TREATMENT METHODS USING ATOXIC NEUROTOXIN DERIVATIVES

The present invention relates to a treatment method. This method involves contacting a subject with an isolated, physiologically active, atoxic derivative of a Clostridial neurotoxin. Contacting is carried out to treat the subject. The derivative of a Clostridial neurotoxin does not possess a cargo attachment peptide sequence at its N-terminus.

References Cited

U.S. PATENT DOCUMENTS

FOREIGN PATENT DOCUMENTS

OTHER PUBLICATIONS

Abstract

The present invention relates to a treatment method. This method involves contacting a subject with an isolated, physiologically active, atoxic derivative of a Clostridial neurotoxin. Contacting is carried out to treat the subject. The derivative of a Clostridial neurotoxin does not possess a cargo attachment peptide sequence at its N-terminus.

21 Claims, 4 Drawing Sheets
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2006/0024794 A1 2/2006 Li et al.

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Shone et al., “Inactivation of Clostridium Botulinum Type A Neurotoxin by Trypsin and Purification of Two Tryptic Fragments,” European J. of Biochem. 151:75-82 (1985).


* cited by examiner
TREATMENT METHODS USING ATOXIC NEUROTOXIN DERIVATIVES

This application claims the benefit of U.S. Provisional Patent Application Ser. No. 61/757,478, filed Jan. 28, 2013, which is hereby incorporated by reference in its entirety.

The subject matter of this application was made with support from the United States Government under National Institutes of Health grant R01 AI093504. The United States Government has certain rights.

FIELD OF THE INVENTION

This invention relates to treatment methods using atoxic neurotoxin derivatives.

BACKGROUND OF THE INVENTION

The Clostridial neurotoxins are a family of structurally similar proteins that target the neuronal machinery for synaptic vesicle exocytosis. Produced by anaerobic bacteria of the Clostridium genus, botulinum neurotoxins ("BoNT"), seven immunologically distinct subtypes, A-G and Tetanus neurotoxin ("TeNT") are the most poisonous substances known on a per-weight basis, with an LD50 in the range of 0.5-2.5 mg/kg when administered by intravenous or intramuscular routes (National Institute of Occupational Safety and Health, "Registry of Toxic Effects of Chemical Substances (R-TECS)," Cincinnati, Ohio: National Institute of Occupational Safety and Health (1996)). BoNT's target cholinergic nerves at their neuromuscular junction, inhibiting acetylcholine release and causing peripheral neuromuscular blockade (Simpson, "Identification of the Major Steps in Botulinum Toxin Action," Annu. Rev. Pharmacol. Toxicol. 44:167-193 (2004)).

A genetic engineering platform that enables rational design of therapeutic agents based on Clostridial toxin genes was described in U.S. Pat. No. 7,785,606 to Ichtchenko and Band. The genetic engineering scheme was based on a two-step approach. Gene constructs, expression systems, and purification schemes were designed that produce physiologically active, recombinant Clostridial neurotoxin derivatives. The recombinant toxin derivatives retained structural features important for developing therapeutic candidates, or useful biologic reagents. Using the genetic constructs and expression systems developed by this paradigm, selective point mutations were then introduced to create recombinant atoxic Clostridial neurotoxin derivatives.

TREATMENT METHODS USING ATOXIC NEUROTOXIN DERIVATIVES

This invention relates to treatment methods using atoxic neurotoxin derivatives.

The present invention relates to treatment methods using atoxic neurotoxin derivatives. This method involves contacting a subject with an isolated, physiologically active, atoxic derivative of a Clostridial neurotoxin, said contacting being carried out to treat the subject, with the proviso that the neurotoxin derivative does not possess a cargo attachment peptide sequence at its N-terminus.

Genetic constructs and expression systems described herein are shown to produce a family of recombinant BoNT derivatives, with conformational and trafficking properties similar to the wild type BoNT toxins. These derivatives of Clostridial neurotoxins can be produced in toxic forms, having a toxicity comparable to that of the wild type toxin, or with mutations that reduce the metalloprotease activity responsible for toxicity (i.e., atoxic derivatives). The LD50 of the atoxic neurotoxin derivatives is much higher than that of the wild type toxin.

As described herein, the atoxic neurotoxin derivatives (see U.S. Pat. No. 7,785,606 to Ichtchenko et al., which is hereby incorporated by reference in its entirety) unexpectedly have in vivo activity similar to the wild type toxins, as used for pharmaceutical purposes. Yet, atoxic neurotoxin derivatives described herein offer significant treatment benefits over current pharmaceutical preparations of wild type neurotoxins produced from cultures. In particular, the atoxic derivatives described herein are safer, providing distinct advantages for medical uses and production/manufacturing. The improved therapeutic index will enable application to conditions where the toxicity of wild type neurotoxins limit their use because of safety concerns.

SUMMARY OF THE INVENTION

The present invention relates to a treatment method. This method involves contacting a patient with isolated, physiologically active, atoxic, Clostridial neurotoxin derivatives. The present invention is directed to overcoming this and other limitations in the art.

FIGS. 1A-C are a comparative alignment of amino acid sequences of seven wild type botulinum neurotoxin serotypes, including Clostridium botulinum serotype A (wt BoNT A) (SEQ ID NO:1), Clostridium botulinum serotype B (wt BoNT B) (SEQ ID NO:2), Clostridium botulinum serotype C (wt BoNT C) (SEQ ID NO:3), Clostridium botulinum serotype D (wt BoNT D) (SEQ ID NO:4), Clostridium botulinum serotype E (wt BoNT E) (SEQ ID NO:5), Clostridium botulinum serotype F (wt BoNT F) (SEQ ID NO:6), and Clostridium botulinum serotype G (wt BoNT G) (SEQ ID NO:7). Gaps have been introduced to maximize homology. Amino acids identical in 250% of compared sequences are highlighted with stars. Disulfide bridge between neurotoxin cysteine residues of the light and heavy chain are shown as a long horizontal bracket. The amino acid residues constituting the minimal catalytic domain of metalloprotease are marked by stars. Dimeric bridge between neurotoxin cysteine residues of the light and heavy chain are shown as a long horizontal bracket. The amino acid residues constituting the minimal catalytic domain of the light chain are hatched. The first amino acid of the C-terminal part of the protein heavy chain (N872 for BoNT A), is shown with a white arrow, as is the first amino acid considered to constitute the receptor-binding domain. Amino acids absent in the mature dichain BoNT A molecule along with the aligned amino acids of the other BoNT serotypes are boxed. A white arrow is also positioned at the first amino acid of the neurotoxins' light chain.

FIG. 2 is a photograph showing the results of in vivo studies performed by intramuscular injection into the lateral gastrocnemius with 0.5 pig?mouse of BoNT A/ad-0 (experimental) in 3 μl of 0.9% NaCl or by injecting 3 μl of 0.9% of NaCl without BoNT A/ad-0 (control). Muscle paralysis and digital abduction was recorded 48 hours after. The two upper panel photographs show control mice, with the arrow in the upper right photograph indicating the site of injection. The three lower panel photographs show experimental mice. Digital abduction muscle paralysis was only observed in mice...
injected with BoMT A/ad-0. Experimental, n=10. Control,
n=5. Representative results are shown in the photographs in
the three bottom panels.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a treatment method. This
method involves contacting a subject with an isolated, physi-
ologically active, atoxic derivative of a Clostridial neuro-
toxin, said contacting being carried out to treat the subject,
with the proviso that the neurotoxin derivative does not pos-
sess a cargo attachment peptide sequence at its N-terminus.

According to one embodiment, the derivative of a Clostri-
dial neurotoxin of the present invention is a derivative of
a *Clostridium botulinum* neurotoxin. *Clostridium botuli-
num* has multiple serotypes (A-G). Suitable derivatives of a
Clostridial neurotoxin may be derivatives of any of the
*Clostridium botulinum* serotypes. In addition, suitable
derivatives of a Clostridial neurotoxin of the present invention
may be derivatives of more than one *Clostridium botulinum*
serotype. For example, it may be desirable to have a derivative
of a Clostridial neurotoxin constructed of a light chain (LC)
region from one *Clostridium botulinum* serotype (e.g., sero-
type A, BoNT A) and a heavy chain (HC) region from another
*Clostridium botulinum* serotype (e.g., serotype B, BoNT B).
Also, suitable derivatives of a Clostridial neurotoxin of the
present invention include chimeras using other receptor
ligands, e.g., epidermal growth factor ("EGF") for LC deliv-
ery to cancer cells (see U.S. Patent Application Publication
no. 2012/0064059 to Foster et al., which is hereby incorpo-
rated by reference in its entirety).

By "derivative" it is meant that the Clostridial neurotoxin is
substantially similar to the wild type toxin, but has been
modified slightly as described herein. For example, deriv-
atives include Clostridial neurotoxins that are at least 60%,
70%, 80%, 85%, 90%, 95%, 98%, 97%, 98%, or 99% iden-
tical to a wild type neurotoxin.

Isolated derivatives of a Clostridial neurotoxin are physi-
oologically active. This physiological activity includes, but
is not limited to, toxin immunogenicity, trans- and intra-cellular
trafficking, cell recognition and targeting, and paralytic activ-
ity. In one embodiment, the derivative of a Clostridial neuro-
toxin is a derivative of a full-length Clostridial neurotoxin.

The atoxic derivative of a Clostridial neurotoxin design-
ated herein using the "ad-0" designation, does not have an
S6 peptide sequence fused to the N-terminus of the neuro-
toxin derivative, as described in U.S. Patent Application
Publication No. 2011/0206616 to Icthtchenko and Band, which is
hereby incorporated by reference in its entirety.

