia, spasmodic torticollis, laryngeal dystonia, oromandibular dysphonia, lingual dystonia, cervical dystonia, focal hand dystonia, blepharospasm,

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strabismus, hemifacial spasm, eyelid disorder, cerebral palsy, focal spasticity, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions, secretory disorders, pain from muscle spasms, headache pain, or dermatological or aesthetic/cosmetic conditions.

33. Use of the polypeptide or BoNT polypeptide of any one of claims 1-23 or the pharmaceutical composition of claim 27 for treating a condition associated with unwanted neuronal activity, wherein the condition is 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 or other voice disorders, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia or other muscle tone disorders or other disorders characterized by involuntary movements of muscle groups, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions, secretory disorders, pain from muscle spasms, headache pain, or dermatological or aesthetic/cosmetic conditions.

34. The polypeptide or botulinum neurotoxin (BoNT) polypeptide of any one of claims 1-23, for use in binding to the Syt-II receptor.

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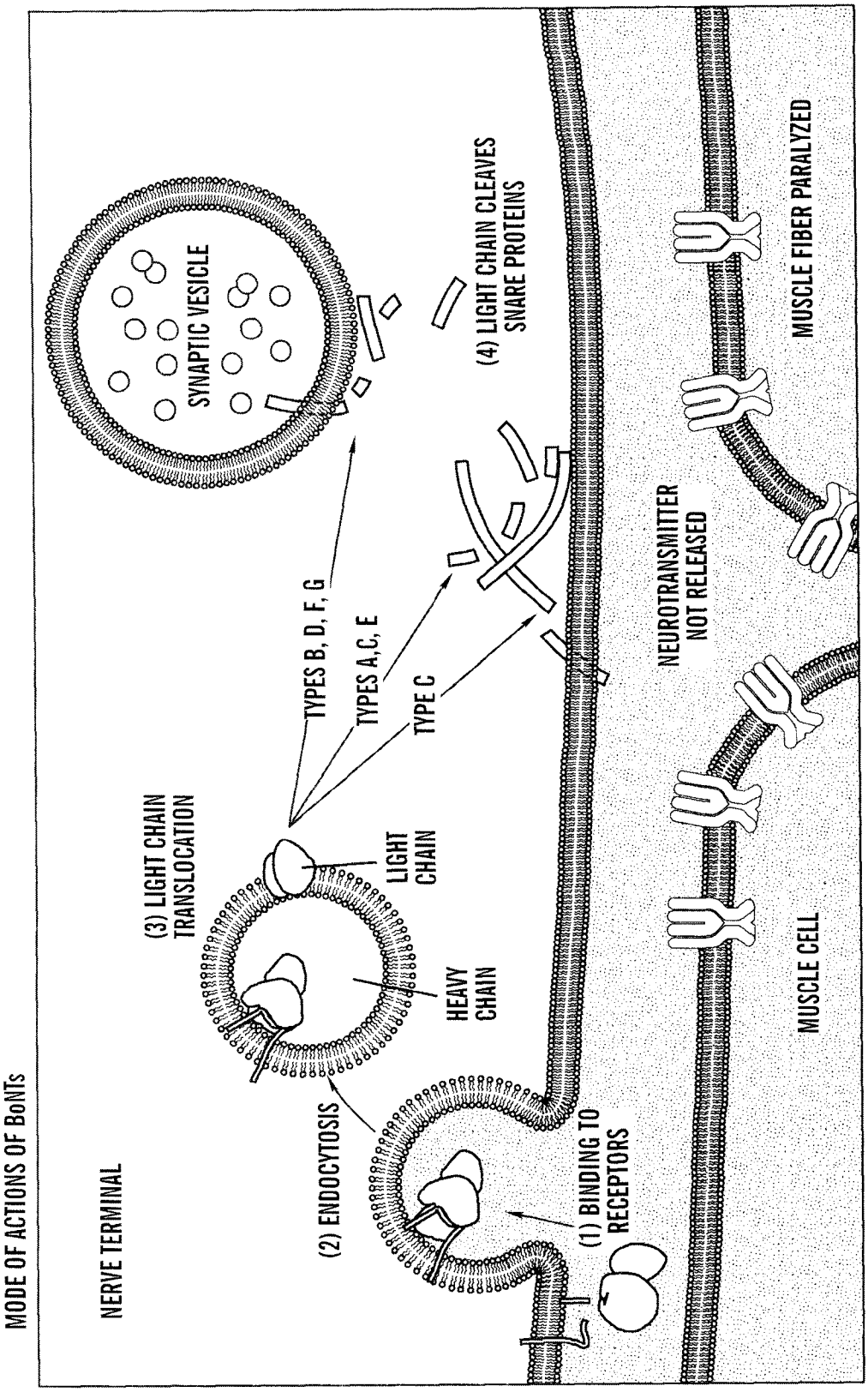

MODE OF ACTIONS OF BoNTs
NERVE TERMINAL
(1) BINDING TO RECEPTORS
(2) ENDOCYTOSIS
(3) LIGHT CHAIN TRANSLOCATION
LIGHT CHAIN
HEAVY CHAIN
TYPES B, D, F, G
TYPES A,C, E
TYPE C
SYNAPTIC VESICLE
(4) LIGHT CHAIN CLEAVES SNARE PROTEINS
NEUROTRANSMITTER NOT RELEASED
MUSCLE CELL
MUSCLE FIBER PARALYZED

FIG. 1A

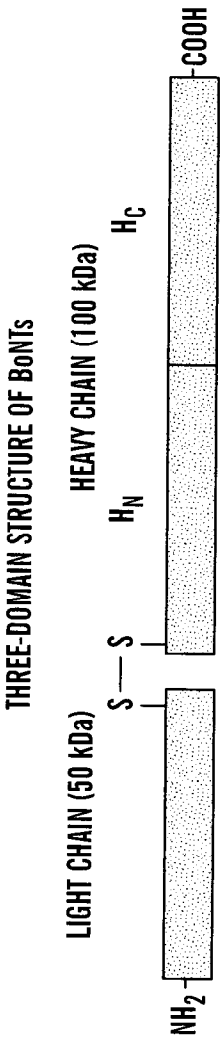


FIG. 1B

TOXINS	PROTEIN RECEPTORS
BoNT/B, G, D-C	SYNAPTOTAGMIN I AND II (Syt I/II)
BoNT/A, E, D, F	SV2A, B, AND C

FIG. 1C

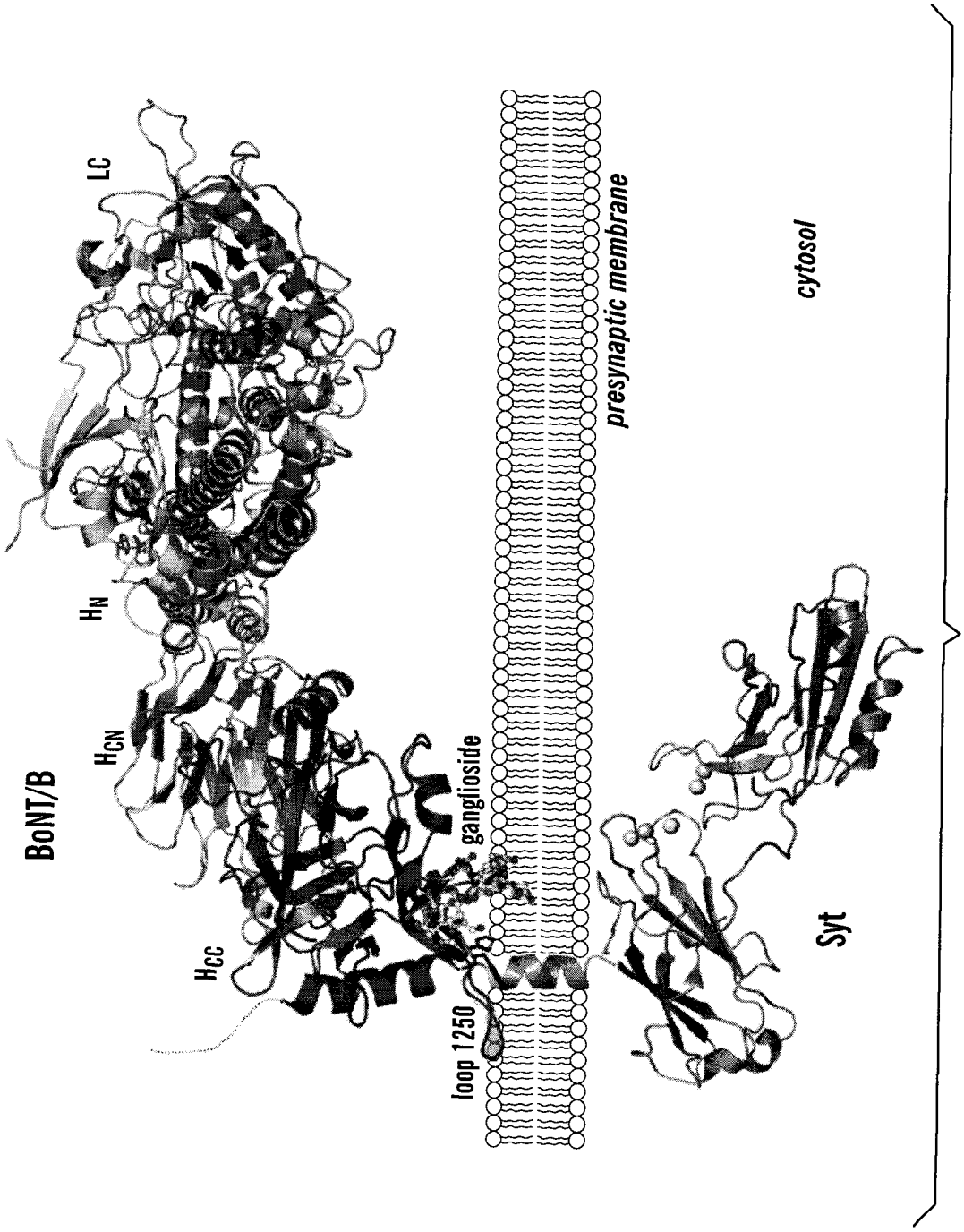

BoNT/B
LC
H_N
H_{CN}
H_{CC}
ganglioside
loop 1250
presynaptic membrane
cytosol
Syt

FIG. 1D

	SYNAPTOTAGMIN I	VS	SYNAPTOTAGMIN II
BINDING AFFINITY TO BoNT/B:	LOW (Kd ~ 2.3 nM)		HIGH (Kd ~ 0.23 nM)
EXPRESSION IN MOTOR NEURONS:	LOW		HIGH

