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Title: BIO THERAPY FOR PAIN

Abstract: The present invention is directed to analgesic Clostridial neurotoxin derivatives comprising polypeptides having a long-lasting SNARE protein-selective endopeptidase activity. These derivatives selectively bind to and are internalized by non-neural cells secreting cytokines or sensory neurons in preference to motor neurons or autonomic neurons. The invention is also directed to nucleic acid constructs encoding such polypeptides, and methods of making such derivatives and nucleic acid constructs, and methods of treating pain, such as chronic pain, by administering such derivatives to a patient suffering from, or at risk of suffering from such pain.
BIOTHERAPY FOR PAIN

[0001] The present invention is drawn to methods and composition involving Clostridial neurotoxin derivatives having an enhanced ability to disrupt exocytosis of pain and/or inflammatory mediators from nociceptors or inducers of inflammation, thus preventing pain.

[0002] The ability of Clostridial toxins such as, e.g., Botulinum neurotoxins (BoNTs) (including the serotypes BoNT/A, BoNT/B, BoNT/Cl, BoNT/D, BoNT/E, BoNT/F and BoNT/G,) to inhibit neuronal transmission are being exploited in a wide variety of therapeutic and cosmetic applications, see e.g., Ward AB and Barnes MP, 2007, Clinical Users of Botulinum Toxins (Cambridge University Press, Cambridge). As an example, the BoNT/A-derived agent BOTOX® is currently approved in one or more countries for the following indications: achalasia, adult spasticity, anal fissure, back pain, blepharospasm, bruxism, cervical dystonia, essential tremor, glabellar lines or hyperkinetic facial lines, headache, hemifacial spasm, hyperactivity of bladder, hyperhidrosis, juvenile cerebral palsy, multiple sclerosis, myoclonic disorders, nasal labial lines, spasmodic dysphonia, strabismus and VII nerve disorder.

[0003] There are Clostridial toxins other than the C. botulinum and C. tetanus derived toxins; these include, without limitation, the toxins of C. perfringins, C. septicum, C. difficile, C. sphirome, C. butyricum and C. barati. However, it will be understood that in this specification a reference to "Clostridial toxins" or a similar reference, concerns the neurotoxins of C. botulinum subtypes and C. tetani subtypes, unless specifically or contextually indicated otherwise.

[0004] In addition, Clostridial toxin therapies are used or are proposed for treating:

[0005] a) neuromuscular disorders, see e.g., Kei Roger Aoki et al., Method for Treating Neuromuscular Disorders and Conditions with Botulinum Toxin Types A and B, U.S. Patent No. 6,872,397 (Mar. 29, 2005); Rhett M. Schiffman, Methods for Treating
Uterine Disorders, U.S. Patent Publication No. 2004/0175399 (Sep. 9, 2004); Richard L.
Barron, Methods for Treating Ulcers and Gastroesophageal Reflux Disease, U.S. Patent
Publication No. 2004/0086531 (May. 7, 2004); and Kei Roger Aoki, et al, Method for
Treating Dystonia with Botulinum Toxin C to G, U.S. Patent No. 6,319,505 (Nov. 20, 2001);

[0006]  

b) eye disorders, see e.g., Eric R. First, Methods and Compositions for
Treating Eye Disorders, U.S. Patent Publication No. 2004/0234532 (Nov. 25, 2004); Kei
Roger Aoki et al., Botulinum Toxin Treatment for Blepharospasm, U.S. Patent Publication
No. 2004/0151740 (Aug. 5, 2004); and Kei Roger Aoki et al., Botulinum Toxin Treatment for
Strabismus, U.S. Patent Publication No. 2004/0126396 (Jul. 1, 2004);

[0007]  

c) pain, see e.g., Kei Roger Aoki et al., Pain Treatment by Peripheral
Administration of a Neurotoxin, U.S. Patent No. 6,869,610 (Mar. 22, 2005); Stephen
Donovan, Clostridial Toxin Derivatives and Methods to Treat Pain, U.S. Patent No.
6,641,820 (Nov. 4, 2003); Kei Roger Aoki, et al., Method for Treating Pain by Peripheral
Administration of a Neurotoxin, U.S. Patent No. 6,464,986 (Oct. 15, 2002); Kei Roger Aoki
and Minglei Cui, Methods for Treating Pain, U.S. Patent No. 6,139,115 (Sep. 5, 2000);
Martin A. Voet, Methods for Treating Fibromyalgia, U.S. Patent 6,623,742 (Sep. 23, 2003);
2004/0062776 (Apr. 1, 2004); and Kei Roger Aoki et al., Botulinum Toxin Therapy for
Lower Back Pain, U.S. Patent Publication No. 2004/0037852 (Feb. 26, 2004);