The mechanism of cellular binding and internalization of
Clostridial neurotoxins is still not completely understood.
The C-terminal portion of the heavy chain of all Clostridial
neurotoxins binds to gangliosides (sialic acid-containing gly-
colipids), with a preference for gangliosides of the *G*$_{1a}$ series
(Montecucco et al., "Structure and Function of Tetanus and
(1995); Montecucco, "How Do Tetanus and Botulinum Toxins
Bind to Neuronal Membranes?" *TIBS* 11:314-317 (1986);
and Van Heyningen et al., "The Fixation of Tetanus Toxin by
are hereby incorporated by reference in their entirety). The
sequence responsible for ganglioside binding has been iden-
tified for the structurally similar TeNT molecule, and is
located within the 34 C-terminal amino acid residues of its
heavy chain. BoNT A, BoNT B, BoNT C, BoNT E, and BoNT
F share a high degree of homology with TeNT in this region
(Fig. 1) (Shapiro et al., "Identification of a Ganglioside Rec-
ognition Domain of Tetanus Toxin Using a Novel Ganglio-
(1997), which is hereby incorporated by reference in its
entirety). Multiple types of evidence suggest the existence of
at least one additional component involved in the binding of
Clostridial neurotoxins to neuronal membranes (Montecucco
et al., "Structure and Function of Tetanus and Botulinum
Neurotoxins," *Q Rev Biophys.* 28:423-472 (1995); Montecuc-
cco, "How Do Tetanus and Botulinum Toxins Bind to Neuronal
Membranes?" *TIBS* 11:314-317 (1986), which are hereby
incorporated by reference in their entirety). In two reports
(Nishiki et al., "The High-Affinity Binding of *Clostridium
Botulinum* Type B Neurotoxin to Synaptotagmin II Asso-
ciated with Gangliosides G$_{2a}$/G$_{12a}$," *FEBS Lett.* 378:
253-257 (1996); Dong et al., "Synaptotagmins I and II Medi-
ate Entry of *Botulinum* Neurotoxin B into Cells," *J. Cell Biol.*
162:1293-1303 (2003), which are hereby incorporated by
reference in their entirety), synaptotagmins were identified as
possible candidates for the auxiliary BoNT B receptor, and
synaptotagmins I and II were implicated as neuronal recep-
tors for BoNT G (Rummel et al., "Synaptotagmins I and II Act
as Nerve Cell Receptors for *Botulinum* Neurotoxin G," *J.
Biol. Chem.* 279:30865-30870 (2004), which is hereby incor-
porated by reference in its entirety). Dong et al., "SV2 is the
Protein Receptor for *Botulinum* Neurotoxin A," *Science* 312:
592-596 (2006), which is hereby incorporated by reference in
its entirety, showed that BoNT A enters neurons by binding to
the synaptic vesicle protein SV2 (isoforms A, B, and C).
However, despite the structural similarity in the putative
receptor-binding domain of Clostridial neurotoxins, other
toxin subtypes show no affinity for SV2 and instead may
target synaptotagmins or synaptotagmin-related molecules.
Lipid rafts (Herreros et al., "Lipid Rafts Act as Specialized
Domains for Tetanus Toxin Binding and Internalization into
is hereby incorporated by reference in its entirety) have been
implicated as a specialized domain involved in TeNT binding
and internalization into neurons, but these domains are
widely distributed on multiple cell types, and therefore
cannot simply explain the high specificity of the toxins for
neurons.

Clostridial neurotoxins are internalized through the
presynaptic membrane by an energy-dependent mechanism
(Montecucco et al., "Structure and Function of Tetanus and
(1995); Matteoli et al., "Synaptic Vesicle Endocytosis Medi-
ates the Entry of Tetanus Neurotoxin into Hippocampal Neu-
rons," *Proc. Natl. Acad. Sci. USA* 93:13310-13315 (1996);
and Mukherjee et al., "Endocytosis," *Physiol. Rev.* 77:759-
803 (1997), which are hereby incorporated by reference in
their entirety), and rapidly appear in vesicles where they are at
least partially protected from degradation (Dolly et al.,
"Acceptors for *Botulinum* Neurotoxin Reside on Motor Nerve
Terminals and Mediate Its Internalization," *Nature* 307:457-
460 (1984); Critchley et al., "Fate of Tetanus Toxin Bound to
the Surface of Primary Neurons in Culture: Evidence for
which are hereby incorporated by reference in their entirety).
The BoNT complex of light and heavy chains interacts with
the endocytic vesicle membrane in a chaperone-like way,
preventing aggregation and facilitating translocation of the
light chain in a fashion similar to the protein conducting/
translocating channels of smooth ER, mitochondria, and
chloroplasts (Korai et al., "Translocation of *Botulinum*
Neurotoxin Light Chain Protease through the Heavy Chain
Channel," *Nat. Struct. Biol.* 10:13-18 (2003), which is hereby
incorporated by reference in its entirety). Acidification of the
endosome is believed to induce pore formation, which allows translocation of the light chain to the cytosol upon reduction of the interchain disulfide bond (Hoch et al., "Channels Formed by Botulinum, Tetanus, and Diphtheria Toxins in Planar Lipid Bilayers: Relevance to Translocation of Proteins Across Membranes," Proc. Natl. Acad. Sci. USA 82:1692-1696 (1985), which is hereby incorporated by reference in its entirety). Within the cytosol, the light chain displays a zinc-endopeptidase activity specific for protein components of the synaptic vesicle exocytosis apparatus. TeNT and BoNT B, BoNT D, BoNT F, and BoNT G recognize VAMP/syntaxin and syntaxin, another protein of the nerve terminal plasmalemma (Montecucco et al., "Structure and Function of neurotoxin. BoNT A, BoNT C, and BoMT E recognize and cleave SNAP-25, a protein of the presynaptic membrane, at the NO:8) motif in the light chain, characteristic of metalloproteases (FIGS. 1A-C). Mutagenesis of BoMT A light chain, followed by microinjection of the corresponding mRNA into presynaptic cholinergic neurons of Aplysia californica, allowed the minimal essential domain responsible for toxicity to be identified (Kurazono et al., "Minimal Essential Domains Specifying Toxicity of the Light Chains of Tetanus and Botulinum Neurotoxins," Q. Rev. Biophys. 28:423-472 (1995); Sutton et al., "Crystal Structure of a SNAPE Complex Involved in Synaptic Exocytosis at 2.4 Å Resolution," Nature 395:347-355 (1998), which are hereby incorporated by reference in their entirety). The cleavage of such components of the synaptic release machinery results in inhibition of acetylcholine release in motor neurons, ultimately leading to neuromuscular paralysis.

The isolated derivative of a Clostridial neurotoxin employed in the method of the present invention is physiologically active and atoxic. The endopeptidase activity responsible for Clostridial neurotoxin toxicity is believed to be associated with the presence of a HExxHxxH (SEQ ID NO:8) motif in the light chain, characteristic of metalloproteases (FIGS. 1A-C). Mutagenesis of BoNT A light chain, followed by microinjection of the corresponding mRNA into presynaptic cholinergic neurons of Aplysia californica, allowed the minimal essential domain responsible for toxicity to be identified (Kurazono et al., "Minimal Essential Domains Specifying Toxicity of the Light Chains of Tetanus and Botulinum Neurotoxins Type A," J. Biol. Chem. 267:14721-14729 (1992), which is hereby incorporated by reference in its entirety). Site-directed mutagenesis of BoNT A light chain pinpointed the amino acid residues involved in Zn²⁺ coordination, and formation of the active metalloendopeptidase core which cleaves SNAP-25 (Rigoni et al., "Site-Directed Mutagenesis Identifies Active-Site Residues of the Light Chain of Botulinum Neurotoxin Type A," Biochem. Biophys. Res. Commun. 288:1231-1237 (2001), which is hereby incorporated by reference in its entirety). The three-dimensional structures of Clostridial neurotoxins and their derivatives confirmed the mutagenesis results, and detailed the spatial organization of the protein domains. For the BoNT A holotoxin, crystal structure was obtained to a resolution of 3.3 Å (Lacy et al., "Crystal Structure of Botulinum Neurotoxin Type A and Implications for Toxicity," Nat. Struct. Biol. 5:898-902 (1998), which is hereby incorporated by reference in its entirety). The BoNT B holotoxin crystal structure was determined at 1.8 and 2.6 Å resolution (Swaminathan et al., "Structural Analysis of the Catalytic and Binding Sites of Clostridium Botulinum Neurotoxin B," Nat. Struct. Biol. 7:693-699 (2000), which is hereby incorporated by reference in its entirety). Recently, a crystal structure for BoNT E catalytic domain was determined to 2.1 Å resolution (Agarwal et al., "Structural Analysis of Botulinum Neurotoxin Type E Catalytic Domain and Its Mutant Glu212>Gln Reveals the Pivotal Role of the Glu212 Carboxylate in the Catalytic Pathway," Biochemistry 43:6637-6644 (2004), which is hereby incorporated by reference in its entirety). The later study provided multiple interesting structural details, and helps explain the complete loss of metalloendoproteolytic activity in the BoNT E LC E212->Q mutant. The availability of this detailed information on the relationship between the amino acid sequence and biological activities of Clostridial toxins enables the design of modified toxin genes with properties specifically altered for therapeutic goals.

Thus, in one embodiment, the physiologically active and atoxic derivative of a Clostridial neurotoxin has a metalloprotease disabling mutation. Specific metalloprotease disabling mutations are described in U.S. Pat. No. 7,785,606 to Ichthchenko and Band, which is hereby incorporated by reference in its entirety. Additional point mutations can be introduced to further modify the characteristics of the atoxic derivative, some of which are also described in U.S. Pat. No. 7,785,606 to Ichthchenko and Band, which is hereby incorporated by reference in its entirety.

The physiologically active and atoxic derivative of a Clostridial neurotoxin may also have a non-native motif (e.g., a SRAE motif) in the light chain region that is capable of inactivating light chain metalloprotease activity in a toxic Clostridial neurotoxin, or otherwise modifying the behavior of the derivative. The sequences of nine non-native motifs that may be substituted for alpha-helix domains are described in U.S. Pat. No. 7,785,606 to Ichthchenko and Band, which is hereby incorporated by reference in its entirety. Atoxic derivatives that incorporate sequences to target other cellular receptors are also possible (e.g., EGF or cancer cells) (see U.S. Patent Application Publication No. 2012/0064059 to Foster et al., which is hereby incorporated by reference in its entirety).

In one embodiment, the physiologically active and atoxic derivative of a Clostridial neurotoxin has an LD₅₀ that is at least 1,000; 2,000; 5,000; 7,000; 9,000; 10,000; 20,000; 30,000; 40,000; 50,000; 60,000; 70,000; 80,000; 90,000; 100,000; or 500,000-fold higher than the LD₅₀ of wild type Clostridial neurotoxin. The particular mode of administration may affect the LD₅₀ of the derivative of a Clostridial neurotoxin.

In one embodiment, the derivative of a Clostridial neurotoxin of the present invention is produced by cleaving a propeptide. The propeptide is cleaved at the highly specific protease cleavage site to form a light and heavy chain, with molecular weights of approximately 50 kD and 100 kD, respectively. The light and heavy chain regions are linked by a disulfide bond.

In one embodiment, the propeptide is contacted with a highly specific protease (e.g., enterokinase or TEV protease) under conditions effective to enable cleavage at the intermediate region of the propeptide of the present invention. Preferably, the expressed propeptide has one or more disulfide bridges.