FIG. 2A

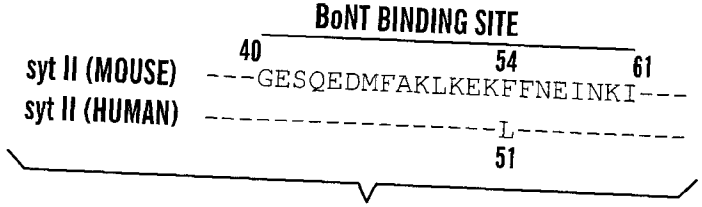


FIG. 2B

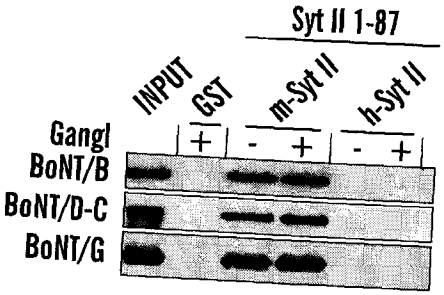


FIG. 2C

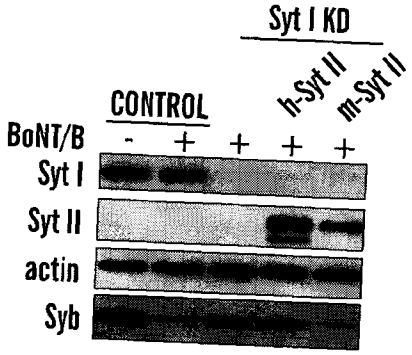


FIG. 2D

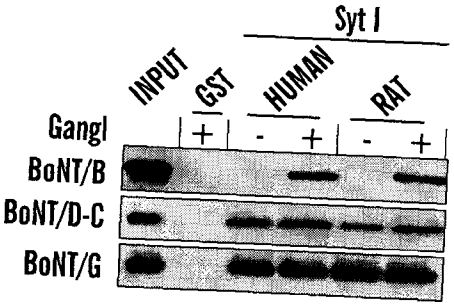


FIG. 2E

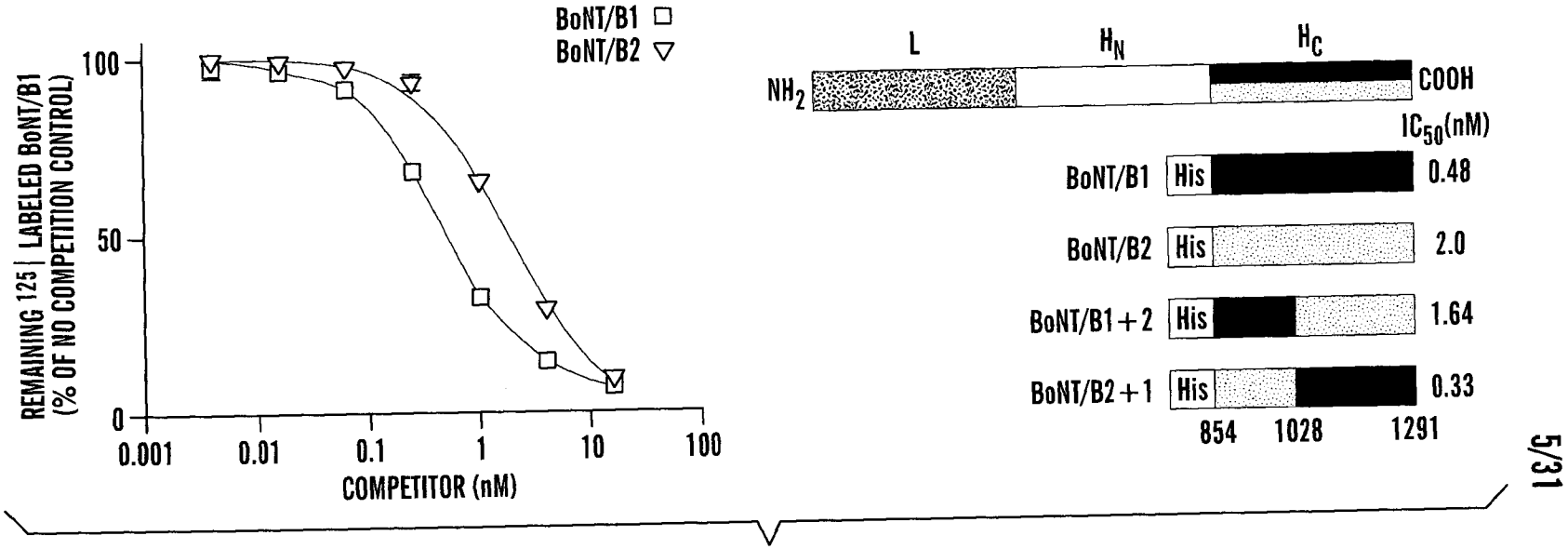


FIG. 2F

RESIDUES THAT ARE DIFFERENT BETWEEN BoNT/B1 AND BoNT/B2 WITHIN THE RECEPTOR BINDING REGION 1028-1291	1117, 1132, 1138, 1174, 1176, 1182, 1185, 1188, 1191 1197, 1199, 1202, 1206, 1250, 1251, 1252, 1255, 1262, 1275

FIG. 2G

TABLE 1. ACTIVITY OF WILD-TYPE AND MUTATED BoNT/A AND B IN THE MPN TOXICITY ASSAY.

RECOMBINANT scBoNT	PARALYTIC HALFTIME $t_{1/2}$[a] *min* 661.8 pM		PARALYTIC HALFTIME $t_{1/2}$[a] *min*	% TOXICITY VERSUS WILD-TYPE scBoNT[b]
scBoNT/A WILD TYPE	35.5±5.0			100[c]
scBoNT/A E1203L	55.0±2.8			17.4±0.9
scBoNT/A S1264A	39.0±1.4			63.3±2.3
scBoNT/A W1266L	126.5±7.8			0.7±0.1
scBoNT/A Y1267F	101.5±12.0			1.7±0.2
scBoNT/A Y1267S	185.5±6.4			0.2±0.1
scBoNT/A H1253A	49.5±6.4			25.9±3.3
scBoNT/A H1253W	n.d.[d]			
	1954 pM		6515 pM	
scBoNT/B wild type	53.5±4.9			100[c]
scBoNT/B E1189L	77.0±1.4			27.5±1.4
scBoNT/B E1190L	>180		160.0±2.8	0.5±0.1
scBoNT/B S1260A	>180	A	138.0±1.4	0.9±0.1
scBoNT/B W1262L	>180		179.5±3.5	0.3±0.1
scBoNT/B Y1263F	154.5±2.1			2.0±0.1
scBoNT/B Y1263S	>180		174.0±8.5	0.4±0.1
scBoNT/B H1241A	64.0±5.7			28.0±3.3
scBoNT/B H1241W	>180		175.5±15.7	0.4±0.1

FIG. 2H

RESIDUES THAT FORM THE BINDING POCKET FOR SYT II	1113, 1115, 1116, 1117, 1118, 1178, 1181, 1183, 1191, 1192, 1194, 1196, 1197, 1199, 1201, 1204

FIG. 2I

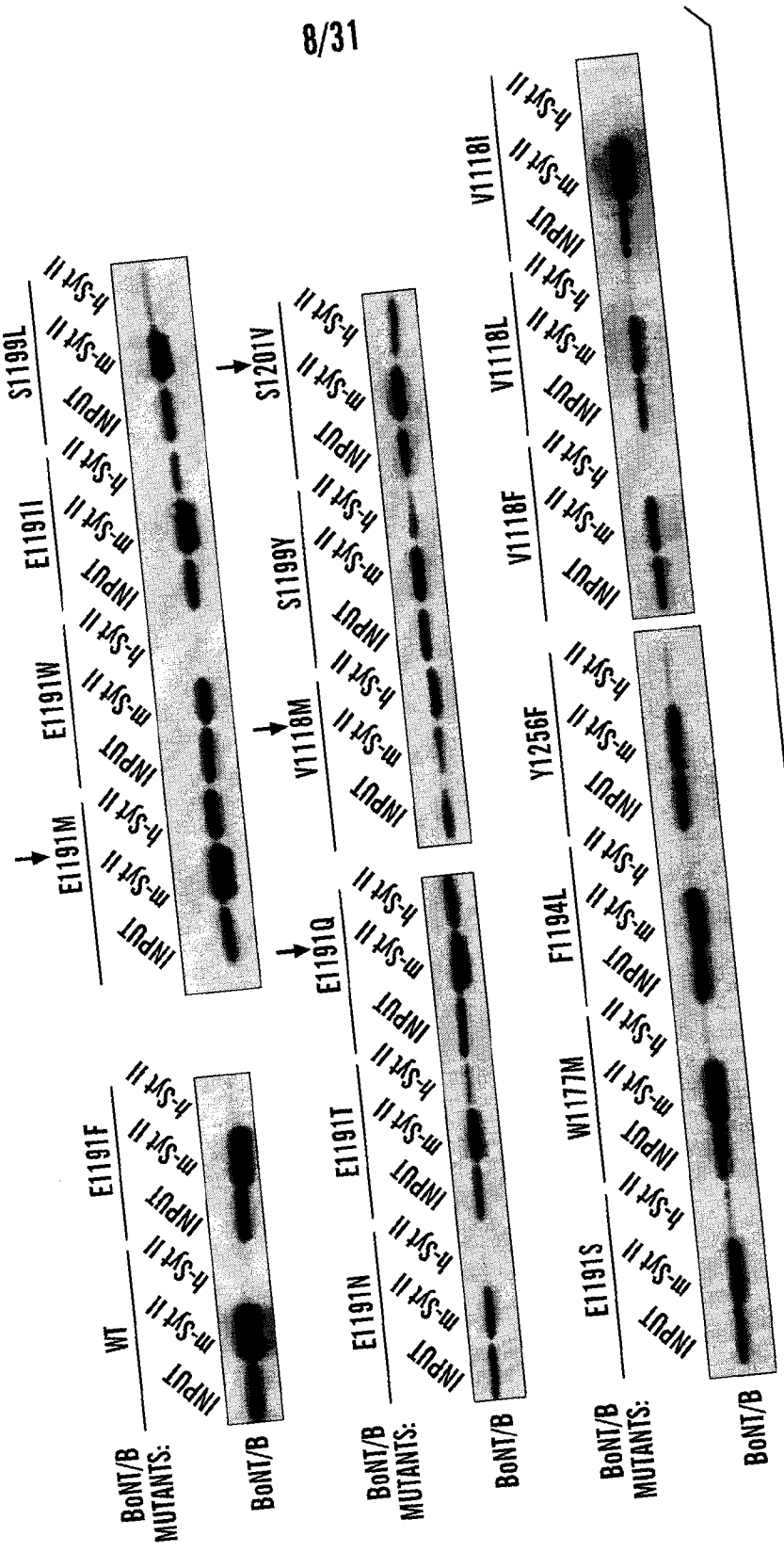

BoNT/B MUTANTS:
WT
E1191F
E1191M
E1191W
E1191I
S1199L
E1191N
E1191T
E1191Q
V1118M
S1199Y
S1201V
E1191S
W1177M
F1194L
Y1256F
V1118F
V1118L
V1118I
BoNT/B
INPUT
m-Syt II
h-Syt II

FIG. 3A

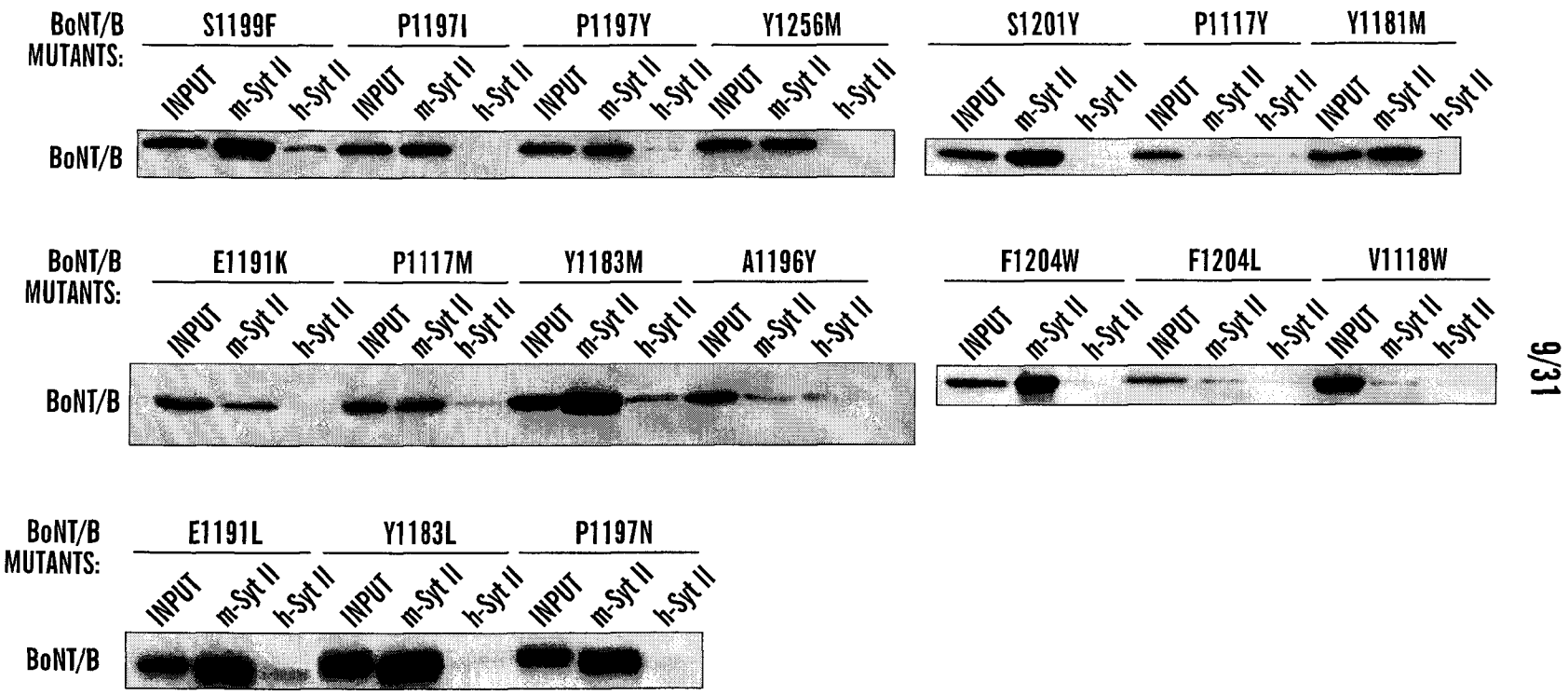


FIG. 3A Continued

STRONG BINDING TO h-Syt II	WEAK BINDING TO h-Syt II	BIND m-Syt-II, WITH MINIMAL OR NO BINDING TO h-Syt II		DO NOT BIND TO EITHER m-Syt II OR h-Syt II
E1191M/Q	E1191I/T	E1191F/W/N/S/K/L	Y12561M	A1196Y
V1118M	S1199Y/F/L		V1118F/L/I	P1117Y
S1201V	Y1183M	W1177M	P1117M	F1204L
		F1195L	S1201Y	V1118W
		Y1256F	F1204W	
		P1197I/Y/N	Y1183L	