[0008]  

d) muscle injuries, see e.g., Gregory F. Brooks, Methods for Treating
Muscle Injuries, U.S. Patent No. 6,423,319 (Jul. 23, 2002);

[0009]  

e) headache, see e.g., Martin Voet, Methods for Treating Sinus
Headache, U.S. Patent No. 6,838,434 (Jan. 4, 2005); Kei Roger Aoki et al, Methods for
Treating Tension Headache, U.S. Patent No. 6,776,992 (Aug. 17, 2004); and Kei Roger Aoki
et al, Method for Treating Headache, U.S. Patent No. 6,458,365 (Oct. 1, 2002); William J.
Binder, Method for Reduction of Migraine Headache Pain, U.S. Patent 5,714,469 (Feb. 3,
1998);
f) cardiovascular diseases, see e.g., Gregory F. Brooks and Stephen Donovan, *Methods for Treating Cardiovascular Diseases with Botulinum Toxin*, U.S. Patent No. 6,767,544 (Jul. 27, 2004);


Table 2, below, provides the amino acid sequences of isotypes of various currently known botulinum-related (BoNT and TeTX) Clostridial toxins. These toxins possess a minimum of approximately 35% amino acid identity with each other and share the same general functional domain organization and overall structural architecture. These Clostridial toxins are each naturally 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 Clostridial toxin protease or a naturally-occurring protease produced in the environment. This posttranslational
processing yields a mature di-chain molecule comprising an approximately 50 kDa light chain (LC) and an approximately 100 kDa heavy chain (HC) held together by a single inter-chain disulfide bond and noncovalent interactions.

[00019] Each mature di-chain Clostridial toxin molecule comprises three functionally distinct domains: 1) an enzymatic 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 (the so-called SNARE ("Soluble NSF Attachment Protein Receptors") proteins that mediate the fusion of the synaptic vesicle with the cell membrane); 2) a translocation domain contained within the amino-terminal half of the H chain (termed "¾") that facilitates release of at least the LC chain of the toxin from an endosome into the cytoplasm of the target cell; and 3) a binding domain found within the carboxyl-terminal half of the H chain (He) that determines the binding activity and binding specificity of the toxin. He comprises ¾ N and ¾ c subdomains (the N- and C-terminal portions of He, respectively). There is now substantial evidence that most or all BoNT/X toxins bind a target cell using a "dual receptor", wherein the He portion of the toxin comprising both ¾ N and ¾ c subdomains binds certain cell surface gangliosides and a protein receptor (perhaps glycosylated); binding of the protein receptor facilitates the internalization of the toxin within the cell. By "X" is meant any serotype of botulinum toxin. Although the term "BoNT/X" is generally used to indicate subtypes of botulinum toxin, the term may also include TeTX regions thereof. ¾ c binds the receptor complex located at the surface of the target cell.

[00020] It will be understood that there exist strains of each of these toxins that may vary somewhat in their amino acid sequences in non-critical (so called variable) regions without a substantial change in the identity or activity characteristic of the indicated toxin or toxin domain.

[00021] In Table 1 below, the one-letter and three letter amino acid codes are provided:
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Three letter code</th>
<th>One letter code</th>
</tr>
</thead>
<tbody>
<tr>
<td>alanine</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td>arginine</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td>asparagine</td>
<td>Asn</td>
<td>N</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>asparagine or</td>
<td>Asx</td>
<td>B</td>
</tr>
<tr>
<td>aspartic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cysteine</td>
<td>Cys</td>
<td>C</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td>glutamine</td>
<td>Gin</td>
<td>Q</td>
</tr>
<tr>
<td>glutamine or</td>
<td>Glx</td>
<td>Z</td>
</tr>
<tr>
<td>glutamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glycine</td>
<td>Gly</td>
<td>G</td>
</tr>
<tr>
<td>histidine</td>
<td>His</td>
<td>H</td>
</tr>
<tr>
<td>isoleucine</td>
<td>Ile</td>
<td>I</td>
</tr>
<tr>
<td>leucine</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>lysine</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td>methionine</td>
<td>Met</td>
<td>M</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>Phe</td>
<td>F</td>
</tr>
<tr>
<td>proline</td>
<td>Pro</td>
<td>P</td>
</tr>
<tr>
<td>serine</td>
<td>Ser</td>
<td>S</td>
</tr>
<tr>
<td>threonine</td>
<td>Thr</td>
<td>T</td>
</tr>
<tr>
<td>tryptophan</td>
<td>Try</td>
<td>W</td>
</tr>
<tr>
<td>tyrosine</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>valine</td>
<td>Val</td>
<td>V</td>
</tr>
</tbody>
</table>
Those of ordinary skill in the art recognize that naturally occurring Clostridial domain variants having variations in the amino acid shown above (or in the nucleotide sequences encoding these amino acid sequences) may occur in nature. As used herein, the term "naturally-occurring Clostridial domain variant" means any Clostridial domain (endopeptidase, translocation, and/or binding domains) produced by a naturally-occurring process, including, without limitation, Clostridial domain isoforms produced from alternatively-spliced transcripts, Clostridial domain isoforms produced by spontaneous mutations and Clostridial domain subtypes. As used herein, a naturally-occurring Clostridial domain variant functions in substantially the same manner as the reference Clostridial domain on which the naturally-occurring Clostridial domain variant is based, and can be substituted for the reference Clostridial domain in any aspect of the present invention. A naturally-occurring Clostridial domain variant may substitute one or more amino acids, two