As discussed infra, Clostridial neurotoxins and their derivatives described herein are synthesized as single chain propeptides which are later activated by a specific proteolysis cleavage event, generating a dimer joined by a disulfide bond. These structural features can be illustrated using BoNT A as an example, and are generally applicable to all Clostridium botulinum serotypes. The mature BoNT A is composed of three functional domains of Mr=50,000, where the catalytic function responsible for toxicity is confined to the light chain (residues 1-437), the translocation activity is associated with the N-terminal half of the heavy chain (residues 448-872), and cell binding is associated with its C-terminal half (residues 873-1,295) (Johnson, "Clostridial Toxins as Therapeutic Agents: Benefits of Nature's Most Toxic Proteins," Annu. Rev. Microbiol. 53:551-575 (1999); Montecucco et al.,
Joining the involved Cys residues. The outlined box in FIG. 1A-C represents amino acid residues approximately in the 420 to 450 range for all seven BoNT serotypes, as illustrated in FIGS. 1A-C. These two Cys residues form the single disulfide bond holding the HC and LC polypeptides together in the mature neurotoxin. This disulfide bond enables the mature neurotoxin to accomplish its native physiological activities by permitting the HC and LC to carry out their respective biological roles in concert. The disulfide bond between HC and LC polypeptides in all seven serotypes is illustrated in FIG. 1A by the solid line joining the involved Cys residues. The outlined box in FIG. IA illustrates the intermediate region defined by amino acid residues Lys448-Lys458 of wt BoNT A. This intermediate region identifies the amino acids eliminated during maturation of wt BoNT A, and believed to be excised by a protease endogenous to the host microorganism. This cleavage event, described infra, generates the biologically active BoNT HC-LC dimer. The outlined amino acid residues in FIGS. 1A-C, representing amino acid residues approximately in the 420 to 450 range for all seven BoNT serotypes, can be considered as a region "non-essential" to the toxins' physiological activity and, therefore, represents targets for directed mutagenesis in all seven BoNT serotypes.

All seven wt BoNT serotypes referred to herein contain Lys or Arg residues in the intermediate region defined by the box in FIG. 1A, which make the propeptides susceptible to activation by trypsin. Native BoNT A propeptide recovered from young bacterial cultures can be activated by trypsinolysis, with production of intact, S-S bound light and heavy chain. Though multiple additional trypsin-susceptible sites are present in the propeptides, they are resistant to proteolysis due to their spatial positions within the native toxin molecule (Dekleva et al., "Nicking of Single Chain Clostridium botulinum Type A Neurotoxin by an Endogenous Protease," Biochem. Biophys. Res. Commun. 162:767-772 (1989); Lacy et al., "Crystal Structure of Botulinum Neurotoxin Type A and Implications for Toxicity," Nat. Struct. Biol. 5:989-902 (1998), which are hereby incorporated by reference in their entirety). A second site in the native propeptide of several BoNT serotypes can be susceptible to trypsin cleavage when subjected to higher enzyme concentrations or incubation times (Chaddock et al., "Expression and Purification of Catalytically Active, Non-Toxic Endopeptidase Derivatives of Clostridium botulinum Toxin Type A," Protein Expr. Purif. 25:219-228 (2002), which is hereby incorporated by reference in its entirety). This trypsin-susceptible site is located in the region adjacent to the toxin receptor binding domain. This region of the HC peptide is found to be exposed to solvent in BoNT serotypes for which information is available on their 3-D crystal structure (Lacy et al., "Crystal Structure of Botulinum Neurotoxin Type A and Implications for Toxicity," Nat. Struct. Biol. 5:989-902 (1998); Swaminathan et al., "Structural Analysis of the Catalytic and Binding Sites of Clostridium botulinum Neurotoxin B," Nat. Struct. Biol. 7:693-699 (2000), which are hereby incorporated by reference in their entirety).

In one embodiment, the propeptide has an intermediate region connecting the light and heavy chain regions which has a highly specific protease cleavage site and no low-specificity protease cleavage sites. For purposes of the present invention, a highly specific protease cleavage site has three or more specific adjacent amino acid residues that are recognized by the highly specific protease in order to permit cleavage (e.g., an enterokinase cleavage site or a TEV recognition sequence). In contrast, a low-specificity protease cleavage site has two or less adjacent amino acid residues that are recognized by a protease in order to enable cleavage (e.g., a trypsin cleavage site).

In all seven BoNT serotypes, the amino acid preceding the N-terminus of the heavy chain is a Lys or Arg residue which is susceptible to proteolysis with trypsin. This trypsin-susceptible site can be replaced with a five amino acid enterokinase cleavage site (i.e., DDDDK (SEQ ID NO:9)) upstream of the heavy chain's N-terminus. Alternatively, the trypsin-susceptible site can be replaced with a tobacco etch virus protease recognition ("TEV") sequence. Use of a TEV sequence enables a one-step heterodimer-forming cleavage event. See U.S. Patent Application Publication No. 2011/0206616 to Ichtchenko et al., which is hereby incorporated by reference in its entirety. Either of these modifications enables standardization activation with specific enzymes. In serotypes A and C, additional Lys residues within this region may be mutated to either Glu or His, thereby eliminating additional trypsin-susceptible sites. Trypsin-susceptible recognition sequences also occur upstream of the heavy chain's receptor-binding domain in serotypes A, E, and F. This region's susceptibility to proteolysis is consistent with its exposure to solvent in the toxin's 3-D structure, as shown by X-ray crystallography analysis. Therefore, in serotypes A, E, and F, the susceptible residues are modified to Asn. These modifications enable standardization activation with either enterokinase or TEV.

Signal peptides and N-terminal affinity tags are also preferably introduced, as required, to enable secretion and recovery and to eliminate truncated variants. The affinity tags can be separated from the N-terminus and C-terminus of the neurotoxin by cleavage using the same specific proteases used to cleave the heavy and light chain.

In one embodiment, the derivative of a Clostridial neurotoxin is from a propeptide that has a metalloprotease disabling mutation. The amino acid residues constituting the minimal catalytic domain of the light chain of the propeptide are illustrated in FIG. 1A by hatching. Specific amino acid residues constituting the active site of the catalytic domain of the metalloprotease are marked by stars in FIG. 1A.

A variety of Clostridial neurotoxin propeptides with light chain regions containing non-native motifs (e.g., SNARE motif peptides) in place of surface alpha-helix domains can be created. These non-native motif bearing propeptides are generated by altering the nucleotide sequences of nucleic acids encoding the propeptides.

In one embodiment, the light and heavy chains of the propeptide are not truncated.
nucleic acid molecule and a 3’ regulatory region operably linked to a native gene into a mammalian host is facilitated by first introducing the nucleic acid molecule encoding an isolated, physiologically active, atoxic derivative of a Clostridial neurotoxin suitable for use in the method of the present invention described in U.S. Pat. No. 7,785,606 to Ichtchenko and Band, which is hereby incorporated by reference in its entirety, other than the functional elements of the present invention. Propeptides that encode atoxic derivatives of a Clostridial neurotoxin suitable for use in the method of the present invention may have any of the structural features of the propeptides described in U.S. Patent Application Publication No. 2011/0206616 to Ichtchenko and Band, which is hereby incorporated by reference in its entirety, other than the cargo attachment peptide sequence at the N-terminus. As described in U.S. Patent Application Publication No. 2011/0206616 to Ichtchenko and Band, which is hereby incorporated by reference in its entirety, a single protease cleavage step can be used for activation and removal of affinity tags.

Isolated nucleic acid molecules that encode atoxic derivatives of a Clostridial neurotoxin suitable for use in the method of the present invention are described in U.S. Pat. No. 7,785,606 to Ichtchenko and Band, which is hereby incorporated by reference in its entirety. In one embodiment, the nucleic acid molecule has a metalloprotease disabling mutation, as described supra.

In one embodiment, the derivative of a Clostridial neurotoxin is a recombinant protein. Expression systems having a nucleic acid molecule encoding an isolated, physiologically active, atoxic derivative of a Clostridial neurotoxin in a heterologous vector, and host cells having a heterologous nucleic acid molecule encoding an isolated, physiologically active, atoxic derivative of a Clostridial neurotoxin are described in U.S. Pat. No. 7,785,606 to Ichtchenko and Band, which is hereby incorporated by reference in its entirety. Expressing a recombinant, physiologically active, atoxic derivative of a Clostridial neurotoxin is carried out by providing a nucleic acid construct having a nucleic acid molecule encoding a propeptide as described herein. The nucleic acid construct has a heterologous promoter operably linked to the nucleic acid molecule. The nucleic acid construct is then introduced into a host cell under conditions to which the molecule is heterologous (i.e., not normally present). The introduction of a particular foreign or native gene into a mammalian host is facilitated by first introducing the gene sequence into a suitable nucleic acid vector. “Vector” is used herein to mean any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, virus, virion, etc., which is capable of replication when associated with the proper control elements and which is capable of transferring gene sequences between cells.

Thus, the term includes cloning and expression vectors, as well as viral vectors. The heterologous nucleic acid molecule is inserted into the expression system or vector in proper sense (5’→3’) orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted Clostridial neurotoxin propeptide-encoding sequences.

U.S. Pat. No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference in its entirety, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replication in unicellular cultures including prokaryotic organisms and eukaryotic cells grown in culture.

Recombinant genes may also be introduced into viruses, including vaccinia virus, adenovirus, and retroviruses, including lentivirus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.1B, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pBR290, pKC37, pKC101, SV 40, pBlue-script II SK+/- or KS+/- (see “Stratagene Cloning Systems” Catalog (1993) from Stratagene, La Jolla, Calif., which is hereby incorporated by reference in its entirety), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, N.Y. (1989), which is hereby incorporated by reference in its entirety.

A variety of host-vector systems may be utilized to express the propeptide-encoding sequence in a cell. Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (“mRNA”) translation). Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eukaryotic promoters differ from those of prokaryotic promoters. Furthermore, eukaryotic promoters and accompanying genetic signals may not be recog-
nized in or may not function in a prokaryotic system, and, further, prokaryotic promoters are not recognized and do not function in eukaryotic cells.

Similarly, translation of mRNA in prokaryotes depends upon the presence of the proper prokaryotic signals which differ from those of eukaryotes. Efficient translation of mRNA in prokaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3′-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression see Roberts and Lauer, Methods in Enzymology 68:473 (1979), which is hereby incorporated by reference in its entirety.

Promoters vary in their "strength" (i.e., their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in E. coli, its bacteriophages, or plasmids, promoters such as the PL promoter, T7 phage promoter, lac promoter, trp promoter, recA promoter, ribosomal RNA promoter, the P5 and P7 promoters of coliphage lambda and others, including but not limited to, lacl5UV5, ompF, bla, lpp, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid trp-lacl5 promoter may be used. Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operons, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the lac operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as trp, pro, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in prokaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strength" transcription and/or translation initiation signals. For instance, efficient translation in E. coli requires a Shine-Dalgarno ("SD") sequence about 7-9 bases 5′ to the initiation codon (ATG) to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the cro gene or the N gene of coliphage lambda, or from the E. coli tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Depending on the vector system and host utilized, any number of suitable transcription and/or translation elements, including constitutive, inducible, and repressible promoters, as well as minimal 5′ promoter elements may be used.