FIG. 3B

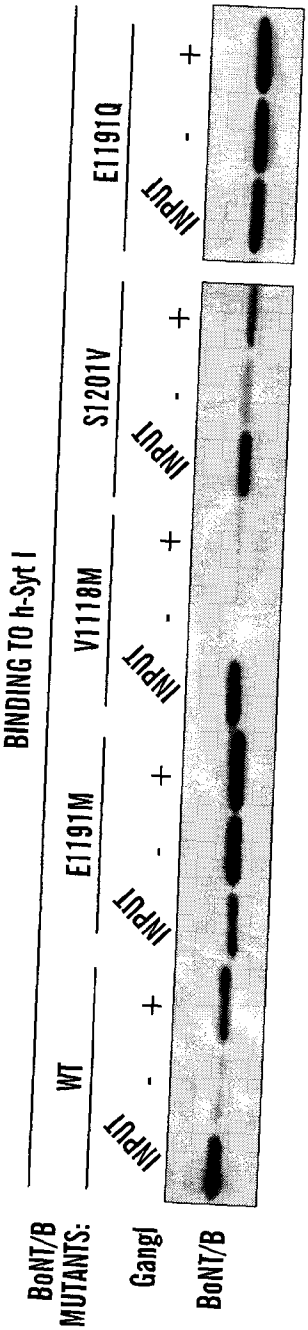

BINDING TO h-Syt I
BoNT/B MUTANTS:
WT
E1191M
V1118M
S1201V
E1191Q
INPUT
-
+
Gangl
BoNT/B

FIG. 4A

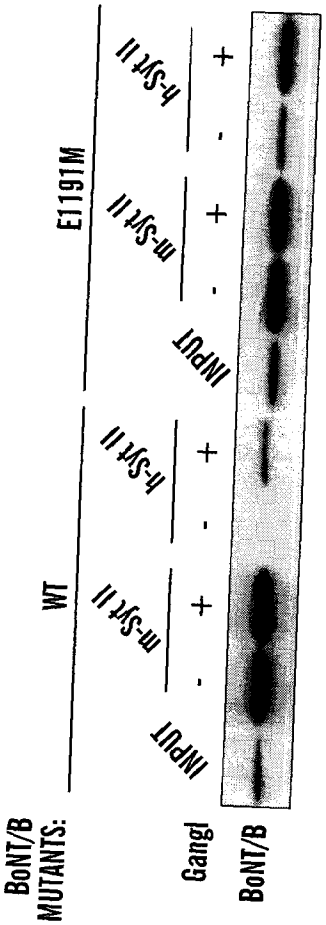

BoNT/B MUTANTS:
WT
E1191M
m-Syt II
h-Syt II
INPUT
-
+
Gangl
BoNT/B

FIG. 4B

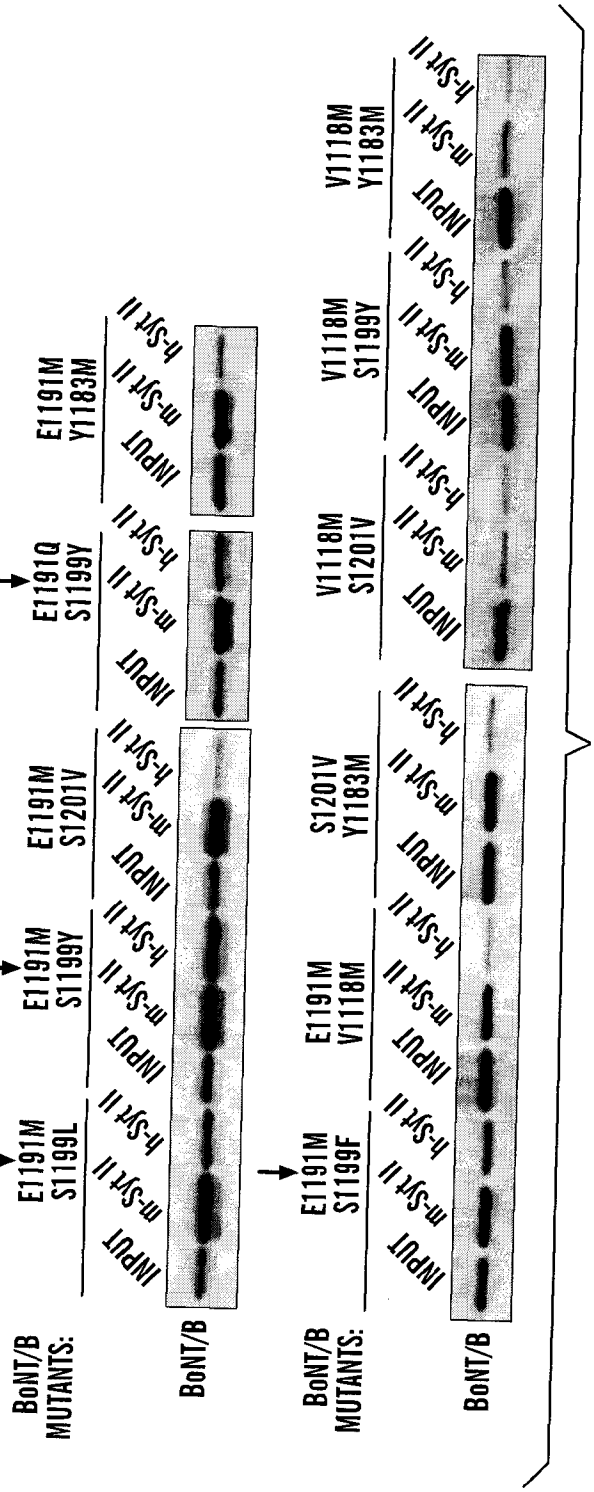

BoNT/B MUTANTS:
E1191M S1199L
E1191M S1199Y
E1191Q S1199Y
E1191M Y1183M
E1191M V1118M
E1191M S1201V
S1201V Y1183M
V1118M S1201V
V1118M S1199Y
V1118M Y1183M
INPUT
m-Syt II
h-Syt II
BoNT/B

FIG. 5A

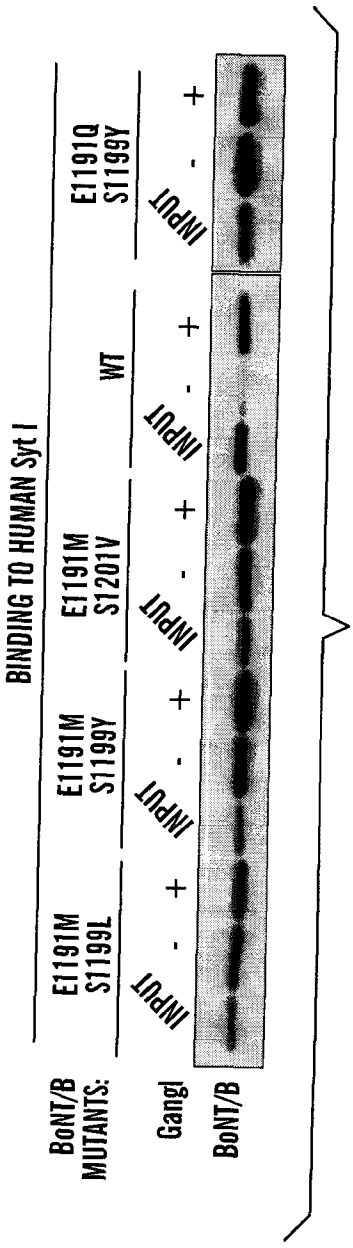

BINDING TO HUMAN Syt I
BoNT/B MUTANTS:
E1191M S1199L
E1191M S1199Y
E1191M S1201V
WT
E1191Q S1199Y
Ganglioside
INPUT
BoNT/B

FIG. 5B

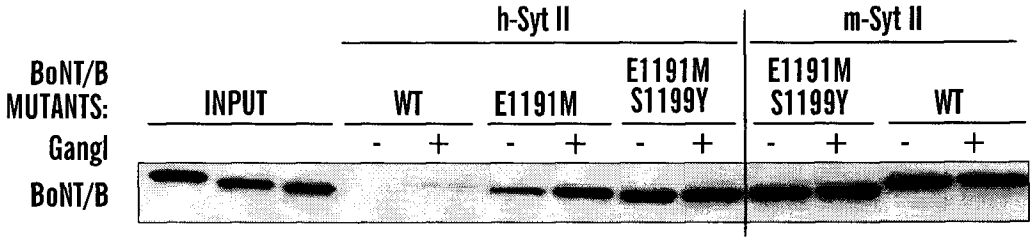


FIG. 6A

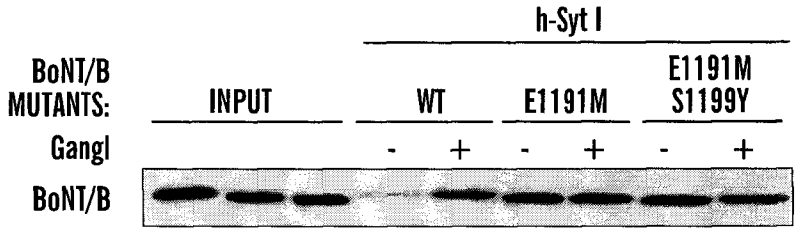


FIG. 6B

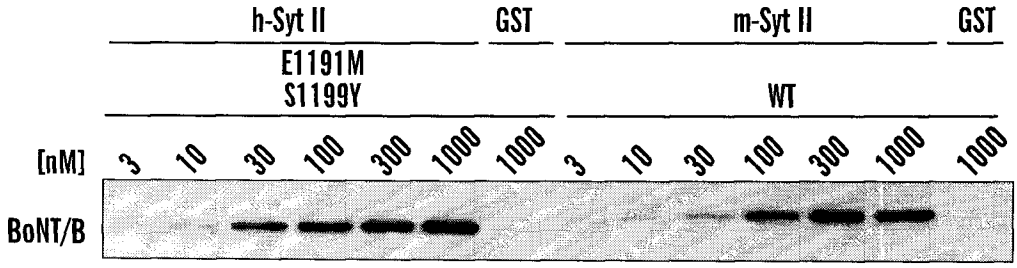


FIG. 6C

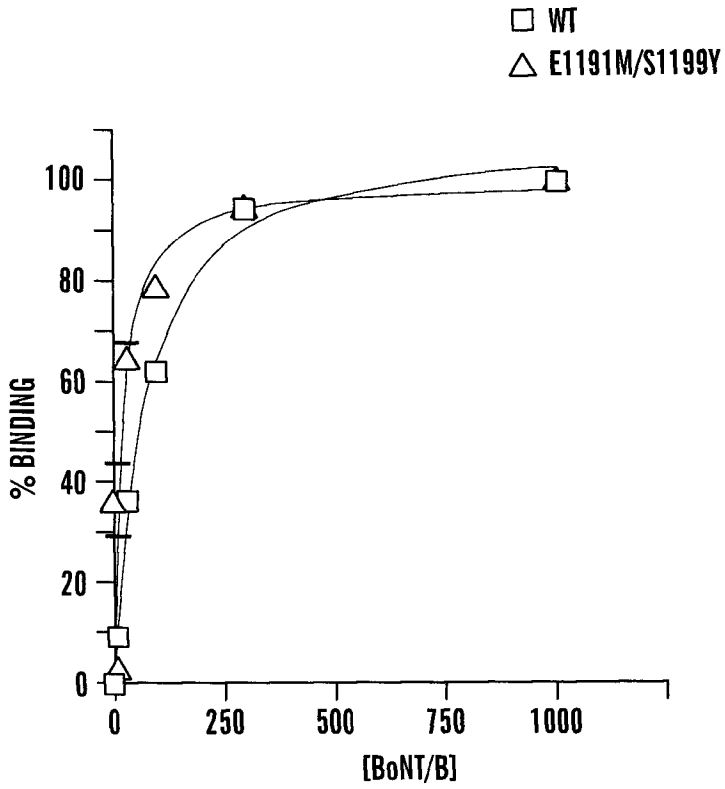

WT
E1191M/S1199Y
100
80
60
40
20
0
% BINDING
0
250
500
750
1000
[BoNT/B]