<table>
<thead>
<tr>
<th>Toxin</th>
<th>SEQ ID NO:</th>
<th>LC</th>
<th>H_W</th>
<th>H_C</th>
</tr>
</thead>
<tbody>
<tr>
<td>BoNT/A</td>
<td>1</td>
<td>M1-K448</td>
<td>A449-K871</td>
<td>N872-L1296</td>
</tr>
<tr>
<td>BoNT/B</td>
<td>2</td>
<td>M1-K441</td>
<td>A442-S858</td>
<td>E859-E1291</td>
</tr>
<tr>
<td>BoNT/C</td>
<td>3</td>
<td>M1-K449</td>
<td>T450-N866</td>
<td>N867-E1291</td>
</tr>
<tr>
<td>BoNT/D</td>
<td>4</td>
<td>M1-R445</td>
<td>D446-N862</td>
<td>S863-E1276</td>
</tr>
<tr>
<td>BoNT/E</td>
<td>5</td>
<td>M1-R422</td>
<td>K423-K845</td>
<td>R846-K1252</td>
</tr>
<tr>
<td>BoNT/F</td>
<td>6</td>
<td>M1-K439</td>
<td>A440-K864</td>
<td>K865-E1274</td>
</tr>
<tr>
<td>BoNT/G</td>
<td>7</td>
<td>M1-K446</td>
<td>S447-S863</td>
<td>N864-E1297</td>
</tr>
<tr>
<td>TeNT</td>
<td>8</td>
<td>M1-A457</td>
<td>S458-V879</td>
<td>I880-D1315</td>
</tr>
</tbody>
</table>
or more amino acids, three or more amino acids, four or more amino acids, five or more
amino acids, ten or more amino acids, 20 or more amino acids, 30 or more amino acids, 40 or
more amino acids, 50 or more amino acids or 100 or more amino acids from the reference
Clostridial domain on which the naturally-occurring Clostridial domain variant is based. A
naturally-occurring Clostridial domain variant can also substitute at least 10 contiguous
amino acids, at least 15 contiguous amino acids, at least 20 contiguous amino acids, or at
least 25 contiguous amino acids from the reference Clostridial domain on which the
naturally-occurring Clostridial domain variant is based, that possess at least 50% amino acid
identity, 65% amino acid identity, 75% amino acid identity, 85% amino acid identity or 95%
amino acid identity to the reference Clostridial domain on which the naturally-occurring
Clostridial domain variant is based. It will also be understood that conservative amino acid
insertions and deletions can also be made so long as the characteristic function and identity of
the domain is not substantially altered.

[00025] Due to the degeneracy of the genetic code, one of ordinary skill in the
art will recognize that these amino acid sequences may be encoded by a finite set of different
DNA molecules having different, but defined, nucleotide sequences. For example,
degenerate nucleotide sequences encoding a given peptide or protein may have different
codons adapted or selected to favor expression in a particular host cell. Using this
information one can construct an expressible open nucleic acid reading frame for assembly of
a nucleic acid molecule comprising any combination of these amino acid domain-encoding
regions, either alone or with additional nucleic acid sequences, inserted into a suitable
expression vector and subsequent expression within a chosen host cell. For example,
International Patent Publication WO91/14570 discloses methods of making single-chain,
cleavable recombinant modified or unmodified Clostridial neurotoxin derivatives and
chimeric and hybrid forms thereof using such methods. Additional publications disclosing
methods of making expressible recombinant neurotoxins and derivatives thereof include U.S.
Patents 5,989,545; 6,203,794; 6,395,513; U.S. Publication Numbers U.S. 2003/0166238;
99/55359; W096/33273; W098/07864; W099/17806; W098/07864; W002/44199;
W002/40506. These and all other patents, patent publications, and non-patent publications
cited in this patent application, whether or not specifically indicated as such, are hereby individually incorporated by reference as part of this specification.

