# ENGINEERED BOTULINUM NEUROTOXIN

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/653,214, filed May 30, 2012, the contents of which is incorporated herein by reference in its entirety.

## 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], the contents of which are incorporated herein by reference.

**[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, each of which is incorporated herein by reference.

**[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, the contents of which are herein incorporated by reference.

**[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, the contents of which are incorporated herein in their entirety.

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. The disclosure of Borodic is incorporated in its entirety herein by reference. 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. The disclosure of these two patents is incorporated in its entirety herein by reference.

**[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. The disclosure of Sander et al. is incorporated in its entirety by reference herein. 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. The disclosures of Binder are incorporated in its entirety herein by reference. 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. The disclosures of Foster et al. are incorporated in its entirety by reference herein. 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 ±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 expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications 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-$_S$yt 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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**FURTHER ASPECTS**

Further aspects and embodiments of the invention are now set out in the following numbered Paragraphs; it is to be understood that the invention encompasses these aspects:

Paragraph 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-$H_c$), 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.

Paragraph 2. The BoNT polypeptide of Paragraph 1, wherein the modified (B-$H_c$) comprises two substitution mutations.

Paragraph 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.

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

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

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

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

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

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

Paragraph 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-$H_c$), comprising a substitution mutation at a position corresponding to S1199 or S1201 of serotype B, strain 1.

Paragraph 11. The BoNT polypeptide of Paragraph 10 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.

Paragraph 12. The BoNT polypeptide of Paragraph 10 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.

Paragraph 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.

Paragraph 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.

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

Paragraph 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.

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

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

Paragraph 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.

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

Paragraph 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.

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

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

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

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

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

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

Paragraph 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.

Paragraph 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.

Paragraph 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.

Paragraph 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.

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

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

Paragraph 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.

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

Paragraph 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.

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

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

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

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

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

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

Paragraph 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.

Paragraph 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.

Paragraph 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.

Paragraph 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.

Paragraph 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.

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

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

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

Paragraph 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.

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

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

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

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

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

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

Paragraph 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.

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

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

Paragraph 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.

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

Paragraph 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.

Paragraph 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.

Paragraph 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.

Paragraph 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.

**CLAIMS**

1. A polypeptide comprising a modified receptor binding domain of *Clostridial botulinum* serotype B (B-$H_c$) comprising one or more substitution mutations corresponding to an E1191M substitution mutation in serotype B, strain 1.

2. A polypeptide according to Claim 1, comprising two substitution mutations selected from the group consisting of: E1191M and S1199L, E1191M and S1199Y, and E1191M and S1199F.

3. A polypeptide according to Claim 1 or 2, comprising two substitution mutations corresponding to E1191M and S1199F.

4. 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-$H_c$), comprising one or more substitution mutations corresponding to an E1191M substitution mutation in serotype B, strain 1.

5. A BoNT polypeptide according to Claim 4, in which the modified (B-$H_c$) comprises two substitution mutations selected from the group consisting of: E1191M and S1199L, E1191M and S1199Y, and E1191M and S1199F.

6. A BoNT polypeptide according to Claim 4 or 5, in which the modified (B-$H_c$) comprises two substitution mutations corresponding to E1191M and S1199F.

7. A BoNT polypeptide according to Claim 4, 5 or 6, in which the protease domain, translocation domain, and protease cleavage site are from a serotype selected from the group consisting of A, B, C, D, E, F, G, and combinations thereof, such as in which the protease domain, translocation domain, and protease cleavage site are from serotype B, strain 1 or serotype A, strain 1.

8. 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, in which the

modified B-$H_c$ comprises an E to M substitution mutation at a position corresponding to E1191 in serotype B, strain 1.

9. A chimeric molecule according to Claim 8, in which the modified B-$H_c$ comprises two substitution mutations selected from the group consisting of: E1191M and S1199L, E1191M and S1199Y, and E1191M and S1199F.

10. A chimeric molecule according to Claim 8 or 9, in which the first portion and the second portion are linked covalently or are linked non-covalently; in which the second portion is selected from the group consisting of a small molecule, a nucleic acid, a short polypeptide and a protein; in which the second portion is a bioactive molecule; or in which the second portion is a therapeutic polypeptide or non-polypeptide drug.

11. A nucleic acid such as a vector comprising a nucleotide sequence that encodes a polypeptide, BoNT polypeptide or a chimeric molecule according to any preceding claim.

12. A cell expressing a polypeptide, BoNT polypeptide or chimeric molecule according to any of Claims 1 to 10 or comprising a nucleic acid or vector according to Claim 11.

13. A pharmaceutical composition comprising a polypeptide, BoNT polypeptide or chimeric molecule according to any of Claims 1 to 10, a nucleic acid according to Claim 11 or a cell according to Claim 12.

14. A pharmaceutical composition according to Claim 13, further comprising a pharmaceutically acceptable excipient.

15. A method of producing a botulinum neurotoxin (BoNT) polypeptide, the method comprising the steps of culturing a host cell according to Claim 12 under conditions in which the BoNT polypeptide is produced, and optionally recovering the BoNT polypeptide from the culture.

16. A polypeptide, botulinum neurotoxin (BoNT) polypeptide or chimeric molecule according to any of Claims 1 to 10, a nucleic acid according to Claim 11 or a cell according to Claim 12, for use in therapy.

17. A polypeptide, botulinum neurotoxin (BoNT) polypeptide or chimeric molecule according to any of Claims 1 to 10, a nucleic acid according to Claim 11 or a cell according to Claim 12, for use in treating a condition associated with unwanted neuronal activity, such as a

condition 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.

18. A polypeptide, botulinum neurotoxin (BoNT) polypeptide or chimeric molecule according to Claim 16 or 17 for a use as specified therein, in which the method comprises administering a therapeutically effective amount of the BoNT polypeptide to a subject to thereby contact one or more neurons exhibiting unwanted neuronal activity, to thereby treat the condition.

19. Use of a polypeptide, botulinum neurotoxin (BoNT) polypeptide or chimeric molecule according to any of Claims 1 to 10, a nucleic acid according to Claim 11 or a cell according to Claim 12 in the preparation of a medicament for the treatment or prevention of a condition associated with unwanted neuronal activity.