The nucleic acid, a promoter molecule of choice, a suitable 3′ regulatory region, and if desired, a reporter gene, are incorporated into a vector-expression system of choice to prepare a nucleic acid construct using standard cloning procedures known in the art, such as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, New York (2001), which is hereby incorporated by reference in its entirety.

The nucleic acid molecule encoding a derivative of a Clostridial neurotoxin is inserted into a vector in the sense (i.e., 5′→3′) direction, such that the open reading frame is properly oriented for the expression of the encoded prepeptide under the control of a promoter of choice. Single or multiple nucleic acids may be ligated into an appropriate vector in this way, under the control of a suitable promoter, to prepare a nucleic acid construct.

Once the isolated nucleic acid molecule encoding the prepeptide has been inserted into an expression vector, it is ready to be incorporated into a host cell. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, lipofection, protoplast fusion, mobilization, particle bombardment, or electroporation. The DNA sequences are incorporated into the host cell using standard cloning procedures known in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Springs Laboratory, Cold Springs Harbor, N.Y. (1989), which is hereby incorporated by reference in its entirety. Suitable hosts include, but are not limited to, bacteria, virus, yeast, fungal, mammalian cells, insect cells, plant cells, and the like. Preferable host cells of the present invention include, but are not limited to, Escherichia coli, insect cells, and Pichia pastoris cells.

Typically, an antibiotic or other compound useful for selective growth of the transformed cells only is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element present in the plasmid with which the host cell was transformed. Suitable genes are those which confer resistance to gentamycin, G418, hygromycin, puromycin, streptomycin, spectinomycin, tetracycline, chloramphenicol, and the like. Similarly, “reporter genes” which encode enzymes providing for production of an identifiable compound, or other markers which indicate relevant information regarding the outcome of gene delivery, are suitable. For example, various luminescent or phosphorescent reporter genes are also appropriate, such that the presence of the heterologous gene may be ascertained visually.

In carrying out the method of the present invention, contacting a subject with the isolated, physiologically active, atoxic derivative of a Clostridial neurotoxin can be carried out by administering the isolated derivative of a Clostridial neurotoxin to a subject inhalationally, parenterally, for example, subcutaneously, intravenously, intramuscularly, intraperitoneal, by intranasal instillation, or by application to mucous membranes, such as, that of the nose, throat, and bronchial tubes. The neurotoxin derivative may be administered alone or with suitable pharmaceutical carriers, and can be in solid or liquid form such as, tablets, capsules, elixirs, suspensions, syrups, and the like. In one embodiment, the formulation includes hemagglutinin protein similar to those produced by Clostridium species to protect the neurotoxin in the gastrointestinal tract. Such compositions and preparations
should contain at least 0.1% of active compound. The percentage of the compound in these compositions may, of course, be varied and may conveniently be between about 2% to about 60% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, capsules, and the like may also contain a binder such as gum tragacanth, acacia, corn starch, or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose, or saccharin. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a fatty oil.

Various other materials may be present as coatings or to modify the physical form of the dosage unit. For instance, tablets may be coated with sugar, glucose, or both. A syrup may contain, in addition to active ingredient, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye, and flavoring such as cherry or orange flavor.

The neurotoxin derivative may also be administered parenterally. Solutions or suspensions can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that syringability is possible. It must be stable under the conditions of manufacture and storage and can be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), vegetable oils, hyaluronic acid, and suitable mixtures thereof.

The neurotoxin derivative may also be administered directly to the airways in the form of an aerosol. For use as aerosols, the neurotoxin derivative in solution or suspension may be packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutane with conventional adjuvants. The neurotoxin derivative may also be administered in a non-pressurized form such as in a nebulizer or atomizer.

BoNTs pass across epithelial surfaces without being destroyed or causing local toxicity. Passage across epithelia is believed to occur by specific binding and transcytosis. The ability of intact BoNT A to pass though pulmonary epithelia and resist proteolytic inactivation was demonstrated in rat primary alveolar epithelial cells and in immortalized human pulmonary adenocarcinoma (Calu-3) cells. The rate of transport was greater in the apical-to-basolateral direction than in the basolateral-to-apical direction, and it was blocked by serotype-specific toxin antibodies (Park et al., “Inhalational Poisoning by Botulinum Toxin and Inhalation Vaccination with Its Heavy-Chain Component,” Infect. Immun. 71:1147-1154 (2003), which is hereby incorporated by reference in its entirety).

Targeting the CNS may require intra-thecal or intra-ventricular administration. Administration may occur directly to the CNS. Alternatively, administration to the CNS may involve retrograde transport from peripheral neurons (motor neurons, nociceptors) to spinal ganglia (see Caleo et al., “A Reappraisal of the Central Effects of Botulinum Neurotoxin Type A: By What Mechanism?” Journal of Neurochemistry 109:15-24 (2009), which is hereby incorporated by reference in its entirety).

Derivatives of a Clostridial neurotoxin of the present invention can be used to augment the endogenous pharmaceutical activity of wild type Clostridial neurotoxins (e.g., BOTOX®, e.g., as a combination therapy.

Derivatives of Clostridial neurotoxins may be administered as a conjugate with a pharmacologically acceptable water-soluble polymer moiety. By way of example, a polyethylene glycol conjugate is useful to increase the circulating half-life of the treatment compound, and to reduce the immunogenicity of the molecule. Specific PEG conjugates are described in U.S. Patent Application Publ. No. 2006/0074200 to Dungs et al., which is hereby incorporated by reference in its entirety.

Other conjugates include HA, which are described in U.S. Pat. No. 7,879,341 to Taylor and U.S. Patent Application Publication No. 2012/0141532 to Blenda et al., each of which is hereby incorporated by reference in its entirety. Liquid forms, including liposome-encapsulated formulations, are illustrated by injectable solutions and suspensions. Exemplary solid forms include capsules, tablets, and controlled-release forms, such as a microsomal pump or an implant. Other dosage forms can be devised by those skilled in the art, as shown, for example, by Ansel and Popovich, Pharmaceutical Dosage Forms and Drug Delivery Systems, 5th Edition (Lea & Febiger 1990), Gennaro (ed.), Remington’s Pharmaceutical Sciences, 19th Edition (Mack Publishing Company 1995), and by Ranade and Holllinger, Drug Delivery Systems (CRC Press 1996), each of which is hereby incorporated by reference in its entirety.


Subjets to be treated pursuant to the method of the present invention include, without limitation, human and non-human primates, or other animals such as dog, cat, horse, cow, goat, sheep, rabbit, or rodent (e.g., mouse or rat).


Neurologic treatment includes, but is not limited to, treatment for tourettes syndrome (Porta et al., “Treatment of Phonie Tics in Patients with Tourette’s Syndrome Using Botulinum Toxin Type A,” Neurol. Sci. 24:420-423 (2004), which is hereby incorporated by reference in its entirety) and focal muscular spasticity or dystonias (MacKinnon et al., “Corticospinal Excitability Accompanying Ballistic Wrist Movements in Primary Dystonia,” Mov. Disord. 19:273-284 (2004), which is hereby incorporated by reference in its entirety), including, but not limited to, treatment for cervical dystonia (Haussermann et al., “Long-Term Follow-Up of Cervical Dystonia Patients Treated with Botulinum Toxin A,” Mov. Disord. 19:303-308 (2004), which is hereby incorporated by reference in its entirety), primary blepharospasm (Defazio et al., “Primary Blepharospasm: Diagnosis and Management,” Drugs 64:237-244 (2004), which is hereby incorporated by reference in its entirety), hemifacial spasm (Post-Stroke (Bakheit, “Optimising the Methods of Evaluation in Primary Dystonia,” Mov. Disord. 19:273-284 (2004), which is hereby incorporated by reference in its entirety), spasmodic dysphonia (Bender et al., “Speech Intelligibility in Severe Adductor Spasmoc Dys-


The derivative of a Clostridial neurotoxin may also be used, pursuant to the treatment method of the present invention, to treat diseases influenced by activity-dependent changes in synaptic structure (e.g., synaptopathologies) or hyperactivity of synapse forming apparatus (e.g., tubulin polymerization), and conditions associated with the proliferation of microtubules. For example, Alzheimer’s Disease, Parkinson’s Disease, and neuronal cancers (of both neural and glial origin). Other conditions that may be treated by the method of the present invention include conditions where the synaptic complex is a disease target.

In one embodiment, neurotoxin derivatives of the present invention accumulate within neuronal cytosol in higher amounts than wild-type Clostridial neurotoxin.

**EXAMPLES**

**Example 1**

In-vivo Pharmaceutical Activity Experiments for BoNT A/ad-0

**Material and Methods**

An atoxic derivative of Clostridium botulinum serotype A ("BoNT A/ad"), as described in U.S. Pat. No. 7,785,606 to Ichtchenko and Band (which is hereby incorporated by reference in its entirety), was expressed as described. Since this
neurotoxin derivative is atoxic and does not possess a cargo attachment peptide sequence at its N-terminus, it was designated "BoNT A/ad-0," where "ad-0" means atoxic derivative with no cargo site (0), as described herein. BoNT A/ad-0 was purified to electrophoretic homogeneity and activated by specific protease cleavage as described in Band et al., "Recombinant Derivatives of Botulinum Neurotoxin A Engineered for Trafficking Studies and Neuronal Delivery," Protein Expression & Purification 71:62 (2010), which is hereby incorporated by reference in its entirety. The purified protein was prepared as a stock at a concentration of 10 mg/ml in PBS containing 40% glycerol for stabilization. The studies described below, evaluate the recombinant molecule's toxicity and pharmacologic activity.

Animals
Mice: Female Balb/C mice, 5 to 7 weeks old; weight around 19±3 grams.