[00026] The use of recombinant DNA techniques permits the construction of modified Clostridial neurotoxins having different or modified functional properties from the naturally-occurring toxin subtypes and strains thereof. For example, altering the naturally-occurring amino acid sequence of the native neurotoxin light chain and/or adding a different therapeutic moiety permits the construction of transport proteins designed to carry a therapeutic agent within a neuron. See U.S. Patent No. 6,203,794 (hereby incorporated by reference herein). Altering the targeting (cell-binding) domain permits the toxin to be transported within pancreatic cells, such as acinar cells, thereby preventing secretion of activated digestive enzymes by such cells. See U.S. Patent No. 6,843,998 (hereby incorporated by reference herein), or sensory afferent neurons, thereby preventing neurotransmitter release and thus providing relief from pain; see U.S. Patent No. 6,395,513 (hereby incorporated by reference herein.)

[00027] In addition, US Patent No. 7,422,877 (hereby incorporated by reference herein) discloses the creation of chimeric neurotoxin derivatives comprising, for example, the binding domain and the translocation domain (or modified versions thereof) of one neurotoxin subtype for example, BoNT/A, and the light chain region of another neurotoxin subtype, for example, BoNT/E. It will be seen that given the general structural homology between the neurotoxin subtypes, any combination of the three basic Clostridial neurotoxin domains, may be made in a single amino acid chain (or in cleaved di-chain molecules). Therefore, for example, a binding domain from any of neurotoxin subtypes A, B, C1, D, E, F, G, or TeTX may be independently combined with a translocation domain from neurotoxin subtypes A, B, C1, D, E, F, G, or TeTX, and further independently combined with an endopeptidase domain from any of neurotoxin subtypes A, B, C1, D, E, F, G or TeTX. This can be done, for example, by recombinant construction and expression of a single chimeric chain which is subsequently cleaved to yield the dichain toxin, or by separate expression of single H and L chains, which are then combined by, for example, creation of an interchain disulfide bond and subsequently purified. Furthermore, using such techniques, the activity of various domains may be altered (for example, mutations can be introduced in an
LC domain to destroy the protease activity of the LC), or the naturally-occurring domains may be replaced with other moieties, as described elsewhere herein, where for example, the HC domain of BoNT/A (or a portion thereof) is mutated or deleted and a targeting ligand (TL) appended.

[00028] When discussing the three general neurotoxin domains of each Clostridial neurotoxin subtype (binding, translocation and endopeptidase), it will be understood that Clostridial neurotoxin research is a well-developed field, and the correlation of the amino acid sequences comprising each of these domains with their functions is well known. Additionally, the subdivision of these general domains into subdomains is also known. For example, the subdivision of binding domain Hc into subdomains HCN (the amino-terminal portion of the domain, corresponding approximately to amino acids 871-1091 of BoNT/A) and Hcc (the carboxy-terminal portion of the Hc domain, corresponding approximately to amino acids 1092-1296 of BoNT/A) is also well known. See e.g., Lacy DB and Stevens RC, Sequence Homology and Structural Analysis of the Clostridial Neurotoxins, 1999, J. Mol. Biol. 291:1091-1104. Subdomain HCN is highly conserved among botulinum toxin subtypes, however, little is known about its function. The Hcc subdomain is less conserved.

[00029] Additionally, the nucleotide and amino acid sequences of each of these domains and subdomains are known and have been disclosed in this specification, and therefore using this disclosure in combination with knowledge of the genetic code, nucleotide sequences encoding a protein to be expressed can be made. It would, of course, be a matter of routine for a person of ordinary skill in the art to immediately envision other nucleotide sequences encoding the indicated polypeptides. Also, due to the redundancy of the genetic code, a finite number of nucleotide sequences are possible for each polypeptide. Further, it is clear that nucleic acids can be synthesized that comprise conservatively modified variants of these nucleotide sequences (or unique portions of them) in the region of homology containing no more than 10%, 8% or 5% base pair differences from a reference sequence.

[00030] Further, it will be understood that the amino acid sequences set forth in Table 2 and elsewhere in this specification (SEQ ID NO: 1-8, 10, 12, 14, 16 and 18) provide
a full disclosure of any and all nucleotide sequences encoding these amino acid sequences and indicated regions thereof. A nucleotide sequence encoding an endopeptidase domain, translocation domain, or binding domain (including any subdomain) of a given neurotoxin subtype may respectively have 60% or greater, or 65% or greater, or 70% or greater, or 75% or greater, or 80% or greater, or 85% or greater, or 90% or greater, or 95% or greater, or 100% identity to any of such reference amino acid sequence regions listed in Table 2 and/or SEQ ID NO: 1-8, 10, 12, 14, 16 and 18.