FIG. 6D

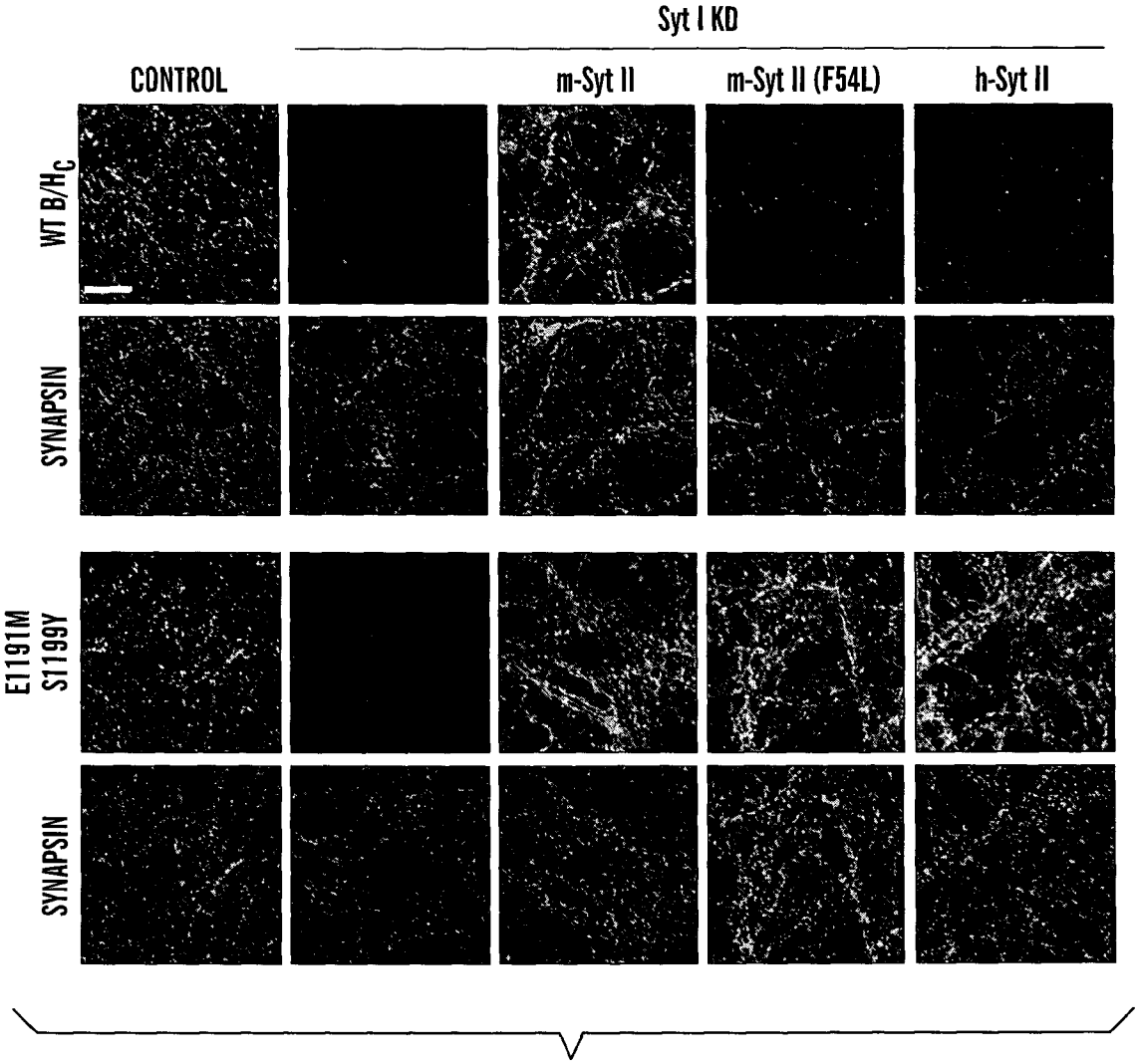

Syt I KD
CONTROL
m-Syt II
m-Syt II (F54L)
h-Syt II
WT B/H$_C$
SYNAPSIN
E1191M
S1199Y
SYNAPSIN

FIG. 7

Protein sequence of BoNT/B-H_C (strain 1) (residues 857-1291 of BoNT/B, strain 1, GenBank: AB232927.1)

NSEILNNIILNLRYKDNNLIDLSGYGAKVEVYDGVELNDKNQFKLTSSANSKIRVTQNQN
IIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIINCMKNNSGWKISIRGNRIIWTLIDIN
GKTKSVFFEYNIREDISEYINRWFFVTITNNLNNAKIYINGKLESNTDIKDIREVIANGEIIF
KLDGDIDRTQFIWMKYFSIFNTELSQSNIEERYKIQSYSEYLKDFWGNPLMYNKEYYMF
NAGNKNSYIKLKKDSPVGEILTRSKYNQNSKYINYRDLYIGEKFIIRRKSNSQSINDDIVR
KEDYIYLDFFNLNQEWRVYTYKYFKKEEEKLFLAPISDSDEFYNTIQIKEYDEQPTYSCQ
LLFKKDEESTDEIGLIGIHRFYESGIVFEEYKDYFCISKWYLKEVKRKPYNLKLGCNWQFI
PKDEGWTE

FIG. 8

The nucleic acid sequence encoding BoNT/B-H_C (strain B1) (residues 857-1291 of BoNT/B,strain 1, based on GenBank: AB232927.1). Note: the sequence has been optimized for expression in E.coli

AACAGCGAAATCCTGAACAACATTATTCTGAACCTGCGCTATAAAGATAACAACCTGATTGA
TCTGAGCGGCTATGGCGCGAAAGTGGAAGTGTATGATGGCGTGGAACTGAACGATAAAAA
CCAGTTCAAACTGACCAGCTCTGCGAACAGCAAAATTCGTGTGACCCAGAACCAGAACATT
ATCTTCAACAGCGTGTTTCTGGATTTTAGCGTGAGCTTTTGGATTCGCATCCCGAAATATAA
AAACGATGGCATCCAGAACTATATCCACAACGAATACACCATTATCAACTGCATGAAAAACA
ACAGCGGCTGGAAAATTAGCATTCGTGGCAACCGTATTATTTGGACCCTGATCGATATTAA
CGGCAAAACCAAAAGCGTGTTCTTCGAATACAACATCCGCGAAGATATCAGCGAATACATT
AACCGCTGGTTCTTTGTGACCATTACCAACAACCTGAACAACGCGAAAATTTATATCAACGG
TAAACTGGAAAGCAACACCGATATCAAAGATATCCGCGAAGTGATTGCGAACGGCGAAATC
ATCTTTAAACTGGATGGCGATATTGATCGTACCCAGTTCATCTGGATGAAATACTTCAGCAT
CTTCAACACCGAACTGAGCCAGAGCAACATTGAAGAACGCTACAAAATCCAGAGCTATAGC
GAATACCTGAAAGATTTTTGGGGCAATCCGCTGATGTATAACAAAGAGTATTACATGTTCAA
CGCGGGTAACAAAAACAGCTATATCAAACTGAAAAAAGATAGCCCGGTGGGCGAAATTCTG
ACCCGTAGCAAATATAACCAGAACAGCAAATACATCAACTATCGCGATCTGTATATCGGCG
AAAAATTTATCATTCGCCGCAAAAGCAACAGCCAGAGCATTAACGATGATATCGTGCGCAA
AGAAGATTATATCTACCTGGATTTTTTCAACCTGAACCAGGAATGGCGCGTTTATACCTATA
AATATTTCAAAAAAGAGGAAGAGAAACTGTTTCTGGCCCCGATTAGCGATAGCGATGAATTT
TACAACACCATCCAAATTAAAGAATACGATGAACAGCCGACCTATAGCTGCCAGCTGCTGT
TTAAAAAAGATGAAGAAAGCACCGATGAAATTGGCCTGATTGGCATCCATCGTTTCTATGAA
AGCGGCATCGTGTTCGAAGAATATAAAGATTATTTCTGCATCAGCAAATGGTATCTGAAAGA
AGTGAAACGCAAACCGTATAACCTGAAACTGGGCTGCAACTGGCAGTTTATTCCGAAAGAT
GAAGGCTGGACCGAATAA

FIG. 9

Clostridium botulinum serotype A:
(1296 a.a.)

Met Pro Phe Val Asn Lys Gln Phe Asn Tyr Lys Asp Pro Val Asn Gly
Val Asp Ile Ala Tyr Ile Lys Ile Pro Asn Ala Gly Gln Met Gln Pro
Val Lys Ala Phe Lys Ile His Asn Lys Ile Trp Val Ile Pro Glu Arg
Asp Thr Phe Thr Asn Pro Glu Glu Gly Asp Leu Asn Pro Pro Pro Glu
Ala Lys Gln Val Pro Val Ser Tyr Tyr Asp Ser Thr Tyr Leu Ser Thr
Asp Asn Glu Lys Asp Asn Tyr Leu Lys Gly Val Thr Lys Leu Phe Glu
Arg Ile Tyr Ser Thr Asp Leu Gly Arg Met Leu Leu Thr Ser Ile Val
Arg Gly Ile Pro Phe Trp Gly Gly Ser Thr Ile Asp Thr Glu Leu Lys
Val Ile Asp Thr Asn Cys Ile Asn Val Ile Gln Pro Asp Gly Ser Tyr
Arg Ser Glu Glu Leu Asn Leu Val Ile Ile Gly Pro Ser Ala Asp Ile
Ile Gln Phe Glu Cys Lys Ser Phe Gly His Glu Val Leu Asn Leu Thr
Arg Asn Gly Tyr Gly Ser Thr Gln Tyr Ile Arg Phe Ser Pro Asp Phe
Thr Phe Gly Phe Glu Glu Ser Leu Glu Val Asp Thr Asn Pro Leu Leu
Gly Ala Gly Lys Phe Ala Thr Asp Pro Ala Val Thr Leu Ala His Glu
Leu Ile His Ala Gly His Arg Leu Tyr Gly Ile Ala Ile Asn Pro Asn
Arg Val Phe Lys Val Asn Thr Asn Ala Tyr Tyr Glu Met Ser Gly Leu
Glu Val Ser Phe Glu Glu Leu Arg Thr Phe Gly Gly His Asp Ala Lys
Phe Ile Asp Ser Leu Gln Glu Asn Glu Phe Arg Leu Tyr Tyr Tyr Asn
Lys Phe Lys Asp Ile Ala Ser Thr Leu Asn Lys Ala Lys Ser Ile Val
Gly Thr Thr Ala Ser Leu Gln Tyr Met Lys Asn Val Phe Lys Glu Lys
Tyr Leu Leu Ser Glu Asp Thr Ser Gly Lys Phe Ser Val Asp Lys Leu
Lys Phe Asp Lys Leu Tyr Lys Met Leu Thr Glu Ile Tyr Thr Glu Asp
Asn Phe Val Lys Phe Phe Lys Val Leu Asn Arg Lys Thr Tyr Leu Asn
Phe Asp Lys Ala Val Phe Lys Ile Asn Ile Val Pro Lys Val Asn Tyr
Thr Ile Tyr Asp Gly Phe Asn Leu Arg Asn Thr Asn Leu Ala Ala Asn
Phe Asn Gly Gln Asn Thr Glu Ile Asn Asn Met Asn Phe Thr Lys Leu
Lys Asn Phe Thr Gly Leu Phe Glu Phe Tyr Lys Leu Leu Cys Val Arg
Gly Ile Ile Thr Ser Lys Thr Lys Ser Leu Asp Lys Gly Tyr Asn Lys
Ala Leu Asn Asp Leu Cys Ile Lys Val Asn Asn Trp Asp Leu Phe Phe
Ser Pro Ser Glu Asp Asn Phe Thr Asn Asp Leu Asn Lys Gly Glu Glu
Ile Thr Ser Asp Thr Asn Ile Glu Ala Ala Glu Glu Asn Ile Ser Leu
Asp Leu Ile Gln Gln Tyr Tyr Leu Thr Phe Asn Phe Asp Asn Glu Pro
Glu Asn Ile Ser Ile Glu Asn Leu Ser Ser Asp Ile Ile Gly Gln Leu
Glu Leu Met Pro Asn Ile Glu Arg Phe Pro Asn Gly Lys Lys Tyr Glu
Leu Asp Lys Tyr Thr Met Phe His Tyr Leu Arg Ala Gln Glu Phe Glu
His Gly Lys Ser Arg Ile Ala Leu Thr Asn Ser Val Asn Glu Ala Leu
Leu Asn Pro Ser Arg Val Tyr Thr Phe Phe Ser Ser Asp Tyr Val Lys
Lys Val Asn Lys Ala Thr Glu Ala Ala Met Phe Leu Gly Trp Val Glu
Gln Leu Val Tyr Asp Phe Thr Asp Glu Thr Ser Glu Val Ser Thr Thr
Asp Lys Ile Ala Asp Ile Thr Ile Ile Ile Pro Tyr Ile Gly Pro Ala
Leu Asn Ile Gly Asn Met Leu Tyr Lys Asp Asp Phe Val Gly Ala Leu
Ile Phe Ser Gly Ala Val Ile Leu Leu Glu Phe Ile Pro Glu Ile Ala
Ile Pro Val Leu Gly Thr Phe Ala Leu Val Ser Tyr Ile Ala Asn Lys
Val Leu Thr Val Gln Thr Ile Asp Asn Ala Leu Ser Lys Arg Asn Glu
Lys Trp Asp Glu Val Tyr Lys Tyr Ile Val Thr Asn Trp Leu Ala Lys