Digit Abduction Score (DAS) Assay
A modification of the classic mouse Digit Abduction Scoring ("DAS") assay was used to determine local pharmacologic activity in muscle, measured by muscle weakening effectiveness, as described in Aoki, "Preclinical Update on BOTOX® (Botulinum Toxin Type A)-Purified Neurotoxin Complex Relative to Other Botulinum Neurotoxin Preparations," European Journal of Neurology (1999), which is hereby incorporated by reference in its entirety. In the DAS assay, mice are suspended by their tails briefly to elic平 a characteristic startle response in which the animal extends its hind limbs and abducts its hind digits. The DAS assay is especially useful to compare the muscle weakening effectiveness of different BoNT preparations (Aoki, "Preclinical Update on BOTOX® (Botulinum Toxin Type A)-Purified Neurotoxin Complex Relative to Other Botulinum Neurotoxin Preparations," European Journal of Neurology (1999) and Aoki, "A Comparison of the Safety Margins of Botulinum Neurotoxin Serotypes A, B, and F In Mice," Toxicol 39:1815-1820 (2001), which are hereby incorporated by reference in their entirety).

This test was utilized to define pharmacological activity of BoNT A/ad-0 in mice. Mice were scored as having a positive DAS response when they were unable to fully extend all digits on the injected leg. A negative score is given to mice that spread the toes of the injected leg comparable to that of the non-injected leg.

Female Balb/C mice were given unilateral gastrocnemius intramuscular injections with the concentration described in a volume of 3 μl of 0.9% NaCl using a 25 μl Hamilton syringe. Muscle weakness was assessed from day 1 until 5 days post injection by suspending the mice in order to elicit a characteristic startle response and observing whether the toes on the injected leg appeared collapsed (digital abduction). Definite muscle paralysis was initially observed for muscle paralysis induced by administration of BoNT A/ad-0. First, by the inability of the mouse to use the injected leg to walk (muscle paralysis). Second, by observing whether the toes on the injected leg appeared collapsed (digital abduction). Definite muscle paralysis was initially observed.

Results: Toxicity, LD50
The BoNT A/ad-0 preparation described above was used for the following toxicity study. The study was designed to approximate the standard murine LD50 test for wild type BoNT A ("wt BoNT A").

A total of 30 female mice were used in this study. Each mouse was injected intraperitoneally with the indicated dose of BoNT A/ad-0 in 200 μl of PBS (Table 1), and observed for 24 hours.

Doses ranging from 0.5 μg/mouse to 2 μg/mouse, based on the LD50 published by Pellett et al., "Neuromonal Targeting, Internalization, and Biological Activity of a Recombinant Atoxic Derivative of Botulinum Neurotoxin A," Biochemical & Biophysical Research Communications 405(4):673-677 (2011), which is hereby incorporated by reference in its entirety), using BoNT A/ad (1.2 μg per mouse or 50 μg/kg body weight. The LD50 for BoNT A/ad-0 was found to be very similar to that for BoNT A/ad (Table 1). Briefly, 50% or 5 out of 10 mice injected with a dose of 50 μg/kg body weight showed symptoms of botulism intoxication by 36 hours. All mice injected with a dose of 2 μg, which is approximately 83.3 μg/kg body weight, expired within 48 hours. From this study it is concluded that 50 μg/kg body weight is the approximate LD50 of BoNT A/ad-0.

<table>
<thead>
<tr>
<th>Injected Dose</th>
<th>No. Mice</th>
<th>Dead</th>
<th>Survive</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 μg</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1.2 μg</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1 μg</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>0.5 μg</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

The LD50 of wt BoNT A is approximately 0.5 ng/kg (Aoki, “A Comparison of the Safety Margins of Botulinum Neurotoxin Serotypes A, B, and F In Mice,” Toxicol 39:1815-1820 (2001), which is hereby incorporated by reference in its entirety), or 100,000-fold lower than that of BoNT A/ad-0. Because of this toxicity, the effectiveness of wt BoNT A at extremely low doses, and the variability in potency for BoNTs produced from a wild type bacterial source, pharmacological doses of wt BoNT A are generally specified in terms of “activity units,” with 1 mouse LD50 of wt BoNT A considered to be 1 activity unit, or approximately 0.5 ng/kg of wt BoNT A (Aoki, “A Comparison of the Safety Margins of Botulinum Neurotoxin Serotypes A, B, and F In Mice,” Toxicol 39:1815-1820 (2001), which is hereby incorporated by reference in its entirety). This takes into account concentration variations in the level of active toxin between preparations and manufacturers. Harmonized standards across producers remain undefined. This is due to both different manufacturing methods and batch-to-batch variation, but is also related to marketing claims. The final pharmaceutical preparations are formulated with albumin (BOTOX®) and/or lactose (DYSPORT®). From the LD50 results described here, it can be concluded that 1 LD50 Unit (IU) of BoNT A/ad-0 corresponds to a dose of approximately 50 μg/kg, or approximately 1.2 μg per mouse.

Results: Muscle Paralysis Study/DAS Assay for Pharmacologic Activity In Vivo
BoNT A/ad-0 described above was tested in the murine DAS to determine if BoNT A/ad-0 possesses pharmacological activity at doses significantly below its LD50 and whether it displays typical dose-response activity. Mice were injected in the gastrocnemius muscle with 3 μl of BoNT A/ad-0 in 0.9% NaCl using a 25 μl Hamilton Syringe. The doses administered are expressed as the μg administered per mouse, or units of BoNT A/ad-0 activity administered per mouse (Table 2).

Two observations are noted to categorize a mouse as positive for muscle paralysis induced by administration of BoNT A/ad-0. First, by the inability of the mouse to use the injected leg to walk (muscle paralysis). Second, by observing whether the digits on the injected leg appeared collapsed (digital abduction). Definite muscle paralysis was initially observed.
and recorded 24 hours after the initial administration. Mice were daily evaluated for definitive muscle paralysis for a maximum of 5 days.

The results of this pharmacologic study of BoNT A/ad-0 are shown in Table 2 and FIG. 2. Mice administered doses ranging from 0.008 LD₅₀ units (0.01 µg) to 0.42 LD₅₀ units (0.5 µg) of BoNT A/ad-0 showed definitive muscle paralysis and digital abduction (FIG. 2 and Table 2), without any signs of mortality. In fact, 4 out of 5 animals injected with 0.01 µg presented with muscle paralysis and some degree of digital abduction (Table 2), indicating that the ED₅₀ for BoNT A/ad-0, the lowest dose at which 50% of the injected animals demonstrated the intended pharmacologic activity, is 0.01 µg or lower, which corresponds to 0.008 LD₅₀ units or lower. All mice that presented paralysis on day 1 continued to present paralysis to the end of the study, day 5. No signs of systemic toxicity were observed in any of the mice in this study.

These data confirm that BoNT A/ad-0 has similar pharmacological properties compared to wt BoNT A, albeit with a dose-response profile, a significantly increased range of safe therapeutic activity and, therefore, an improved therapeutic index, and an improved safety margin. This comparison of BoNT A/ad-0 to pharmaceutical preparations of wt BoNT is illustrated in Table 3, and contrasted to the data reported by Aoki, “A Comparison of the Safety Margins of Botulinum Neurotoxin Serotypes A, B, and F In Mice,” Toxicol 39:1815-1820 (2001), which is hereby incorporated by reference in its entirety. For instance, Aoki, “A Comparison of the Safety Margins of Botulinum Neurotoxin Serotypes A, B, and F In Mice,” Toxicol 39:1815-1820 (2001), which is hereby incorporated by reference in its entirety, reported that the safety margin for BOTOX® is about 13.94/–1.7 and for DYSPORT® is about 13.94/–1.7 and for DYSPORT® is about 13.94/–1.7. Here it is shown that at the lowest dose of BoNT A/ad-0 studied, 0.01 µg, definite paralysis was observed in 4/5 mice. This dose can be considered a conservative estimate of the ED₅₀. Therefore, for BoNT A/ad-0, the safety margin is approximately 120, or expressed differently, 10-fold better than that for BOTOX® or DYSPORT® (Table 3).

### TABLE 2

<table>
<thead>
<tr>
<th>Dose Injected per Mouse</th>
<th>LD₅₀ Units</th>
<th>No. Mice</th>
<th>No. with Definitive Paralysis</th>
<th>No. Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (placebo)</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.01 µg</td>
<td>0.008</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>0.1 µg</td>
<td>0.08</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>0.5 µg</td>
<td>0.42</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1 µg</td>
<td>0.83</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>1.2 µg</td>
<td>1.25</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Naive mice were administered BoNT A/ad-0 in the left gastrocnemius via intramuscular injection with 3 µl containing the indicated mass or units of BoNT A/ad-0.

### TABLE 3

<table>
<thead>
<tr>
<th>LD₅₀ and ED₅₀ of BoNT A/ad-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD₅₀ = ~1.2 µg</td>
</tr>
<tr>
<td>ED₅₀ = ~0.01 µg (ED₅₀ = 0.01 µg or lower)</td>
</tr>
<tr>
<td>LD₅₀/ED₅₀ = safety margin = 120</td>
</tr>
</tbody>
</table>

If expressed as units, the ED₅₀ of BoNT A/ad-0 is 0.008 LD₅₀ units, or lower.

### TABLE 4

<table>
<thead>
<tr>
<th>Dose</th>
<th>No. Mice</th>
<th>No. with Definitive Paralysis</th>
<th>No. Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (placebo)</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5 µg</td>
<td>38</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>1 µg</td>
<td>15</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>1.2 µg</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

1.2 µg is the apparent LD₅₀ for intramuscular injections of BoNT A/ad estimated from this experiment.

### TABLE 5

<table>
<thead>
<tr>
<th>Repeat Dose</th>
<th>No. Mice</th>
<th>No. with Definitive Paralysis</th>
<th>No. Dead (within 48 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg</td>
<td>18</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>2 µg</td>
<td>20</td>
<td>17</td>
<td>15 dead, with 3 appearing sick</td>
</tr>
<tr>
<td>3 µg</td>
<td>20</td>
<td>17</td>
<td>2 mice appeared normal at 48 hrs</td>
</tr>
</tbody>
</table>

Comparison to Prior Studies and Conclusions

Prior studies have found that mutations introduced into the light chain of recombinant BoNT A/ad (a molecule containing a cargo attachment peptide as described in U.S. Patent Application Publication No. 2011/0206616 to Ichthchenko and Band, which is hereby incorporated by reference in its entirety) increased the LD₅₀ of the toxin by 100,000-fold. In particular, injections of 0.5 µg (n=25) or 1 µg (n=15) of BoNT A/ad (in the absence of any therapeutic agent) were made into the tibialis muscle two months prior to administration of the repeat dose to each animal. The repeat dose, consisting of 3 µl containing the indicated quantities of BoNT A/ad, 1 µg (n=18) or 2 µg (n=20), were similarly injected into the tibialis muscle. These data (Table 4 and Table 5) suggest that immune resistance to BoNT A/ad is not developing with repeat treatment.