**Statements of the Invention**

According to a general aspect of the invention, there is provided a composition comprising a Clostridial neurotoxin derivative, said composition comprising:

a) a first active Clostridial toxin-derived endopeptidase domain effective to cleave a SNARE protein under physiological conditions;

b) a Clostridial toxin-derived translocation domain effective to facilitate the movement of said first, and optionally a second, endopeptidase domains across a cellular membrane into the cytosol under physiological conditions; and

c) a non-Clostridial toxin derived binding domain comprising a first targeting ligand (TL) selectively binding, under physiological conditions, to a first cell surface receptor displayed by a target cell selected from the group consisting of a sensory neuron and a cell that secretes at least one inflammatory cytokine, and substantially not displayed by a motor or autonomic neuron; wherein at least a light chain protease of said Clostridial neurotoxin derivative is internalized by said target cell upon binding of said TL to the target cell, and wherein the neurotoxin derivative possesses a functional Clostridial neurotoxin-derived H$_{C_N}$ domain and lacks a functional Clostridial neurotoxin-derived H$_{C_C}$ targeting domain activity.

According to another general aspect of the invention, there is provided a composition comprising a Clostridial neurotoxin derivative, said composition comprising:

a) a first active Clostridial toxin-derived endopeptidase domain effective to cleave a SNARE protein under physiological conditions;

b) a second, different, Clostridial toxin-derived endopeptidase domain;
c) a Clostridial toxin-derived translocation domain effective to facilitate the movement of said first and second endopeptidase domains across a cellular membrane into the cytosol under physiological conditions; and
d) a non-Clostridial toxin derived binding domain comprising a first targeting ligand (TL) selectively binding, under physiological conditions, to a first cell surface receptor displayed by a target cell selected from the group consisting of a sensory neuron and a cell that secretes at least one inflammatory cytokine, and substantially not displayed by a motor or autonomic neuron; wherein at least a light chain protease of said Clostridial neurotoxin derivative is internalized by said target cell upon binding of said TL to the target cell, and wherein the neurotoxin derivative possesses a functional Clostridial neurotoxin-derived HCN domain and an Hcc targeting domain is either absent or is mutated to impede binding of the Hcc domain to its natural protein receptor.

According to yet another general aspect of the invention, there is provided an analgesic Clostridial neurotoxin derivative comprising:

a) A first active Clostridial toxin-derived endopeptidase domain effective to cleave a SNARE protein under physiological conditions and having an enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological conditions;
b) a Clostridial toxin-derived translocation domain effective to facilitate the movement of said first endopeptidase domain across a cellular membrane into the cytosol under physiological conditions; and
c) a binding domain comprising a first targeting ligand (TL) selectively binding, under physiological conditions, to a first cell surface receptor displayed by a target cell type selected from the group consisting of sensory neurons and cytokine secreting cells in preference to a non-target cell type selected from the group consisting of motor neurons and autonomic neurons; wherein said neurotoxin derivative lacks a functional Clostridial toxin HCC domain and wherein a target cell internalizes at least the endopeptidase domain of said Clostridial neurotoxin derivative upon binding of said TL to the target cell.

According to a first aspect of the invention, there is provided a composition comprising a Clostridial neurotoxin derivative, said composition comprising:
a) a first active Clostridial toxin-derived endopeptidase domain which cleaves a SNARE protein under physiological conditions;

b) a Clostridial toxin-derived translocation domain which facilitates the movement of said first endopeptidase domain across a cellular membrane into the cytosol under physiological conditions

c) a Clostridial toxin-derived functional HCC domain; and

d) a non-Clostridial toxin derived binding domain comprising a first targeting ligand (TL) selectively binding, under physiological conditions, to a first cell surface receptor displayed by a target cell, said target cell selected from the group consisting of

i) a sensory neuron, and

ii) a cell that secretes at least one inflammatory cytokine;

said cell surface receptor being substantially absent from motor or autonomic neurons;

wherein said light chain protease of said Clostridial neurotoxin derivative is internalized by said target cell upon binding of said TL to the target cell, and wherein an Hcc targeting domain is either absent or is mutated to impede binding of the Hcc domain to its natural protein receptor.

Optionally, the neurotoxin derivative lacks a Clostridial neurotoxin-derived Hcc targeting domain. Alternatively, the neurotoxin derivative contains a Clostridial neurotoxin-derived Hcc domain mutated to impede binding of the Hcc domain to its natural protein receptor.