FIG. 10

Val Asn Thr Gln Ile Asp Leu Ile Arg Lys Lys Met Lys Glu Ala Leu
Glu Asn Gln Ala Glu Ala Thr Lys Ala Ile Ile Asn Tyr Gln Tyr Asn
Gln Tyr Thr Glu Glu Glu Lys Asn Asn Ile Asn Phe Asn Ile Asp Asp
Leu Ser Ser Lys Leu Asn Glu Ser Ile Asn Lys Ala Met Ile Asn Ile
Asn Lys Phe Leu Asn Gln Cys Ser Val Ser Tyr Leu Met Asn Ser Met
Ile Pro Tyr Gly Val Lys Arg Leu Glu Asp Phe Asp Ala Ser Leu Lys
Asp Ala Leu Leu Lys Tyr Ile Tyr Asp Asn Arg Gly Thr Leu Ile Gly
Gln Val Asp Arg Leu Lys Asp Lys Val Asn Asn Thr Leu Ser Thr Asp
Ile Pro Phe Gln Leu Ser Lys Tyr Val Asp Asn Gln Arg Leu Leu Ser
Thr Phe Thr Glu Tyr Ile Lys Asn Ile Ile Asn Thr Ser Ile Leu Asn
Leu Arg Tyr Glu Ser Asn His Leu Ile Asp Leu Ser Arg Tyr Ala Ser
Lys Ile Asn Ile Gly Ser Lys Val Asn Phe Asp Pro Ile Asp Lys Asn
Gln Ile Gln Leu Phe Asn Leu Glu Ser Ser Lys Ile Glu Val Ile Leu
Lys Asn Ala Ile Val Tyr Asn Ser Met Tyr Glu Asn Phe Ser Thr Ser
Phe Trp Ile Arg Ile Pro Lys Tyr Phe Asn Ser Ile Ser Leu Asn Asn
Glu Tyr Thr Ile Ile Asn Cys Met Glu Asn Asn Ser Gly Trp Lys Val
Ser Leu Asn Tyr Gly Glu Ile Ile Trp Thr Leu Gln Asp Thr Gln Glu
Ile Lys Gln Arg Val Val Phe Lys Tyr Ser Gln Met Ile Asn Ile Ser
Asp Tyr Ile Asn Arg Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu
Asn Asn Ser Lys Ile Tyr Ile Asn Gly Arg Leu Ile Asp Gln Lys Pro
Ile Ser Asn Leu Gly Asn Ile His Ala Ser Asn Asn Ile Met Phe Lys
Leu Asp Gly Cys Arg Asp Thr His Arg Tyr Ile Trp Ile Lys Tyr Phe
Asn Leu Phe Asp Lys Glu Leu Asn Glu Lys Glu Ile Lys Asp Leu Tyr
Asp Asn Gln Ser Asn Ser Gly Ile Leu Lys Asp Phe Trp Gly Asp Tyr
Leu Gln Tyr Asp Lys Pro Tyr Tyr Met Leu Asn Leu Tyr Asp Pro Asn
Lys Tyr Val Asp Val Asn Asn Val Gly Ile Arg Gly Tyr Met Tyr Leu
Lys Gly Pro Arg Gly Ser Val Met Thr Thr Asn Ile Tyr Leu Asn Ser
Ser Leu Tyr Arg Gly Thr Lys Phe Ile Ile Lys Lys Tyr Ala Ser Gly
Asn Lys Asp Asn Ile Val Arg Asn Asn Asp Arg Val Tyr Ile Asn Val
Val Val Lys Asn Lys Glu Tyr Arg Leu Ala Thr Asn Ala Ser Gln Ala
Gly Val Glu Lys Ile Leu Ser Ala Leu Glu Ile Pro Asp Val Gly Asn
Leu Ser Gln Val Val Val Met Lys Ser Lys Asn Asp Gln Gly Ile Thr
Asn Lys Cys Lys Met Asn Leu Gln Asp Asn Asn Gly Asn Asp Ile Gly
Phe Ile Gly Phe His Gln Phe Asn Asn Ile Ala Lys Leu Val Ala Ser
Asn Trp Tyr Asn Arg Gln Ile Glu Arg Ser Ser Arg Thr Leu Gly Cys
Ser Trp Glu Phe Ile Pro Val Asp Asp Gly Trp Gly Glu Arg Pro Leu

FIG. 10 Continued

Clostridium botulinum serotype B
(1291 a.a.)

Met Pro Val Thr Ile Asn Asn Phe Asn Tyr Asn Asp Pro Ile Asp Asn
Asn Asn Ile Ile Met Met Glu Pro Pro Phe Ala Arg Gly Thr Gly Arg
Tyr Tyr Lys Ala Phe Lys Ile Thr Asp Arg Ile Trp Ile Ile Pro Glu
Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly
Ile Phe Asn Arg Asp Val Cys Glu Tyr Tyr Asp Pro Asp Tyr Leu Asn
Thr Asn Asp Lys Lys Asn Ile Phe Leu Gln Thr Met Ile Lys Leu Phe
Asn Arg Ile Lys Ser Lys Pro Leu Gly Glu Lys Leu Leu Glu Met Ile
Ile Asn Gly Ile Pro Tyr Leu Gly Asp Arg Arg Val Pro Leu Glu Glu
Phe Asn Thr Asn Ile Ala Ser Val Thr Val Asn Lys Leu Ile Ser Asn
Pro Gly Glu Val Glu Arg Lys Lys Gly Ile Phe Ala Asn Leu Ile Ile
Phe Gly Pro Gly Pro Val Leu Asn Glu Asn Glu Thr Ile Asp Ile Gly
Ile Gln Asn His Phe Ala Ser Arg Glu Gly Phe Gly Gly Ile Met Gln
Met Lys Phe Cys Pro Glu Tyr Val Ser Val Phe Asn Asn Val Gln Glu
Asn Lys Gly Ala Ser Ile Phe Asn Arg Arg Gly Tyr Phe Ser Asp Pro
Ala Leu Ile Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr
Gly Ile Lys Val Asp Asp Leu Pro Ile Val Pro Asn Glu Lys Lys Phe
Phe Met Gln Ser Thr Asp Ala Ile Gln Ala Glu Glu Leu Tyr Thr Phe
Gly Gly Gln Asp Pro Ser Ile Ile Thr Pro Ser Thr Asp Lys Ser Ile
Tyr Asp Lys Val Leu Gln Asn Phe Arg Gly Ile Val Asp Arg Leu Asn
Lys Val Leu Val Cys Ile Ser Asp Pro Asn Ile Asn Ile Asn Ile Tyr
Lys Asn Lys Phe Lys Asp Lys Tyr Lys Phe Val Glu Asp Ser Glu Gly
Lys Tyr Ser Ile Asp Val Glu Ser Phe Asp Lys Leu Tyr Lys Ser Leu
Met Phe Gly Phe Thr Glu Thr Asn Ile Ala Glu Asn Tyr Lys Ile Lys
Thr Arg Ala Ser Tyr Phe Ser Asp Ser Leu Pro Pro Val Lys Ile Lys
Asn Leu Leu Asp Asn Glu Ile Tyr Thr Ile Glu Glu Gly Phe Asn Ile
Ser Asp Lys Asp Met Glu Lys Glu Tyr Arg Gly Gln Asn Lys Ala Ile
Asn Lys Gln Ala Tyr Glu Glu Ile Ser Lys Glu His Leu Ala Val Tyr
Lys Ile Gln Met Cys Lys Ser Val Lys Ala Pro Gly Ile Cys Ile Asp
Val Asp Asn Glu Asp Leu Phe Phe Ile Ala Asp Lys Asn Ser Phe Ser
Asp Asp Leu Ser Lys Asn Glu Arg Ile Glu Tyr Asn Thr Gln Ser Asn
Tyr Ile Glu Asn Asp Phe Pro Ile Asn Glu Leu Ile Leu Asp Thr Asp
Leu Ile Ser Lys Ile Glu Leu Pro Ser Glu Asn Thr Glu Ser Leu Thr
Asp Phe Asn Val Asp Val Pro Val Tyr Glu Lys Gln Pro Ala Ile Lys
Lys Ile Phe Thr Asp Glu Asn Thr Ile Phe Gln Tyr Leu Tyr Ser Gln
Thr Phe Pro Leu Asp Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp
Asp Ala Leu Leu Phe Ser Asn Lys Val Tyr Ser Phe Phe Ser Met Asp
Tyr Ile Lys Thr Ala Asn Lys Val Val Glu Ala Gly Leu Phe Ala Gly
Trp Val Lys Gln Ile Val Asn Asp Phe Val Ile Glu Ala Asn Lys Ser
Asn Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile
Gly Leu Ala Leu Asn Val Gly Asn Glu Thr Ala Lys Gly Asn Phe Glu
Asn Ala Phe Glu Ile Ala Gly Ala Ser Ile Leu Leu Glu Phe Ile Pro
Glu Leu Leu Ile Pro Val Val Gly Ala Phe Leu Leu Glu Ser Tyr Ile
Asp Asn Lys Asn Lys Ile Ile Lys Thr Ile Asp Asn Ala Leu Thr Lys
Arg Asn Glu Lys Trp Ser Asp Met Tyr Gly Leu Ile Val Ala Gln Trp
Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile Lys Glu Gly Met Tyr

FIG. 11

Lys Ala Leu Asn Tyr Gln Ala Gln Ala Leu Glu Glu Ile Ile Lys Tyr
Arg Tyr Asn Ile Tyr Ser Glu Lys Glu Lys Ser Asn Ile Asn Ile Asp
Phe Asn Asp Ile Asn Ser Lys Leu Asn Glu Gly Ile Asn Gln Ala Ile
Asp Asn Ile Asn Asn Phe Ile Asn Gly Cys Ser Val Ser Tyr Leu Met
Lys Lys Met Ile Pro Leu Ala Val Glu Lys Leu Leu Asp Phe Asp Asn
Thr Leu Lys Lys Asn Leu Leu Asn Tyr Ile Asp Glu Asn Lys Leu Tyr
Leu Ile Gly Ser Ala Glu Tyr Glu Lys Ser Lys Val Asn Lys Tyr Leu
Lys Thr Ile Met Pro Phe Asp Leu Ser Ile Tyr Thr Asn Asp Thr Ile
Leu Ile Glu Met Phe Asn Lys Tyr Asn Ser Glu Ile Leu Asn Asn Ile
Ile Leu Asn Leu Arg Tyr Lys Asp Asn Asn Leu Ile Asp Leu Ser Gly
Tyr Gly Ala Lys Val Glu Val Tyr Asp Gly Val Glu Leu Asn Asp Lys
Asn Gln Phe Lys Leu Thr Ser Ser Ala Asn Ser Lys Ile Arg Val Thr
Gln Asn Gln Asn Ile Ile Phe Asn Ser Val Phe Leu Asp Phe Ser Val
Ser Phe Trp Ile Arg Ile Pro Lys Tyr Lys Asn Asp Gly Ile Gln Asn
Tyr Ile His Asn Glu Tyr Thr Ile Ile Asn Cys Met Lys Asn Asn Ser
Gly Trp Lys Ile Ser Ile Arg Gly Asn Arg Ile Ile Trp Thr Leu Ile
Asp Ile Asn Gly Lys Thr Lys Ser Val Phe Phe Glu Tyr Asn Ile Arg
Glu Asp Ile Ser Glu Tyr Ile Asn Arg Trp Phe Phe Val Thr Ile Thr
Asn Asn Leu Asn Asn Ala Lys Ile Tyr Ile Asn Gly Lys Leu Glu Ser
Asn Thr Asp Ile Lys Asp Ile Arg Glu Val Ile Ala Asn Gly Glu Ile
Ile Phe Lys Leu Asp Gly Asp Ile Asp Arg Thr Gln Phe Ile Trp Met
Lys Tyr Phe Ser Ile Phe Asn Thr Glu Leu Ser Gln Ser Asn Ile Glu
Glu Arg Tyr Lys Ile Gln Ser Tyr Ser Glu Tyr Leu Lys Asp Phe Trp
Gly Asn Pro Leu Met Tyr Asn Lys Glu Tyr Tyr Met Phe Asn Ala Gly
Asn Lys Asn Ser Tyr Ile Lys Leu Lys Lys Asp Ser Pro Val Gly Glu
Ile Leu Thr Arg Ser Lys Tyr Asn Gln Asn Ser Lys Tyr Ile Asn Tyr
Arg Asp Leu Tyr Ile Gly Glu Lys Phe Ile Ile Arg Arg Lys Ser Asn
Ser Gln Ser Ile Asn Asp Asp Ile Val Arg Lys Glu Asp Tyr Ile Tyr
Leu Asp Phe Phe Asn Leu Asn Gln Glu Trp Arg Val Tyr Thr Tyr Lys
Tyr Phe Lys Lys Glu Glu Glu Lys Leu Phe Leu Ala Pro Ile Ser Asp
Ser Asp Glu Phe Tyr Asn Thr Ile Gln Ile Lys Glu Tyr Asp Glu Gln
Pro Thr Tyr Ser Cys Gln Leu Leu Phe Lys Lys Asp Glu Glu Ser Thr
Asp Glu Ile Gly Leu Ile Gly Ile His Arg Phe Tyr Glu Ser Gly Ile
Val Phe Glu Glu Tyr Lys Asp Tyr Phe Cys Ile Ser Lys Trp Tyr Leu
Lys Glu Val Lys Arg Lys Pro Tyr Asn Leu Lys Leu Gly Cys Asn Trp
Gln Phe Ile Pro Lys Asp Glu Gly Trp Thr Glu

FIG. 11 Continued

Clostridium botulinum serotype C1
(1291 a.a.)