In the present study it was found that the LD₅₀ of BoNT A/ad-0, which has identical toxin-disabling mutations as BoNT A/ad, is likewise elevated ~100,000-fold relative to wt BoNT A. But surprisingly, it was observed that BoNT A/ad-0 still possessed pharmacologic activity similar to that observed for wt BoNT A, and that a therapeutic agent need not be delivered via the cargo site of BoNT A/ad to render it therapeutic. By comparing the dose-response of BoNT A/ad-0 to that reported for pharmaceutical preparations of wt BoNT A, it can be concluded that BoNT A/ad-0 can be used for pharmaceutical treatments in the same way as wt BoNTs, but with significantly reduced danger of systemic toxicity, and thus significant improved safety advantages for clinical use.

Although the invention has been described in detail for the purposes of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.
160 NUMBER OF SEQ ID NOS: 10
210 SEQ ID NO 1
211 LENGTH: 1296
212 TYPE: PRT
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Arg Tyr Thr Phe Gly Tyr Lys Pro Glu Asp Phe Asn Lys Ser Ser Gly 50 55 60
Ile Phe Asn Asp Val Cys Glu Tyr Tyr Asp Pro Asp Tyr Leu Asn 65 70 75 80
Thr Asn Asp Lys Lys Asn Ile Phe Leu Glu Thr Met Ile Lys Leu Phe 85 90 95
Asn Arg Ile Lys Ser Lys Pro Leu Gly Glu Lys Leu Leu Glu Met Ile 100 105 110
Ile Asn Gly Ile Pro Tyr Leu Gly Asp Arg Arg Val Leu Glu Glu 110 120 125
Phe Asn Thr Asn Ile Ala Ser Val Thr Val Asn Lys Leu Ile Ser Asn 130 135 140
Pro Gly Glu Val Glu Arg Lys Gly Ile Phe Ala Asn Leu Ile Ile 145 150 155 160
Phe Gly Pro Gly Pro Val Leu Asn Glu Asn Thr Ile Asp Ile Gly 165 170 175
Ile Gln Asn His Phe Ala Ser Arg Glu Gly Phe Gly Gly Ile Met Gln 180 185 190
Met Lys Phe Cys Pro Glu Tyr Val Ser Val Phe Asn Asn Val Gln Glu 195 200 205
Asn Lys Gly Ala Ser Ile Phe Asn Arg Arg Gly Tyr Phe Ser Asp Pro 210 215 220
Ala Leu Ile Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr 225 230 235 240
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| Tyr Asp Lys Val Leu Gln Asn Phe Arg Gly Ile Val Asp Arg Leu Asn | 290 |
| Lys Val Leu Val Cys Ile Ser Asp Pro Asn Asn Ile Asn Asn Ile Tyr | 310 |
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| Asp Asp Leu Ser Lys Asn Glu Arg Ile Glu Tyr Asn Thr Gln Ser Asn | 470 |
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| Thr Phe Pro Leu Asp Ile Arg Asp Ile Ser Leu Thr Ser Ser Phe Asp | 570 |
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| Trp Val Lys Gin Ile Val Asp Asp Phe Val Ile Glu Ala Asn Lys Ser | 630 |
| Asn Thr Met Asp Lys Ile Ala Asp Ile Ser Leu Ile Val Pro Tyr Ile | 650 |
| Gly Leu Ala Leu Asn Val Gly Asn Thr Ala Lys Gin Gly Asn Phe Glu | 670 |
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Phe Asn Ile Met Lys Asn Ile Thr Ile Lys Pro Glu Arg Asn Val Ile
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Asp Ser Ser Tyr Tyr Asp Pro Asn Tyr Leu Gin Ser Asp Glu Glu Lys
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Asp Arg Phe Leu Lys Ile Val Thr Lys Ile Phe Asn Arg Ile Asn Asn
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Asn Leu Ser Gly Gly Ile Leu Leu Glu Glu Leu Ser Lys Ala Asn Pro
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Tyr Leu Gly Asn Asp Asn Thr Pro Asp Asn Gin Phe His Ile Gly Asp
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Ala Ser Ala Val Glu Ile Lys Phe Ser Asn Gly Ser Glu Asn Ile Leu
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Glu Asn Asp Leu Asp Gln Val Ile Leu Asn Phe Asn Ser Glu Ser Ala
465 470 475 480
Pro Gly Leu Ser Asp Glu Lys Leu Asn Leu Thr Ile Gln Asn Asp Ala
485 490 495
Tyr Ile Pro Lys Tyr Asp Ser Asn Gly Thr Ser Asp Ile Glu Gln His
500 505 510
Asp Val Asn Glu Leu Asn Val Phe Phe Tyr Leu Asp Ala Gln Lys Val
515 520 525
Pro Glu Gly Glu Asn Asn Val Asn Leu Thr Ser Ser Ile Asp Thr Ala
530 535 540
Leu Leu Glu Gln Pro Lys Ile Tyr Thr Phe Phe Ser Ser Glu Phe Ile
545 550 555 560
Asn Asn Val Asn Lys Pro Val Gln Ala Ala Leu Phe Val Ser Trp Ile
565 570 575
Gln Gln Val Leu Val Asp Phe Thr Thr Glu Ala Asn Gln Lys Ser Thr
580 585 590
Val Asp Lys Ile Ala Asp Ile Ser Ile Val Val Pro Tyr Ile Gly Leu
595 600 605
Ala Leu Asn Ile Gly Asn Glu Ala Gln Lys Gly Asn Asp Ala
610 615 620
Leu Glu Leu Leu Gly Ala Gly Leu Leu Glu Phe Glu Pro Glu Leu
625 630 635 640
Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe Leu Gly Ser
645 650 655
Ser Asp Asn Lys Asn Lys Val Ile Lys Ala Ile Asn Ala Leu Lys
660 665 670
Glu Arg Asp Glu Lys Thr Lys Glu Val Tyr Ser Phe Ile Val Ser Asn
675 680 685
Trp Met Thr Lys Ile Asn Thr Glu Phe Asn Lys Arg Lys Glu Gln Met
690 695 700
Tyr Gln Ala Leu Gln Asn Glu Val Asn Ala Ile Lys Thr Ile Ile Glu
705 710 715 720
Ser Lys Tyr Asn Ser Tyr Thr Leu Glu Lys Asn Glu Leu Thr Asn
725 730 735
Lys Tyr Asp Ile Lys Glu Ile Glu Asn Glu Leu Asn Gln Lys Val Ser
740 745 750
Ile Ala Met Asn Asn Ile Asp Arg Phe Leu Thr Glu Ser Ser Ile Ser
745 760 765
Tyr Leu Met Lys Leu Ile Asn Glu Val Lys Ile Asn Lys Leu Arg Glu
770 775 780
Tyr Asp Glu Asn Val Lys Thr Tyr Leu Asn Tyr Ile Ile Gln His
785 790 795 800
Gly Ser Ile Leu Gly Glu Ser Gln Glu Leu Asn Ser Met Val Thr
805
Asp Thr Leu Asn Asn Ser Ile Pro Phe Lys Leu Ser Ser Tyr Thr Asp
820
Asp Lys Ile Leu Ser Tyr Phe Asn Lys Phe Lys Arg Ile Lys
835
Ser Ser Ser Val Leu Asn Met Arg Tyr Lys Asn Asp Lys Tyr Val Asp
850
Thr Ser Gly Tyr Asp Ser Asn Ile Asn Ile Asn Glu Asp Val Tyr Lys
865
Tyr Pro Thr Asn Lys Asn Gln Phe Gly Ile Tyr Asn Asp Lys Leu Ser
880
Glu Val Asn Ile Ser Gln Asn Tyr Ile Ile Tyr Asp Asn Tyr Asn Phe
900
Lys Asn Phe Ser Ile Ser Phe Thr Val Arg Ile Pro Asn Tyr Asp Asn
915
Lys Ile Val Asn Val Asn Glu Tyr Thr Ile Ile Asn Cys Met Arg
930
Asp Asn Ser Ser Gly Thr Lys Val Ser Leu Asn His Asn Glu Ile Ile
945
Trp Thr Leu Glu Asp Asn Ala Gly Ile Asn Gin Lys Leu Ala Phe Asn
965
Tyr Gly Asn Ala Asn Gly Ile Ser Asp Tyr Ile Asn Lys Trp Ile Phe
980
Val Thr Ile Thr Asn Asp Arg Leu Gly Asp Ser Lys Leu Tyr Ile Asn
995
Gly Asn Leu Ile Asp Gln Lys Ser Ile Leu Asn Leu Gly Asn Ile
1010
His Val Ser Asp Asn Ile Leu Phe Lys Ile Val Arg Cys Ser Tyr
1025
Thr Arg Tyr Ile Gly Ile Arg Tyr Phe Asn Ile Phe Asp Lys Glu
1040
Leu Asp Glu Thr Glu Ile Gln Thr Leu Tyr Ser Asn Glu Pro Asn
1055
Thr Asn Ile Leu Lys Asp Phe Trp Gly Asn Tyr Leu Leu Tyr Asp
1070
Lys Glu Tyr Tyr Leu Leu Asn Val Leu Lys Pro Asn Asn Phe Ile
1085
Asp Arg Arg Lys Ser Thr Leu Ser Ile Asn Asn Ile Asn Ser
1100
Thr Ile Leu Leu Ala Asn Arg Leu Tyr Ser Gly Ile Lys Val Lys
1115
Ile Gln Arg Val Asn Ser Thr Thr Thr Thr Arg Ser
1130
Lys Asp Gln Val Tyr Ile Asn Phe Val Ala Ser Lys Thr His
1145
Leu Phe Asp Lys Tyr Thr Ala Thr Ser Thr Tyr Asp
1160
Thr Ile Val Ser Ser Ser Gly Asn Arg Phe Asn Glu Val Val
1175
Val Met Asn Ser Val Gly Asn Asn Thr Met Asn Phe Lys Asn Asn
1190
Val Ala Ser Thr Trp Tyr Tyr Thr His Met Arg Asp His Thr Asn
1220 1225 1230

<210> SEQ ID NO 6
<211> LENGTH: 1277
<212> TYPE: PRT
<213> ORGANISM: Clostridium botulinum (serotype F)