According to one embodiment the mutated Hcc domain comprises a glutamic acid residue at a position corresponding to amino acid 1192 of BoNT/B. According to another embodiment the mutated Hcc domain comprises a lysine residue at a position corresponding to amino acid 1196 of BoNT/B. According to another embodiment the mutated Hcc domain also comprises a glutamic acid residue at a position corresponding to amino acid 1192 of BoNT/B.

According to one embodiment the mutated Hcc domain is derived from BoNT/B. According to another embodiment the mutated Hcc domain is derived from BoNT/B. According to another embodiment the mutated Hcc domain is derived from BoNT/B.
embodiment the mutated Hcc domain is derived from BoNT/B.

According to another embodiment the neurotoxin derivative comprises a second, different, Clostridial toxin-derived endopeptidase domain. According to one embodiment, the neurotoxin derivative comprises a second active Clostridial toxin-derived endopeptidase domain. According to one embodiment the neurotoxin derivative comprises a second Clostridial toxin-derived endopeptidase domain containing a mutation rendering it substantially proteolytically inactive. According to another embodiment the neurotoxin derivative comprises a second Clostridial toxin-derived endopeptidase domain derived from BoNT/A. According to another embodiment the first Clostridial toxin-derived endopeptidase domain comprises an endopeptidase derived from BoNT/E. According to another embodiment the Clostridial toxin-derived translocation domain is derived from a BoNT/X subtype selected from the group consisting of BoNT/A, BoNT/Cl, BoNT/D and BoNT/E. According to another embodiment the Clostridial toxin-derived translocation domain is derived from a BoNT/X subtype selected from the group consisting of BoNT/A, BoNT/Cl, BoNT/D and BoNT/E.

According to another embodiment the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP-selective or CGRP-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof.

According to another embodiment the composition of the invention comprises a polypeptide comprising an active Clostridial toxin-derived endopeptidase domain comprising an endopeptidase derived from BoNT/E, a Clostridial toxin-derived translocation domain is derived from BoNT/A, and a TL domain.

According to another embodiment the TL comprises a targeting component comprising a CGRP antagonist.
According to another embodiment said Clostridial toxin-derived endopeptidase domain comprises an endopeptidase having an enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological conditions.

According to another embodiment the first or second Clostridial toxin-derived endopeptidase domain comprises an endopeptidase having an enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological conditions.

According to another embodiment the neurotoxin derivative of the invention comprises a BoNT/D light chain, a BoNT/D translocation domain, and a targeting ligand comprising CGRP$_{8-37}$, and lacking a functional Hcc domain.

According to another embodiment the neurotoxin derivative of the invention contains a Clostridial neurotoxin-derived HCC domain mutated to impede binding of the Hcc domain to its natural protein receptor.

According to another embodiment the neurotoxin derivative of the invention comprises BoNT/D(-Hcc)-CGRP$_{8-37}$.

According to another embodiment the neurotoxin derivative of the invention comprises a BoNT/D light chain, a BoNT/D translocation domain, and a targeting ligand comprising human IL-IRA, and lacking a functional Hcc domain.

According to another embodiment the neurotoxin derivative of the invention contains a Clostridial neurotoxin-derived Hcc domain mutated to impede binding of the Hcc domain to its natural protein receptor.

According to another embodiment the neurotoxin derivative of the invention comprises BoNT/D(-Hcc)-human IL-IRA.

According to another embodiment the neurotoxin derivative of the invention comprises a BoNT/A light chain, a BoNT/A translocation domain, and a targeting ligand comprising a
purinergic receptor ligand, and lacking a functional HCC domain.

According to another embodiment the targeting ligand is a P2X3 receptor ligand. According to another embodiment the targeting ligand comprises a purotoxin 1 or a selectively binding fragment thereof.

According to another embodiment the neurotoxin derivative of the invention comprises LC.H$_N$.HC$_N$/A-PT- 1.

According to another embodiment the TL specifically binds, under physiological conditions, to a first cell surface receptor displayed by a sensory neuron in preference to motor or autonomic neurons.

According to another embodiment the neurotoxin derivative of the invention comprises at least two TL domains.

According to another embodiment the neurotoxin derivative of the invention comprises a Clostridial neurotoxin translocation domain is selected from the group consisting of

a) a BoNT-A translocation domain;

b) a BoNT-B translocation domain;

c) a BoNT-Cl translocation domain;

d) a BoNT-D translocation domain;

e) a BoNT-E translocation domain;

f) a BoNT-F translocation domain;

g) a BoNT-G translocation domain, and

h) conservatively modified variants and isoforms of any of the above.