Met Pro Ile Thr Ile Asn Asn Phe Asn Tyr Ser Asp Pro Val Asp Asn
Lys Asn Ile Leu Tyr Leu Asp Thr His Leu Asn Thr Leu Ala Asn Glu
Pro Glu Lys Ala Phe Arg Ile Thr Gly Asn Ile Trp Val Ile Pro Asp
Arg Phe Ser Arg Asn Ser Asn Pro Asn Leu Asn Lys Pro Pro Arg Val
Thr Ser Pro Lys Ser Gly Tyr Tyr Asp Pro Asn Tyr Leu Ser Thr Asp
Ser Asp Lys Asp Pro Phe Leu Lys Glu Ile Ile Lys Leu Phe Lys Arg
Ile Asn Ser Arg Glu Ile Gly Glu Glu Leu Ile Tyr Arg Leu Ser Thr
Asp Ile Pro Phe Pro Gly Asn Asn Asn Thr Pro Ile Asn Thr Phe Asp
Phe Asp Val Asp Phe Asn Ser Val Asp Val Lys Thr Arg Gln Gly Asn
Asn Trp Val Lys Thr Gly Ser Ile Asn Pro Ser Val Ile Ile Thr Gly
Pro Arg Glu Asn Ile Ile Asp Pro Glu Thr Ser Thr Phe Lys Leu Thr
Asn Asn Thr Phe Ala Ala Gln Glu Gly Phe Gly Ala Leu Ser Ile Ile
Ser Ile Ser Pro Arg Phe Met Leu Thr Tyr Ser Asn Ala Thr Asn Asp
Val Gly Glu Gly Arg Phe Ser Lys Ser Glu Phe Cys Met Asp Pro Ile
Leu Ile Leu Met His Glu Leu Asn His Ala Met His Asn Leu Tyr Gly
Ile Ala Ile Pro Asn Asp Gln Thr Ile Ser Ser Val Thr Ser Asn Ile
Phe Tyr Ser Gln Tyr Asn Val Lys Leu Glu Tyr Ala Glu Ile Tyr Ala
Phe Gly Gly Pro Thr Ile Asp Leu Ile Pro Lys Ser Ala Arg Lys Tyr
Phe Glu Glu Lys Ala Leu Asp Tyr Tyr Arg Ser Ile Ala Lys Arg Leu
Asn Ser Ile Thr Thr Ala Asn Pro Ser Ser Phe Asn Lys Tyr Ile Gly
Glu Tyr Lys Gln Lys Leu Ile Arg Lys Tyr Arg Phe Val Val Glu Ser
Ser Gly Glu Val Thr Val Asn Arg Asn Lys Phe Val Glu Leu Tyr Asn
Glu Leu Thr Gln Ile Phe Thr Glu Phe Asn Tyr Ala Lys Ile Tyr Asn
Val Gln Asn Arg Lys Ile Tyr Leu Ser Asn Val Tyr Thr Pro Val Thr
Ala Asn Ile Leu Asp Asp Asn Val Tyr Asp Ile Gln Asn Gly Phe Asn
Ile Pro Lys Ser Asn Leu Asn Val Leu Phe Met Gly Gln Asn Leu Ser
Arg Asn Pro Ala Leu Arg Lys Val Asn Pro Glu Asn Met Leu Tyr Leu
Phe Thr Lys Phe Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn
Lys Thr Leu Asp Cys Arg Glu Leu Leu Val Lys Asn Thr Asp Leu Pro
Phe Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys
Asp Ile Asn Glu Glu Thr Glu Val Ile Tyr Tyr Pro Asp Asn Val Ser
Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu
Asp Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly
Glu Asn Gln Val Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu
Asn Ser Tyr Tyr Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu
Asp Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala
Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly
Val Gln Gly Gly Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp
Phe Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp
Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn
Ser Val Arg Arg Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val
Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly
Ala Phe Val Ile Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys
Thr Ile Asp Asn Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser
Tyr Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe
Asn Asn Ile Ser Tyr Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly

FIG. 12

Ala Ile Lys Ala Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser
Asp Lys Glu Asn Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu
Asp Val Lys Ile Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg
Glu Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile
Asp Glu Leu Asn Glu Phe Asp Arg Asn Thr Lys Ala Lys Leu Ile Asn
Leu Ile Asp Ser His Asn Ile Ile Leu Val Gly Glu Val Asp Lys Leu
Lys Ala Lys Val Asn Asn Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile
Phe Ser Tyr Thr Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr
Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys
Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu
Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly
Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn
Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile
Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp
Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe
Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn
Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe
Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile
Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile
Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr
Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp
Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu
Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn
Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu
Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg
Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg
Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu
Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys
Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala
Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe
Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe
Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly
Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu
Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr
Ser Thr His Trp Gly Phe Val Pro Val Ser Glu

FIG. 12 Continued

Clostridium botulinum serotype D
(1276 a.a.)

Met Thr Trp Pro Val Lys Asp Phe Asn Tyr Ser Asp Pro Val Asn Asp
Asn Asp Ile Leu Tyr Leu Arg Ile Pro Gln Asn Lys Leu Ile Thr Thr
Pro Val Lys Ala Phe Met Ile Thr Gln Asn Ile Trp Val Ile Pro Glu
Arg Phe Ser Ser Asp Thr Asn Pro Ser Leu Ser Lys Pro Pro Arg Pro
Thr Ser Lys Tyr Gln Ser Tyr Tyr Asp Pro Ser Tyr Leu Ser Thr Asp
Glu Gln Lys Asp Thr Phe Leu Lys Gly Ile Ile Lys Leu Phe Lys Arg
Ile Asn Glu Arg Asp Ile Gly Lys Lys Leu Ile Asn Tyr Leu Val Val
Gly Ser Pro Phe Met Gly Asp Ser Ser Thr Pro Glu Asp Thr Phe Asp
Phe Thr Arg His Thr Thr Asn Ile Ala Val Glu Lys Phe Glu Asn Gly
Ser Trp Lys Val Thr Asn Ile Ile Thr Pro Ser Val Leu Ile Phe Gly
Pro Leu Pro Asn Ile Leu Asp Tyr Thr Ala Ser Leu Thr Leu Gln Gly
Gln Gln Ser Asn Pro Ser Phe Glu Gly Phe Gly Thr Leu Ser Ile Leu
Lys Val Ala Pro Glu Phe Leu Leu Thr Phe Ser Asp Val Thr Ser Asn
Gln Ser Ser Ala Val Leu Gly Lys Ser Ile Phe Cys Met Asp Pro Val
Ile Ala Leu Met His Glu Leu Thr His Ser Leu His Gln Leu Tyr Gly
Ile Asn Ile Pro Ser Asp Lys Arg Ile Arg Pro Gln Val Ser Glu Gly
Phe Phe Ser Gln Asp Gly Pro Asn Val Gln Phe Glu Glu Leu Tyr Thr
Phe Gly Gly Leu Asp Val Glu Ile Ile Pro Gln Ile Glu Arg Ser Gln
Leu Arg Glu Lys Ala Leu Gly His Tyr Lys Asp Ile Ala Lys Arg Leu
Asn Asn Ile Asn Lys Thr Ile Pro Ser Ser Trp Ile Ser Asn Ile Asp
Lys Tyr Lys Lys Ile Phe Ser Glu Lys Tyr Asn Phe Asp Lys Asp Asn
Thr Gly Asn Phe Val Val Asn Ile Asp Lys Phe Asn Ser Leu Tyr Ser
Asp Leu Thr Asn Val Met Ser Glu Val Val Tyr Ser Ser Gln Tyr Asn
Val Lys Asn Arg Thr His Tyr Phe Ser Arg His Tyr Leu Pro Val Phe
Ala Asn Ile Leu Asp Asp Asn Ile Tyr Thr Ile Arg Asp Gly Phe Asn
Leu Thr Asn Lys Gly Phe Asn Ile Glu Asn Ser Gly Gln Asn Ile Glu
Arg Asn Pro Ala Leu Gln Lys Leu Ser Ser Glu Ser Val Val Asp Leu
Phe Thr Lys Val Cys Leu Arg Leu Thr Lys Asn Ser Arg Asp Asp Ser
Thr Cys Ile Lys Val Lys Asn Asn Arg Leu Pro Tyr Val Ala Asp Lys
Asp Ser Ile Ser Gln Glu Ile Phe Glu Asn Lys Ile Ile Thr Asp Glu
Thr Asn Val Gln Asn Tyr Ser Asp Lys Phe Ser Leu Asp Glu Ser Ile
Leu Asp Gly Gln Val Pro Ile Asn Pro Glu Ile Val Asp Pro Leu Leu
Pro Asn Val Asn Met Glu Pro Leu Asn Leu Pro Gly Glu Glu Ile Val
Phe Tyr Asp Asp Ile Thr Lys Tyr Val Asp Tyr Leu Asn Ser Tyr Tyr
Tyr Leu Glu Ser Gln Lys Leu Ser Asn Asn Val Glu Asn Ile Thr Leu
Thr Thr Ser Val Glu Glu Ala Leu Gly Tyr Ser Asn Lys Ile Tyr Thr
Phe Leu Pro Ser Leu Ala Glu Lys Val Asn Lys Gly Val Gln Ala Gly
Leu Phe Leu Asn Trp Ala Asn Glu Val Val Glu Asp Phe Thr Thr Asn
Ile Met Lys Lys Asp Thr Leu Asp Lys Ile Ser Asp Val Ser Val Ile
Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Gly Asn Ser Ala Leu Arg
Gly Asn Phe Asn Gln Ala Phe Ala Thr Ala Gly Val Ala Phe Leu Leu
Glu Gly Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly Val Phe Thr Phe
Tyr Ser Ser Ile Gln Glu Arg Glu Lys Ile Ile Lys Thr Ile Glu Asn
Cys Leu Glu Gln Arg Val Lys Arg Trp Lys Asp Ser Tyr Gln Trp Met
Val Ser Asn Trp Leu Ser Arg Ile Thr Thr Gln Phe Asn His Ile Asn

FIG. 13

Tyr Gln Met Tyr Asp Ser Leu Ser Tyr Gln Ala Asp Ala Ile Lys Ala
Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser Asp Lys Glu Asn
Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu Asp Val Lys Ile
Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg Glu Cys Ser Val
Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile Asp Glu Leu Asn
Lys Phe Asp Leu Arg Thr Lys Thr Glu Leu Ile Asn Leu Ile Asp Ser
His Asn Ile Ile Leu Val Gly Glu Val Asp Arg Leu Lys Ala Lys Val
Asn Glu Ser Phe Glu Asn Thr Met Pro Phe Asn Ile Phe Ser Tyr Thr
Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe Asn Ser Ile
Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Lys Lys Asn Ala Leu Val
Asp Thr Ser Gly Tyr Asn Ala Glu Val Arg Val Gly Asp Asn Val Gln
Leu Asn Thr Ile Tyr Thr Asn Asp Phe Lys Leu Ser Ser Ser Gly Asp
Lys Ile Ile Val Asn Leu Asn Asn Asn Ile Leu Tyr Ser Ala Ile Tyr
Glu Asn Ser Ser Val Ser Phe Trp Ile Lys Ile Ser Lys Asp Leu Thr
Asn Ser His Asn Glu Tyr Thr Ile Ile Asn Ser Ile Glu Gln Asn Ser
Gly Trp Lys Leu Cys Ile Arg Asn Gly Asn Ile Glu Trp Ile Leu Gln
Asp Val Asn Arg Lys Tyr Lys Ser Leu Ile Phe Asp Tyr Ser Glu Ser
Leu Ser His Thr Gly Tyr Thr Asn Lys Trp Phe Phe Val Thr Ile Thr
Asn Asn Ile Met Gly Tyr Met Lys Leu Tyr Ile Asn Gly Glu Leu Lys
Gln Ser Gln Lys Ile Glu Asp Leu Asp Glu Val Lys Leu Asp Lys Thr
Ile Val Phe Gly Ile Asp Glu Asn Ile Asp Glu Asn Gln Met Leu Trp
Ile Arg Asp Phe Asn Ile Phe Ser Lys Glu Leu Ser Asn Glu Asp Ile
Asn Ile Val Tyr Glu Gly Gln Ile Leu Arg Asn Val Ile Lys Asp Tyr
Trp Gly Asn Pro Leu Lys Phe Asp Thr Glu Tyr Tyr Ile Ile Asn Asp
Asn Tyr Ile Asp Arg Tyr Ile Ala Pro Glu Ser Asn Val Leu Val Leu
Val Gln Tyr Pro Asp Arg Ser Lys Leu Tyr Thr Gly Asn Pro Ile Thr
Ile Lys Ser Val Ser Asp Lys Asn Pro Tyr Ser Arg Ile Leu Asn Gly
Asp Asn Ile Ile Leu His Met Leu Tyr Asn Ser Arg Lys Tyr Met Ile
Ile Arg Asp Thr Asp Thr Ile Tyr Ala Thr Gln Gly Gly Glu Cys Ser
Gln Asn Cys Val Tyr Ala Leu Lys Leu Gln Ser Asn Leu Gly Asn Tyr
Gly Ile Gly Ile Phe Ser Ile Lys Asn Ile Val Ser Lys Asn Lys Tyr
Cys Ser Gln Ile Phe Ser Ser Phe Arg Glu Asn Thr Met Leu Leu Ala
Asp Ile Tyr Lys Pro Trp Arg Phe Ser Phe Lys Asn Ala Tyr Thr Pro
Val Ala Val Thr Asn Tyr Glu Thr Lys Leu Leu Ser Thr Ser Ser Phe
Trp Lys Phe Ile Ser Arg Asp Pro Gly Trp Val Glu

FIG. 13 Continued

Clostridium botulinum serotype E
(1252 a.a.)