<400> SEQUENCE: 6

Met Pro Val Val Ile Asn Ser Phe Asn Tyr Asn Asp Pro Val Asn Asp
1 5 10 15

Asp Thr Ile Leu Tyr Met Gln Ile Pro Tyr Glu Glu Ser Lys Lys
20 25 30

Tyr Tyr Lys Ala Phe Glu Ile Met Arg Asp Val Trp Ile Ile Pro Glu
35 40 45

Arg Asn Thr Ile Gly Thr Pro Ser Asp Phe Asp Pro Pro Ala Ser
50 55 60

Leu Glu Asn Gly Ser Ser Ala Tyr Asp Pro Tyr Leu Thr Thr
65 70 75 80

Arg Ala Glu Lys Asp Arg Tyr Leu Lys Thr Thr Ile Lys Lys Lys
95 100 105 110

Asn Lys Pro Tyr Leu Gly Asn Glu His Thr Pro Ile Asn Glu Phe
115 120 125

His Pro Val Thr Arg Thr Ser Val Asn Ile Lys Ser Ser Thr Asn
130 135 140

Val Lys Ser Ser Ile Ile Leu Asn Leu Val Leu Gly Ala Gly Pro
145 150 155 160

Asp Ile Phe Glu Asn Ser Ser Tyr Pro Val Arg Lys Leu Met Asp Ser
165 170 175

Gly Val Thr Tyr Asp Pro Ser Asn Phe Gly Ser Ser Ile Asn Ile
180 185 190

Val Thr Phe Ser Pro Glu Tyr Glu Tyr Thr Phe Asn Asp Ile Ser Gly
195 200 205

Gly Tyr Asn Ser Ser Thr Glu Ser Phe Ile Ala Asp Pro Ala Ile Ser
210 215 220

Leu Ala His Glu Leu Ile His Ala Leu His Gly Leu Tyr Gly Ala Arg
225 230 235 240

Gly Val Thr Tyr Lys Glu Thr Ile Lys Val Lys Gin Ala Pro Leu Met
245 250 255

Ile Ala Ile Lys Pro Ile Arg Leu Glu Phe Leu Thr Phe Gly Gly
260 265 270

Gln Asp Leu Asn Ile Ile Thr Ser Ala Met Lys Glu Lys Ile Tyr Asn
275 280 285

Asn Leu Leu Ala Asn Tyr Glu Ile Ala Thr Arg Leu Ser Arg Val
290 295 300

Asn Ser Ala Pro Pro Glu Tyr Asp Ile Asn Glu Tyr Lys Asp Tyr Phe
305 310 315 320
|      | Gln Trp Lys Tyr Gly Leu Asp Lys Asn Ala Asp Gly Ser Tyr Thr Val | Asn Glu Asn Lys Phe Asn Glu Ile Tyr Lys Leu Tyr Ser Phe Thr | Glu Ile Asp Leu Ala Asn Lys Phe Lys Val Lys Cys Arg Asn Thr Tyr | Phe Ile Lys Tyr Gly Phe Leu Lys Leu Pro Asn Leu Leu Asp Asp Asp | Asn Tyr Thr Val Ser Glu Gly Phe Asn Ile Gly Asn Ala Val Asn | Asn Arg Gly Gln Asn Ile Lys Leu Asn Pro Lys Ile Ile Asp Ser Ile | Pro Asp Lys Gly Leu Val Glu Lys Ile Val Lys Phe Cys Lys Ser Val | Ile Pro Arg Lys Gly Thr Thr Ala Pro Pro Arg Leu Cys Ile Arg Val | Asn Asn Arg Glu Leu Phe Phe Val Ala Ser Glu Ser Ser Tyr Asn Glu | Asn Asp Ile Asn Thr Pro Lys Glu Ile Asp Asp Thr Thr Asn Leu Asn | Asn Asn Tyr Arg Asn Asn Leu Asp Glu Val Ile Leu Asp Tyr Asn Ser | Glu Thr Ile Pro Gln Ile Ser Asn Gln Thr Leu Asn Thr Leu Val Gln | Asp Asp Ser Tyr Val Pro Arg Tyr Asp Ser Asn Gly Thr Ser Glu Ile | Glu Glu His Asn Val Val Asp Leu Asn Val Phe Phe Tyr Leu His Ala | Gln Lys Val Pro Glu Gly Glu Thr Asn Ile Ser Leu Thr Ser Ser Ile | Asp Thr Ala Leu Ser Glu Ser Gln Val Tyr Thr Phe Phe Ser Ser | Glu Phe Ile Asn Thr Ile Asn Lys Pro Val His Ala Ala Leu Phe Ile | Ser Trp Ile Asn Gln Val Ile Arg Asp Phe Thr Glu Ala Thr Gln | Lys Ser Thr Phe Asp Lys Ile Ala Asp Ile Ser Leu Val Val Pro Tyr | Val Gly Leu Ala Leu Asn Ile Gly Asn Glu Val Gin Lys Glu Asn Phe | Lys Glu Ala Phe Glu Leu Leu Gly Ala Gly Ile Leu Leu Glu Phe Val | Pro Glu Leu Leu Ile Pro Thr Ile Leu Val Phe Thr Ile Lys Ser Phe | Ile Gly Ser Ser Glu Asn Lys Asn Lys Ile Ile Lys Ala Ile Asn Asn | Ser Leu Met Glu Arg Glu Thr Lys Trp Lys Glu Ile Tyr Ser Trp Ile | Val Ser Asn Trp Leu Thr Arg Ile Asn Gin Phe Aen Lys Arg Lys | Glu Gin Met Tyr Gin Ala Leu Gin Gin Val Asp Ala Ile Lys Thr |
Val Ile Glu Tyr Lys Tyr Asn Asn Tyr Thr Ser Asp Glu Arg Asn Arg
Leu Glu Ser Glu Tyr Asn Ile Asn Ile Arg Glu Glu Leu Asn Lys
Lys Val Ser Leu Ala Met Glu Asn Ile Glu Arg Phe Ile Thr Glu Ser
Ser Ile Phe Tyr Leu Met Lys Leu Ile Asn Glu Ala Lys Val Ser Lys
Leu Arg Glu Tyr Asp Glu Gly Val Lys Glu Tyr Leu Asp Tyr Ile
Ser Glu His Arg Ser Ile Leu Gly Asn Ser Val Glu Leu Asn Asp
Leu Val Thr Ser Thr Leu Asn Asn Ser Ile Pro Phe Glu Leu Ser Ser
Tyr Thr Asn Asp Lys Ile Leu Tyr Asp Phe Lys Tyr Asp Lys Tyr Ser Lys
Leu Gly Asp Asn Ser Tyr Thr Tyr Ser Tyr Thr Tyr Ser Tyr Thr Tyr Ser Lys
Gly Asp Ser Val Met Arg Tyr Thr Tyr Ser Tyr Thr Tyr Ser Tyr Thr Tyr Ser Ser
Val Tyr Ile Tyr Ser Thr Asn Arg Asn Ser Val Glu Phe Gly Ile Tyr Ser Ser
Lys Pro Ser Glu Val Asn Ile Ala Glu Asn Asn Asp Ile Ile Tyr Asn
Gly Arg Tyr Gln Asn Phe Ser Ile Ser Phe Trp Val Arg Ile Pro Lys
Tyr Phe Asn Lys Val Asn Leu Asn Glu Tyr Thr Ile Asp Cys
Ile Arg Asn Asn Ser Gly Thr Lys Ile Ser Leu Asn Tyr Asn Lys
Ile Ile Trp Thr Leu Gln Asp Thr Ala Gly Asn Asn Gly Met Leu Val
Phe Asn Tyr Thr Gln Met Ile Ser Ile Ser Asp Tyr Ile Asn Lys Trp
Ile Phe Val Thr Ile Thr Asn Arg Leu Gly Asn Ser Arg Ile
Tyr Ile Asn Gly Asn Leu Ile Asp Glu Lys Ser Ile Ser Asn Leu
Gly Asp Ile His Val Ser Asp Asn Ile Leu Phe Lys Ile Val Gly
Cys Asn Asp Thr Arg Tyr Val Gly Ile Arg Tyr Phe Lys Val Phe
Asp Thr Glu Leu Gly Lys Thr Glu Ile Glu Thr Leu Tyr Ser Asp
Glu Pro Asp Pro Ser Ile Leu Lys Asp Phe Trp Gly Asn Tyr Leu
Leu Tyr Asn Lys Arg Tyr Tyr Leu Leu Asn Asn Leu Leu Arg Thr Asp
Lys Ser Ile Thr Gln Asn Ser Asn Phe Leu Asn Ile Asn Gln Gln
Arg Gly Val Tyr Gln Lys Pro Asn Ile Phe Ser Asn Thr Arg Leu
Tyr Thr Gly Val Glu Val Ile Ile Arg Lys Asn Gly Ser Thr Asp
Ile Ser Thr Asp Asn Phe Val Arg Lys Asn Asp Leu Ala Tyr
Ile Asn Val Val Asp Arg Phe Phe Tyr Arg Leu Tyr Ala Asp
Ile Ser Ile Ala Lys Pro Glu Lys Ile Ile Lys Leu Ile Arg Thr
Ser Asn Ser Asn Asn Ser Leu Gly Glu Ile Ile Val Met Asp Ser
Ile Gly Asn Asn Thr Met Asn Phe Glu Asn Asn Asn Gly Gly Asn
Ile Gly Leu Leu Gly Phe His Ser Asn Asn Leu Val Ala Ser Ser
Trp Tyr Tyr Asn Asn Ile Arg Lys Asn Thr Ser Ser Asn Gly Cys
Phe Trp Ser Phe Ile Ser Lys Glu His Gly Trp Glu Gly Asn