According to a second aspect of the invention, there is provided an analgesic Clostridial neurotoxin derivative comprising:

a) A first active Clostridial toxin-derived endopeptidase domain which cleaves a SNARE protein under physiological conditions and has an enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological
conditions;
  b) a Clostridial toxin-derived translocation domain which facilitates the movement of
  said first endopeptidase domain across a cellular membrane into the cytosol under
  physiological conditions;
  c) a Clostridial toxin-derived functional $H_G \bar{N}$ domain; and
  d) a binding domain comprising a first targeting ligand (TL) selectively binding,
  under physiological conditions, to a first cell surface receptor displayed by a target cell type
  selected from the group consisting of:
    i) sensory neurons, and
    ii) cytokine secreting cells
  in preference to a non-target cell type selected from the group consisting of motor neurons
  and autonomic neurons;

  wherein said neurotoxin derivative lacks a functional Clostridial toxin Hcc domain and
  wherein a target cell internalizes said first endopeptidase domain of said Clostridial
  neurotoxin derivative upon binding of said TL to the target cell.

  Optionally, the neurotoxin derivative contains a Clostridial neurotoxin-derived Hcc domain
  mutated to impede binding of the Hcc domain to its natural protein receptor. According to
  one embodiment the neurotoxin derivative of the invention comprises an active $H_G \bar{N}$ domain.

  Optionally, said first active Clostridial toxin-derived endopeptidase domain is derived from a
  toxin serotype selected from the group consisting of BoNT/ A, BoNT/ E, BoNT/ Cl, and
  BoNT/ D.

According to another embodiment of the invention the Clostridial neurotoxin translocation
domain is selected from the group consisting of
  a) a BoNT-A translocation domain;
  b) a BoNT-B translocation domain;
  c) a BoNT-Cl translocation domain;
  d) a BoNT-D translocation domain;
  e) a BoNT-E translocation domain;
  f) a BoNT-F translocation domain;
g) a BoNT-G translocation domain, and
h) conservatively modified variants and isoforms of any of the above.

According to one embodiment the endopeptidase domain and the first active endopeptidase domain are both derived from the same BoNT serotype.

According to one embodiment the neurotoxin derivative of the invention comprises a second Clostridial toxin-derived endopeptidase domain. Optionally, the second Clostridial toxin-derived endopeptidase domain retains active endopeptidase protease activity. Optionally, the second Clostridial toxin-derived endopeptidase domain lacks endopeptidase protease activity effective to substantially cleave a population of SNARE proteins under physiological conditions.

According to another embodiment the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP-selective or CGRP-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof, and conservatively modified variants and isoforms of any of the above.

Optionally, the neurotoxin derivative comprises at least two TL domains.

According to one embodiment said first endopeptidase domain and said translocation domain are both derived from BoNT/D. According to another embodiment the neurotoxin derivative of the invention said first endopeptidase domain and said translocation domain are both derived from BoNT/A. According to one embodiment said first endopeptidase domain and said translocation domain are both derived from BoNT/Cl. Optionally, the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP-selective or CGRP-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific
antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof, and conservatively modified variants and isoforms of any of the above.

According to a third aspect of the invention, there is provided a nucleic acid encoding a polypeptide comprising the Clostridial neurotoxin derivative of the invention.

According to a fourth aspect of the invention, there is provided a host cell selected from the group consisting of a bacterial host cell, yeast, a mammalian cell, and an insect cell, wherein the host cell contains a nucleic acid vector comprising the nucleic acid the invention.

According to all aspects of the invention, ideally the target cell is a cell that secretes at least one inflammatory cytokine, wherein said cell is selected from the group consisting of a macrophage, a synoviocyte, and a mast cell.

According to all aspects of the invention, ideally the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP receptor-selective or CGPRP receptor-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptorspecific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof, and conservatively modified variants and isoforms of any of the above.

According to a fifth aspect of the invention, there is provided a cleared cell lysate comprising the Clostridial neurotoxin derivative of the invention.

According to a sixth aspect of the invention, there is provided a compound comprising the Clostridial neurotoxin derivative of the invention for use in the treatment of chronic pain.

According to a seventh aspect of the invention, there is provided a Clostridial neurotoxin derivative comprising: a) a first active Clostridial toxin-derived endopeptidase domain which
cleaves a SNARE protein under physiological conditions and has an enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological conditions; b) a second active or inactive Clostridial toxin-derived endopeptidase domain; c) a Clostridial toxin-derived translocation domain which facilitates the movement of said first endopeptidase domain across a cellular membrane into the cytosol under physiological conditions; and d) a Clostridial toxin-derived functional HCC domain.