Met Pro Lys Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp Arg
Thr Ile Leu Tyr Ile Lys Pro Gly Gly Cys Gln Glu Phe Tyr Lys Ser
Phe Asn Ile Met Lys Asn Ile Trp Ile Ile Pro Glu Arg Asn Val Ile
Gly Thr Thr Pro Gln Asp Phe His Pro Pro Thr Ser Leu Lys Asn Gly
Asp Ser Ser Tyr Tyr Asp Pro Asn Tyr Leu Gln Ser Asp Glu Glu Lys
Asp Arg Phe Leu Lys Ile Val Thr Lys Ile Phe Asn Arg Ile Asn Asn
Asn Leu Ser Gly Gly Ile Leu Leu Glu Glu Leu Ser Lys Ala Asn Pro
Tyr Leu Gly Asn Asp Asn Thr Pro Asp Asn Gln Phe His Ile Gly Asp
Ala Ser Ala Val Glu Ile Lys Phe Ser Asn Gly Ser Gln Asp Ile Leu
Leu Pro Asn Val Ile Ile Met Gly Ala Glu Pro Asp Leu Phe Glu Thr
Asn Ser Ser Asn Ile Ser Leu Arg Asn Asn Tyr Met Pro Ser Asn His
Gly Phe Gly Ser Ile Ala Ile Val Thr Phe Ser Pro Glu Tyr Ser Phe
Arg Phe Asn Asp Asn Ser Met Asn Glu Phe Ile Gln Asp Pro Ala Leu
Thr Leu Met His Glu Leu Ile His Ser Leu His Gly Leu Tyr Gly Ala
Lys Gly Ile Thr Thr Lys Tyr Thr Ile Thr Gln Lys Gln Asn Pro Leu
Ile Thr Asn Ile Arg Gly Thr Asn Ile Glu Glu Phe Leu Thr Phe Gly
Gly Thr Asp Leu Asn Ile Ile Thr Ser Ala Gln Ser Asn Asp Ile Tyr
Thr Asn Leu Leu Ala Asp Tyr Lys Lys Ile Ala Ser Lys Leu Ser Lys
Val Gln Val Ser Asn Pro Leu Leu Asn Pro Tyr Lys Asp Val Phe Glu
Ala Lys Tyr Gly Leu Asp Lys Asp Ala Ser Gly Ile Tyr Ser Val Asn
Ile Asn Lys Phe Asn Asp Ile Phe Lys Lys Leu Tyr Ser Phe Thr Glu
Phe Asp Leu Ala Thr Lys Phe Gln Val Lys Cys Arg Gln Thr Tyr Ile
Gly Gln Tyr Lys Tyr Phe Lys Leu Ser Asn Leu Leu Asn Asp Ser Ile
Tyr Asn Ile Ser Glu Gly Tyr Asn Ile Asn Asn Leu Lys Val Asn Phe
Arg Gly Gln Asn Ala Asn Leu Asn Pro Arg Ile Ile Thr Pro Ile Thr
Gly Arg Gly Leu Val Lys Lys Ile Ile Arg Phe Cys Lys Asn Ile Val
Ser Val Lys Gly Ile Arg Lys Ser Ile Cys Ile Glu Ile Asn Asn Gly
Glu Leu Phe Phe Val Ala Ser Glu Asn Ser Tyr Asn Asp Asp Asn Ile
Asn Thr Pro Lys Glu Ile Asp Asp Thr Val Thr Ser Asn Asn Asn Tyr
Glu Asn Asp Leu Asp Gln Val Ile Leu Asn Phe Asn Ser Glu Ser Ala
Pro Gly Leu Ser Asp Glu Lys Leu Asn Leu Thr Ile Gln Asn Asp Ala
Tyr Ile Pro Lys Tyr Asp Ser Asn Gly Thr Ser Asp Ile Glu Gln His
Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val
Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala
Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile
Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile
Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr
Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu
Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Phe Lys Asp Ala
Leu Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Glu Pro Glu Leu
Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser
Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Asn Ala Leu Lys
Glu Arg Asp Glu Lys Trp Lys Glu Val Tyr Ser Phe Ile Val Ser Asn
Trp Met Thr Lys Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu Gln Met
Tyr Gln Ala Leu Gln Asn Gln Val Asn Ala Ile Lys Thr Ile Ile Glu
Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Glu Lys Asn Glu Leu Thr Asn

FIG. 14

Lys Tyr Asp Ile Lys Gln Ile Glu Asn Glu Leu Asn Gln Lys Val Ser
Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser
Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu
Tyr Asp Glu Asn Val Lys Thr Tyr Leu Leu Asn Tyr Ile Ile Gln His
Gly Ser Ile Leu Gly Glu Ser Gln Gln Glu Leu Asn Ser Met Val Thr
Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp
Asp Lys Ile Leu Ile Ser Tyr Phe Asn Lys Phe Phe Lys Arg Ile Lys
Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp
Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Gly Asp Val Tyr Lys
Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser
Glu Val Asn Ile Ser Gln Asn Asp Tyr Ile Ile Tyr Asp Asn Lys Tyr
Lys Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Asn Tyr Asp Asn
Lys Ile Val Asn Val Asn Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg
Asp Asn Asn Ser Gly Trp Lys Val Ser Leu Asn His Asn Glu Ile Ile
Trp Thr Leu Gln Asp Asn Ala Gly Ile Asn Gln Lys Leu Ala Phe Asn
Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe
Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn
Gly Asn Leu Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile His
Val Ser Asp Asn Ile Leu Phe Lys Ile Val Asn Cys Ser Tyr Thr Arg
Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu Leu Asp Glu
Thr Glu Ile Gln Thr Leu Tyr Ser Asn Glu Pro Asn Thr Asn Ile Leu
Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp Lys Glu Tyr Tyr Leu
Leu Asn Val Leu Lys Pro Asn Asn Phe Ile Asp Arg Arg Lys Asp Ser
Thr Leu Ser Ile Asn Asn Ile Arg Ser Thr Ile Leu Leu Ala Asn Arg
Leu Tyr Ser Gly Ile Lys Val Lys Ile Gln Arg Val Asn Asn Ser Ser
Thr Asn Asp Asn Leu Val Arg Lys Asn Asp Gln Val Tyr Ile Asn Phe
Val Ala Ser Lys Thr His Leu Phe Pro Leu Tyr Ala Asp Thr Ala Thr
Thr Asn Lys Glu Lys Thr Ile Lys Ile Ser Ser Ser Gly Asn Arg Phe
Asn Gln Val Val Val Met Asn Ser Val Gly Asn Asn Cys Thr Met Asn
Phe Lys Asn Asn Asn Gly Asn Asn Ile Gly Leu Leu Gly Phe Lys Ala
Asp Thr Val Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp His
Thr Asn Ser Asn Gly Cys Phe Trp Asn Phe Ile Ser Glu Glu His Gly
Trp Gln Glu Lys

FIG. 14 Continued

Clostridium botulinum serotype F
(1274 a.a.)

Met Pro Val Ala Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp
Asp Thr Ile Leu Tyr Met Gln Ile Pro Tyr Glu Glu Lys Ser Lys Lys
Tyr Tyr Lys Ala Phe Glu Ile Met Arg Asn Val Trp Ile Ile Pro Glu
Arg Asn Thr Ile Gly Thr Asn Pro Ser Asp Phe Asp Pro Pro Ala Ser
Leu Lys Asn Gly Ser Ser Ala Tyr Tyr Asp Pro Asn Tyr Leu Thr Thr
Asp Ala Glu Lys Asp Arg Tyr Leu Lys Thr Thr Ile Lys Leu Phe Lys
Arg Ile Asn Ser Asn Pro Ala Gly Lys Val Leu Leu Gln Glu Ile Ser
Tyr Ala Lys Pro Tyr Leu Gly Asn Asp His Thr Pro Ile Asp Glu Phe
Ser Pro Val Thr Arg Thr Thr Ser Val Asn Ile Lys Leu Ser Thr Asn
Val Glu Ser Ser Met Leu Leu Asn Leu Leu Val Leu Gly Ala Gly Pro
Asp Ile Phe Glu Ser Cys Cys Tyr Pro Val Arg Lys Leu Ile Asp Pro
Asp Val Val Tyr Asp Pro Ser Asn Tyr Gly Phe Gly Ser Ile Asn Ile
Val Thr Phe Ser Pro Glu Tyr Glu Tyr Thr Phe Asn Asp Ile Ser Gly
Gly His Asn Ser Ser Thr Glu Ser Phe Ile Ala Asp Pro Ala Ile Ser
Leu Ala His Glu Leu Ile His Ala Leu His Gly Leu Tyr Gly Ala Arg
Gly Val Thr Tyr Glu Glu Thr Ile Glu Val Lys Gln Ala Pro Leu Met
Ile Ala Glu Lys Pro Ile Arg Leu Glu Glu Phe Leu Thr Phe Gly Gly
Gln Asp Leu Asn Ile Ile Thr Ser Ala Met Lys Glu Lys Ile Tyr Asn
Asn Leu Leu Ala Asn Tyr Glu Lys Ile Ala Thr Arg Leu Ser Glu Val
Asn Ser Ala Pro Pro Glu Tyr Asp Ile Asn Glu Tyr Lys Asp Tyr Phe
Gln Trp Lys Tyr Gly Leu Asp Lys Asn Ala Asp Gly Ser Tyr Thr Val
Asn Glu Asn Lys Phe Asn Glu Ile Tyr Lys Lys Leu Tyr Ser Phe Thr
Glu Ser Asp Leu Ala Asn Lys Phe Lys Val Lys Cys Arg Asn Thr Tyr
Phe Ile Lys Tyr Glu Phe Leu Lys Val Pro Asn Leu Leu Asp Asp Asp
Ile Tyr Thr Val Ser Glu Gly Phe Asn Ile Gly Asn Leu Ala Val Asn
Asn Arg Gly Gln Ser Ile Lys Leu Asn Pro Lys Ile Ile Asp Ser Ile
Pro Asp Lys Gly Leu Val Glu Lys Ile Val Lys Phe Cys Lys Ser Val
Ile Pro Arg Lys Gly Thr Lys Ala Pro Pro Arg Leu Cys Ile Arg Val
Asn Asn Ser Glu Leu Phe Phe Val Ala Ser Glu Ser Ser Tyr Asn Glu
Asn Asp Ile Asn Thr Pro Lys Glu Ile Asp Asp Thr Thr Asn Leu Asn
Asn Asn Tyr Arg Asn Asn Leu Asp Glu Val Ile Leu Asp Tyr Asn Ser
Gln Thr Ile Pro Gln Ile Ser Asn Arg Thr Leu Asn Thr Leu Val Gln
Asp Asn Ser Tyr Val Pro Arg Tyr Asp Ser Asn Gly Thr Ser Glu Ile
Glu Glu Tyr Asp Val Val Asp Phe Asn Val Phe Phe Tyr Leu His Ala
Gln Lys Val Pro Glu Gly Glu Thr Asn Ile Ser Leu Thr Ser Ser Ile
Asp Thr Ala Leu Leu Glu Glu Ser Lys Asp Ile Phe Phe Ser Ser Glu
Phe Ile Asp Thr Ile Asn Lys Pro Val Asn Ala Ala Leu Phe Ile Asp
Trp Ile Ser Lys Val Ile Arg Asp Phe Thr Thr Glu Ala Thr Gln Lys
Ser Thr Val Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Val
Gly Leu Ala Leu Asn Ile Ile Ile Glu Ala Glu Lys Gly Asn Phe Glu
Glu Ala Phe Glu Leu Leu Gly Val Gly Ile Leu Leu Glu Phe Val Pro
Glu Leu Thr Ile Pro Val Ile Leu Val Phe Thr Ile Lys Ser Tyr Ile
Asp Ser Tyr Glu Asn Lys Asn Lys Ala Ile Lys Ala Ile Asn Asn Ser
Leu Ile Glu Arg Glu Ala Lys Trp Lys Glu Ile Tyr Ser Trp Ile Val
Ser Asn Trp Leu Thr Arg Ile Asn Thr Gln Phe Asn Lys Arg Lys Glu