<210> SEQ ID NO 7
<211> LENGTH: 1297
<212> TYPE: PRT
<213> ORGANISM: Clostridium botulinum (serotype G)
<400> SEQUENCE: 7
Met Pro Val Asn Ile Lys Asn Phe Asn Tyr Asn Asp Pro Ile Asn Asn
1  5 10 15
Asp Asp Ile Ile Met Met Glu Pro Phe Asn Asp Pro Gly Pro Gly Thr
20 25 30
Tyr Tyr Lys Ala Phe Arg Ile Ile Asp Arg Ile Thr Ile Val Pro Glu
35 40 45
Arg Phe Thr Tyr Gly Phe Glu Pro Asp Glu Phe Asn Ala Ser Thr Gly
50 55 60
Val Phe Ser Lys Asp Val Tyr Glu Tyr Tyr Asp Thr Tyr Tyr Leu Lys
65 70 75 80
Thr Asp Ala Glu Asp Lys Phe Leu Lys Thr Met Ile Lys Leu Phe
85 90 95
Asn Arg Ile Asp Ser Gly Glu Arg Leu Leu Asp Met Ile
100 105 110
Val Asp Ala Ile Pro Tyr Leu Gly Asn Ala Ser Thr Pro Pro Asp Lys
115 120 125
Phe Ala Ala Ann Val Ala Ann Val Ser Ile Ann Lys Ile Ile Gln
130 135 140
Pro Gly Ala Glu Asp Glu Ile Lys Gly Leu Met Thr Ann Leu Ile Ile
145 150 155 160
Phe Gly Pro Gly Pro Val Leu Ser Asp Ann Phe Thr Asp Ser Met Ile
165 170 175
Met Asn Gly His Ser Pro Ile Ser Glu Gly Phe Gly Ala Arg Met Met
180 185 190
Ile Arg Phe Cys Pro Ser Cys Leu Ann Val Phe Ann Ann Val Gln Glu
195 200 205
Asn Lys Asp Thr Ser Ile Phe Ser Arg Ala Tyr Phe Ala Asp Pro
210 215 220
Ala Leu Thr Leu Met His Glu Leu Ile His Val Leu His Gly Leu Tyr
225 230 235 240
Met Glu Phe Ile Pro Glu Leu Ile Val Pro Ile Val Gly Phe Phe Thr
Leu Glu Ser Tyr Val Gly Asn Lys Gly His Ile Ile Met Thr Ile Ser
Asn Ala Leu Lys Lys Arg Asp Gln Leu Trp Thr Asp Met Tyr Gly Leu
Ile Val Ser Gln Trp Leu Ser Thr Val Ile Thr Gln Phe Tyr Thr Ile
Lys Glu Arg Met Tyr Asn Ala Leu Asn Asn Gin Ser Gin Ala Ile Glu
Lys Ile Ile Glu Asp Gln Tyr Asn Arg Tyr Ser Glu Glu Asp Lys Met
Asn Ile Asn Ile Asp Phe Asn Asp Ile Asp Phe Asn Asp Leu Asn Gin Ser
Ile Asn Ala Ile Asn Asn Ile Asp Asp Ile Asp Phe Asn Asp Leu Asn Gin Cys Ser
Ile Ser Tyr Leu Met Asn Arg Met Ile Pro Leu Ala Val Lys Leu
Lys Asp Phe Asp Asp Asn Leu Asp Leu Asp Leu Glu Tyr Ile Asp
Thr Asn Glu Tyr Leu Leu Asp Glu Val Asn Ile Leu Lys Ser Lys
Val Asn Arg His Leu Lys Asp Ser Ile Pro Phe Asp Leu Ser Leu Tyr
Thr Lys Asp Thr Ile Leu Ile Gin Val Phe Asn Asn Tyr Ile Ser Asn
Ile Ser Ser Asn Ala Ile Leu Ser Leu Ser Tyr Arg Gly Gly Arg Leu
Ile Asp Ser Ser Gly Tyr Gly Ala Thr Met Asn Val Gly Ser Asp Val
Ile Phe Asp Asp Ile Gly Asn Gly Gln Phe Lys Leu Asn Asn Ser Glu
Asn Ser Asn Ile Thr Ala His Gin Ser Lys Phe Val Val Tyr Asp Ser
Met Phe Asp Asn Phe Ser Ile Asn Phe Thr Val Arg Thr Pro Lys Tyr
Asn Asn Asn Ile Gin Thr Tyr Leu Gin Asn Gin Glu Tyr Thr Ile Ile
Ser Cys Ile Lys Asn Asp Ser Gly Trp Tyr Val Ser Ile Lys Gly Asn
Arg Ile Ile Thr Leu Ile Asp Val Asn Ala Lys Ser Lys Ser Ile
Phe Phe Glu Tyr Ser Ile Lys Asp Asn Ile Ser Asp Tyr Ile Asn Lys
Trp Phe Ser Ile Thr Ile Thr Asn Asp Arg Leu Gly Asn Ala Asn
Ile Tyr Ile Asn Gly Ser Leu Lys Lys Ser Glu Lys Ile Leu Asn
Leu Asp Arg Ile Asn Ser Ser Asn Asp Ile Asp Phe Lys Leu Ile
Asn Cys Thr Asp Thr Thr Lys Phe Val Trp Ile Lys Asp Phe Asn
Ile Phe Gly Arg Glu Leu Asn Ala Thr Glu Val Ser Ser Leu Tyr
1070 1075 1080
Trp Ile Gln Ser Ser Thr Asn Thr Leu Lys Asp Phe Trp Gly Asn
1085 1090 1095
Pro Leu Arg Tyr Asp Thr Gln Tyr Tyr Leu Phe Asn Gln Gly Met
1100 1105 1110
Gln Asn Ile Tyr Ile Lys Tyr Phe Ser Lys Ala Ser Met Gly Glu
1115 1120 1125
Thr Ala Pro Arg Thr Asn Phe Asn Asn Ala Ala Ile Asn Tyr Gln
1130 1135 1140
Asn Leu Tyr Leu Leu Arg Phe Ile Ile Lys Ala Ser Asn Ser
1145 1150 1155
Arg Asn Ile Asn Asn Asp Asn Ile Val Arg Glu Gly Asp Tyr Ile
1160 1165 1170
Tyr Leu Asn Ile Asp Asn Ile Ser Asp Glu Ser Tyr Arg Val Tyr
1175 1180 1185
Val Leu Val Asn Ser Lys Glu Ile Gln Thr Gln Leu Phe Leu Ala
1190 1195 1200
Pro Ile Asn Asp Asp Pro Thr Phe Tyr Asp Val Leu Gln Ile Gly
1205 1210 1215
Lys Lys Tyr Tyr Glu Lys Thr Thr Tyr Asn Cys Gln Ile Leu Cys
1220 1225 1230
Glu Lys Asp Thr Lys Thr Phe Gly Leu Phe Gly Ile Gly Lys Phe
1235 1240 1245
Val Lys Asp Tyr Gly Tyr Val Trp Asp Thr Tyr Asp Asn Tyr Phe
1250 1255 1260
Cys Ile Ser Gln Trp Tyr Leu Arg Arg Ile Ser Glu Asn Ile Asn
1265 1270 1275
Lys Leu Arg Leu Gly Cys Asn Trp Gln Phe Ile Pro Val Asp Glu
1280 1285 1290
Gly Trp Thr Glu
1295

<210> SEQ ID NO 9
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATRUE:
<223> OTHER INFORMATION: enterokinase cleavage site
What is claimed:

1. A treatment method comprising:
selecting a subject in need of therapeutic treatment involving induction of muscle paralysis and
contacting the subject with an isolated, physiologically active derivative of a wild type Clostridium botulinum neurotoxin, wherein the derivative of a Clostridium botulinum neurotoxin comprises one or more amino acid substitutions relative to the wild type Clostridium botulinum neurotoxin that reduces the metalloprotease activity responsible for the toxicity of wild type Clostridium botulinum neurotoxin and wherein the neurotoxin derivative comprises:
a light chain region and
a heavy chain region, wherein the light and heavy chain regions are linked by a disulfide bond, and wherein the light and heavy chain regions are not truncated, said contacting being carried out to induce muscle paralysis in the subject to treat the subject, with the proviso that the neurotoxin derivative does not possess a cargo attachment peptide sequence at its N-terminus.

2. The method according to claim 1, wherein the derivative of a Clostridium botulinum neurotoxin is a derivative of Clostridium botulinum serotype A, Clostridium botulinum serotype B, Clostridium botulinum serotype C, Clostridium botulinum serotype D, Clostridium botulinum serotype E, Clostridium botulinum serotype F, or Clostridium botulinum serotype G.

3. The method according to claim 1, wherein the derivative of a Clostridium botulinum neurotoxin is a recombinant protein.

4. The method according to claim 1, wherein the treatment is for a dermatologic or aesthetic condition selected from the group consisting of Rhytides, hypertrophic masseter muscles, and focal hyperhydrosis.

5. The method according to claim 1, wherein the treatment is for a gastroenterological condition selected from the group consisting of esophageal motility disorders, pharyngeal-esophageal spasm, and anal fissure.

6. The method according to claim 1, wherein the treatment is for a genitourinary condition selected from the group consisting of neurogenic dysfunction of the urinary tract, overactive bladder, and neuromodulation of urinary urge incontinence.

7. The method according to claim 1, wherein the treatment is for a neurologic condition selected from the group consisting of tourette syndrome, focal muscle spasticity or dystonias, cervical dystonia, primary blepharospasm, hemifacial spasm, spasmodic dysphonia, facial nerve disorders, Ramus syndrome, amputation pain, voice tremor, crocodile tear syndrome, marginal mandibular nerve paralysis, pain, chest pain of esophageal origin, headache, cerebral palsy, hip adductor muscle dysfunction in multiple sclerosis, neurogenic pain and inflammation, arthritis, iatrogenic parotid sialocele, and chronic TMJ pain and displacement.

8. The method according to claim 1, wherein the derivative of a Clostridium botulinum neurotoxin has an LDso that is at least 1,000-fold higher than the LDso of the corresponding wild-type Clostridium botulinum neurotoxin.

9. The method according to claim 1, wherein the derivative of a Clostridium botulinum neurotoxin accumulates within neuronal cytosol in higher amounts than the corresponding wild-type Clostridium botulinum neurotoxin.

10. The method according to claim 1, wherein the derivative of a wild type Clostridium botulinum neurotoxin is produced by cleaving a propeptide, wherein the propeptide comprises:
a light chain region; a heavy chain region; and an intermediate region connecting the light and heavy chain regions and comprising a highly specific protease cleavage site, wherein said highly specific protease cleavage site has three or more specific adjacent amino acid residues that are recognized by the highly specific protease in order to enable cleavage.

11. The method according to claim 10, wherein the highly specific protease cleavage site is selected from an enterokinase cleavage site and a tobacco etch virus protease recognition (TEV) sequence.

12. The method according to claim 10, wherein the propeptide has no low-specificity protease cleavage sites in the intermediate region, said low-specificity protease cleavage sites having two or less adjacent amino acid residues that are recognized by a protease in order to permit cleavage.

13. The method according to claim 10, wherein the propeptide further comprises a signal peptide coupled to the light chain region, wherein the signal peptide is suitable to permit secretion of the neurotoxin propeptide from a eukaryotic cell to a medium.
14. The method according to claim 13, wherein the signal peptide is a gp64 signal peptide.

15. The method according to claim 13, wherein the propeptide further comprises an affinity tag located between the signal peptide and the light chain region.

16. The method according to claim 15, wherein the affinity tag has a sequence of SEQ ID NO:10.

17. The method according to claim 1, wherein the heavy chain has no trypsin-susceptible recognition sequences.

18. The method according to claim 1, wherein the wild type Clostridium botulinum neurotoxin is selected from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7.

19. The method according to claim 1, wherein the derivative of a Clostridium botulinum neurotoxin is selected from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7 comprising an amino acid substitution in the light chain region.

20. The method according to claim 19, wherein the amino acid substitution is in a metalloprotease site.

21. The method according to claim 1, wherein the derivative of a Clostridium botulinum neurotoxin is selected from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7 comprising a non-native motif in the light chain region.