FIG. 15

Gln Met Tyr Gln Ala Leu Gln Asn Gln Val Asp Ala Ile Lys Thr Ala
Ile Glu Tyr Lys Tyr Asn Asn Tyr Thr Ser Asp Glu Lys Asn Arg Leu
Glu Ser Glu Tyr Asn Ile Asn Asn Ile Glu Glu Glu Leu Asn Lys Lys
Val Ser Leu Ala Met Lys Asn Ile Glu Arg Phe Met Thr Glu Ser Ser
Ile Ser Tyr Leu Met Lys Leu Ile Asn Glu Ala Lys Val Gly Lys Leu
Lys Lys Tyr Asp Asn His Val Lys Ser Asp Leu Leu Asn Tyr Ile Leu
Asp His Arg Ser Ile Leu Gly Glu Gln Thr Asn Glu Leu Ser Asp Leu
Val Thr Ser Thr Leu Asn Ser Ser Ile Pro Phe Glu Leu Ser Ser Tyr
Thr Asn Asp Lys Ile Leu Ile Ile Tyr Phe Asn Arg Leu Tyr Lys Lys
Ile Lys Asp Ser Ser Ile Leu Asp Met Arg Tyr Glu Asn Asn Lys Phe
Ile Asp Ile Ser Gly Tyr Gly Ser Asn Ile Ser Ile Asn Gly Asn Val
Tyr Ile Tyr Ser Thr Asn Arg Asn Gln Phe Gly Ile Tyr Asn Ser Arg
Leu Ser Glu Val Asn Ile Ala Gln Asn Asn Asp Ile Ile Tyr Asn Ser
Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Lys His
Tyr Lys Pro Met Asn His Asn Arg Glu Tyr Thr Ile Ile Asn Cys Met
Gly Asn Asn Asn Ser Gly Trp Lys Ile Ser Leu Arg Thr Val Arg Asp
Cys Glu Ile Ile Trp Thr Leu Gln Asp Thr Ser Gly Asn Lys Glu Asn
Leu Ile Phe Arg Tyr Glu Glu Leu Asn Arg Ile Ser Asn Tyr Ile Asn
Lys Trp Ile Phe Val Thr Ile Thr Asn Asn Arg Leu Gly Asn Ser Arg
Ile Tyr Ile Asn Gly Asn Leu Ile Val Glu Lys Ser Ile Ser Asn Leu
Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile Val Gly Cys
Asp Asp Glu Thr Tyr Val Gly Ile Arg Tyr Phe Lys Val Phe Asn Thr
Glu Leu Asp Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asn Glu Pro Asp
Pro Ser Ile Leu Lys Asn Tyr Trp Gly Asn Tyr Leu Leu Tyr Asn Lys
Lys Tyr Tyr Leu Phe Asn Leu Leu Arg Lys Asp Lys Tyr Ile Thr Leu
Asn Ser Gly Ile Leu Asn Ile Asn Gln Gln Arg Gly Val Thr Glu Gly
Ser Val Phe Leu Asn Tyr Lys Leu Tyr Glu Gly Val Glu Val Ile Ile
Arg Lys Asn Gly Pro Ile Asp Ile Ser Asn Thr Asp Asn Phe Val Arg
Lys Asn Asp Leu Ala Tyr Ile Asn Val Val Asp Arg Gly Val Glu Tyr
Arg Leu Tyr Ala Asp Thr Lys Ser Glu Lys Glu Lys Ile Ile Arg Thr
Ser Asn Leu Asn Asp Ser Leu Gly Gln Ile Ile Val Met Asp Ser Ile
Gly Asn Asn Cys Thr Met Asn Phe Gln Asn Asn Asn Gly Ser Asn Ile
Gly Leu Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser Ser Trp Tyr
Tyr Asn Asn Ile Arg Arg Asn Thr Ser Ser Asn Gly Cys Phe Trp Ser
Ser Ile Ser Lys Glu Asn Gly Trp Lys Glu

FIG. 15 Continued

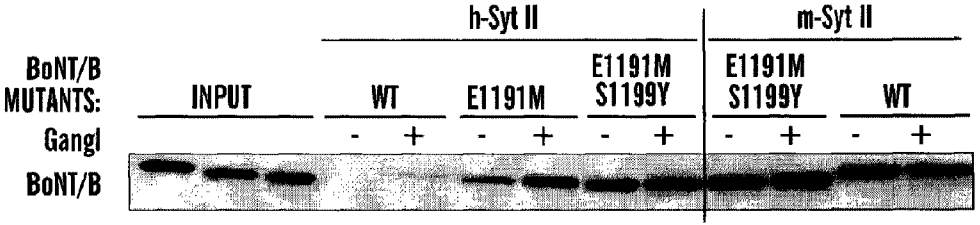
Clostridium botulinum serotype G
(1297 a.a.)

Met Pro Val Asn Ile Lys Asn Phe Asn Tyr Asn Asp Pro Ile Asn Asn
Asp Asp Ile Ile Met Met Glu Pro Phe Asn Asp Pro Gly Pro Gly Thr
Tyr Tyr Lys Ala Phe Arg Ile Ile Asp Arg Ile Trp Ile Val Pro Glu
Arg Phe Thr Tyr Gly Phe Gln Pro Asp Gln Phe Asn Ala Ser Thr Gly
Val Phe Ser Lys Asp Val Tyr Glu Tyr Tyr Asp Pro Thr Tyr Leu Lys
Thr Asp Ala Glu Lys Asp Lys Phe Leu Lys Thr Met Ile Lys Leu Phe
Asn Arg Ile Asn Ser Lys Pro Ser Gly Gln Arg Leu Leu Asp Met Ile
Val Asp Ala Ile Pro Tyr Leu Gly Asn Ala Ser Thr Pro Pro Asp Lys
Phe Ala Ala Asn Val Ala Asn Val Ser Ile Asn Lys Lys Ile Ile Gln
Pro Gly Ala Glu Asp Gln Ile Lys Gly Leu Met Thr Asn Leu Ile Ile
Phe Gly Pro Gly Pro Val Leu Ser Asp Asn Phe Thr Asp Ser Met Ile
Met Asn Gly His Ser Pro Ile Ser Glu Gly Phe Gly Ala Arg Met Met
Ile Arg Phe Cys Pro Ser Cys Leu Asn Val Phe Asn Asn Val Gln Glu
Asn Lys Asp Thr Ser Ile Phe Ser Arg Arg Ala Tyr Phe Ala Asp Pro
Ala Leu Thr Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr
Gly Ile Lys Ile Ser Asn Leu Pro Ile Thr Pro Asn Thr Lys Glu Phe
Phe Met Gln His Ser Asp Pro Val Gln Ala Glu Glu Leu Tyr Thr Phe
Gly Gly His Asp Pro Ser Val Ile Ser Pro Ser Thr Asp Met Asn Ile
Tyr Asn Lys Ala Leu Gln Asn Phe Gln Asp Ile Ala Asn Arg Leu Asn
Ile Val Ser Ser Ala Gln Gly Ser Gly Ile Asp Ile Ser Leu Tyr Lys
Gln Ile Tyr Lys Asn Lys Tyr Asp Phe Val Glu Asp Pro Asn Gly Lys
Tyr Ser Val Asp Lys Asp Lys Phe Asp Lys Leu Tyr Lys Ala Leu Met
Phe Gly Phe Thr Glu Thr Asn Leu Ala Gly Glu Tyr Gly Ile Lys Thr
Arg Tyr Ser Tyr Phe Ser Glu Tyr Leu Pro Pro Ile Lys Thr Glu Lys
Leu Leu Asp Asn Thr Ile Tyr Thr Gln Asn Glu Gly Phe Asn Ile Ala
Ser Lys Asn Leu Lys Thr Glu Phe Asn Gly Gln Asn Lys Ala Val Asn
Lys Glu Ala Tyr Glu Glu Ile Ser Leu Glu His Leu Val Ile Tyr Arg
Ile Ala Met Cys Lys Pro Val Met Tyr Lys Asn Thr Gly Lys Ser Glu
Gln Cys Ile Ile Val Asn Asn Glu Asp Leu Phe Phe Ile Ala Asn Lys
Asp Ser Phe Ser Lys Asp Leu Ala Lys Ala Glu Thr Ile Ala Tyr Asn
Thr Gln Asn Asn Thr Ile Glu Asn Asn Phe Ser Ile Asp Gln Leu Ile
Leu Asp Asn Asp Leu Ser Ser Gly Ile Asp Leu Pro Asn Glu Asn Thr
Glu Pro Phe Thr Asn Phe Asp Asp Ile Asp Ile Pro Val Tyr Ile Lys
Gln Ser Ala Leu Lys Lys Ile Phe Val Asp Gly Asp Ser Leu Phe Glu
Tyr Leu His Ala Gln Thr Phe Pro Ser Asn Ile Glu Asn Leu Gln Leu
Thr Asn Ser Leu Asn Asp Ala Leu Arg Asn Asn Asn Lys Val Tyr Thr
Phe Phe Ser Thr Asn Leu Val Glu Lys Ala Asn Thr Val Val Gly Ala
Ser Leu Phe Val Asn Trp Val Lys Gly Val Ile Asp Asp Phe Thr Ser
Glu Ser Thr Gln Lys Ser Thr Ile Asp Lys Val Ser Asp Val Ser Ile
Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Val Gly Asn Glu Thr Ala
Lys Glu Asn Phe Lys Asn Ala Phe Glu Ile Gly Gly Ala Ala Ile Leu
Met Glu Phe Ile Pro Glu Leu Ile Val Pro Ile Val Gly Phe Phe Thr
Leu Glu Ser Tyr Val Gly Asn Lys Gly His Ile Ile Met Thr Ile Ser
Asn Ala Leu Lys Lys Arg Asp Gln Lys Trp Thr Asp Met Tyr Gly Leu
Ile Val Ser Gln Trp Leu Ser Thr Val Asn Thr Gln Phe Tyr Thr Ile

FIG. 16

Lys Glu Arg Met Tyr Asn Ala Leu Asn Asn Gln Ser Gln Ala Ile Glu
Lys Ile Ile Glu Asp Gln Tyr Asn Arg Tyr Ser Glu Glu Asp Lys Met
Asn Ile Asn Ile Asp Phe Asn Asp Ile Asp Phe Lys Leu Asn Gln Ser
Ile Asn Leu Ala Ile Asn Asn Ile Asp Asp Phe Ile Asn Gln Cys Ser
Ile Ser Tyr Leu Met Asn Arg Met Ile Pro Leu Ala Val Lys Lys Leu
Lys Asp Phe Asp Asp Asn Leu Lys Arg Asp Leu Leu Glu Tyr Ile Asp
Thr Asn Glu Leu Tyr Leu Leu Asp Glu Val Asn Ile Leu Lys Ser Lys
Val Asn Arg His Leu Lys Asp Ser Ile Pro Phe Asp Leu Ser Leu Tyr
Thr Lys Asp Thr Ile Leu Ile Gln Val Phe Asn Asn Tyr Ile Ser Asn
Ile Ser Ser Asn Ala Ile Leu Ser Leu Ser Tyr Arg Gly Gly Arg Leu
Ile Asp Ser Ser Gly Tyr Gly Ala Thr Met Asn Val Gly Ser Asp Val
Ile Phe Asn Asp Ile Gly Asn Gly Gln Phe Lys Leu Asn Asn Ser Glu
Asn Ser Asn Ile Thr Ala His Gln Ser Lys Phe Val Val Tyr Asp Ser
Met Phe Asp Asn Phe Ser Ile Asn Phe Trp Val Arg Thr Pro Lys Tyr
Asn Asn Asn Asp Ile Gln Thr Tyr Leu Gln Asn Glu Tyr Thr Ile Ile
Ser Cys Ile Lys Asn Asp Ser Gly Trp Lys Val Ser Ile Lys Gly Asn
Arg Ile Ile Trp Thr Leu Ile Asp Val Asn Ala Lys Ser Lys Ser Ile
Phe Phe Glu Tyr Ser Ile Lys Asp Asn Ile Ser Asp Tyr Ile Asn Lys
Trp Phe Ser Ile Thr Ile Thr Asn Asp Arg Leu Gly Asn Ala Asn Ile
Tyr Ile Asn Gly Ser Leu Lys Lys Ser Glu Lys Ile Leu Asn Leu Asp
Arg Ile Asn Ser Ser Asn Asp Ile Asp Phe Lys Leu Ile Asn Cys Thr
Asp Thr Thr Lys Phe Val Trp Ile Lys Asp Phe Asn Ile Phe Gly Arg
Glu Leu Asn Ala Thr Glu Val Ser Ser Leu Tyr Trp Ile Gln Ser Ser
Thr Asn Thr Leu Lys Asp Phe Trp Gly Asn Pro Leu Arg Tyr Asp Thr
Gln Tyr Tyr Leu Phe Asn Gln Gly Met Gln Asn Ile Tyr Ile Lys Tyr
Phe Ser Lys Ala Ser Met Gly Glu Thr Ala Pro Arg Thr Asn Phe Asn
Asn Ala Ala Ile Asn Tyr Gln Asn Leu Tyr Leu Gly Leu Arg Phe Ile
Ile Lys Lys Ala Ser Asn Ser Arg Asn Ile Asn Asn Asp Asn Ile Val
Arg Glu Gly Asp Tyr Ile Tyr Leu Asn Ile Asp Asn Ile Ser Asp Glu
Ser Tyr Arg Val Tyr Val Leu Val Asn Ser Lys Glu Ile Gln Thr Gln
Leu Phe Leu Ala Pro Ile Asn Asp Asp Pro Thr Phe Tyr Asp Val Leu
Gln Ile Lys Lys Tyr Tyr Glu Lys Thr Thr Tyr Asn Cys Gln Ile Leu
Cys Glu Lys Asp Thr Lys Thr Phe Gly Leu Phe Gly Ile Gly Lys Phe
Val Lys Asp Tyr Gly Tyr Val Trp Asp Thr Tyr Asp Asn Tyr Phe Cys
Ile Ser Gln Trp Tyr Leu Arg Arg Ile Ser Glu Asn Ile Asn Lys Leu
Arg Leu Gly Cys Asn Trp Gln Phe Ile Pro Val Asp Glu Gly Trp Thr
Glu

FIG. 16 Continued


h-Syt II
m-Syt II
BoNT/B MUTANTS:
INPUT
WT
E1191M
E1191M S1199Y
E1191M S1199Y
WT
GangI
- + - + - + - + - +
BoNT/B