Brief Description of the Drawings

[00031] Fig. 1 is a diagram showing various general embodiments of the present invention, beginning from a BoNT/X dichain toxin (which may be created by proteolytic cleavage of a recombinant single chain (SC) toxin), and showing pathways A or B, respectively. These involve the excision from BoNT/X of the Hcc subdomain (pathway A), or mutation of the Hcc subdomain (pathway B), and the addition of a targeting ligand (TL) selective to bind to sensory neurons and/or non-neuronal cells to block release of affectors in the chronic pain and/or inflammatory pathways to form novel genera of therapeutic biologies BoNT/X(Hcc)-TL and BoNT/X(PrR)-TL. A member of either genus may be further modified as shown in pathway C by the addition of an active type E light chain protease domain (LC/E) to extend the time period of SNARE protein proteolysis; see LC/E-BoNT/X(-Hcc)-TL and LC/E-BoNT/X(PrR)-TL. For example, in the first instance, key residues (Lys\textsuperscript{1192} and/or Ala\textsuperscript{1196} in Hcc of BoNT/B) identified as being essential for binding to its protein receptor (synaptotagmin) could be mutated to Glu\textsuperscript{1192} and Lys\textsuperscript{1196} to ablate their interaction, as data have shown a greater than 300-fold drop of its neuromuscular paralytic activity upon mutation of either of the key residues (Rummel \textit{et al.}, Proc. Nat. Acad. Sci. USA, 2007, 104:359-364 and Jin \textit{et al.}, Nature, 2006, 444:1092-1095). Those of ordinary skill in the art will be aware that an optional second active light chain protease derived from Clostridial serotypes other than type E may alternatively be used.

[00032] Fig. 2A shows schematic diagrammatical structures of recombinant BoNT/A (rA) and a variant (\textit{rA} (\textit{\text坦^4,_{N}})) lacking the HCC subdomain. Both structures illustrate a C-terminal 6 residue histidine tag (H\textsubscript{6}) (SEQ ID NO: 19), appended during the construction of the recombinant nucleic acid to aid in purification of the protein by immobilized metal
affinity chromatography (IMAC), as well as two thrombin cleavage sites, also inserted during
the construction of the recombinant nucleic acid. The first (leftmost) thrombin cleavage site
permits post-expression proteolytic conversion of single chain to the active di-chain form; the
second thrombin site permits the removal of the ⅔ "tag" (SEQ ID NO: 19) after purification
of the polypeptide.

[00033] Fig. 2B shows the purification from a cell lysate of rA ⅔ N by IMAC
on a Coomassie blue-stained SDS-PAGE gel.

[00034] Fig. 2C shows a Coomassie blue-stained SDS-PAGE gel of IMAC-
purified SC and DC proteins under reducing and non-reducing conditions.

[00035] Fig. 3A shows Western blots of SDS-PAGE gels. The lanes of the two
blots on the right are samples of eluates in binding assays between an immobilized
recombinantly expressed fragment of the BoNT/A protein receptor (SV2C) and recombinant
proteins BoNT/A (rA), rA HCN, and rE (recombinant BoNT/E). Two blots on the left are
samples of rA, rA HCN and rE (40 ng/lane) to confirm the specificity of antibodies used.
Blots were developed using antibodies against the light chain of either BoNT/A (anti-LC/A)
or BoNT/E (anti-LC/E).

[00036] Fig. 3B shows Western blots of SDS-PAGE gels in which rat
cerebellar granule neurons (CGNs) pre-treated with varying concentrations (expressed in the
upper axis legend in units of picomolarity (pM)) of either toxin derivative rA or rA HCN. The
gels show protein bands comprising the SNARE proteins syntaxin 1, SNAP-25, or SNAP-
25A (a cleavage product of BoNT/A digestion). The results show that, as expected, the non-
target SNARE syntaxin 1 is not cleaved by either toxin protein; SNAP-25 is only digested by
rA (and not by rA HCN, except at very high concentrations (1 nM) of the toxin). The results
suggest the inability of rA HCN to effectively internalize into the CGN cells.

[00037] Fig. 3C is a table showing the toxicity of rA and rAT4 N upon
intraperitoneal injection of each toxin into mice, expressed as mLDso - the lowest dose of
toxin sufficient to kill 50% of injected mice within 4 days. The table indicates that rA has an
mLD_{50} \text{6.7} \times 10^4 \text{times greater than does rA}_N \text{ in this experiment.}

[00038] Fig. 4 shows a diagrammatic view of various exemplary targeting ligands (TLs) that may be combined with individual members of the toxin-derived therapeutic genera shown in Fig. 1 to make therapeutic embodiments of the invention targeted to pain-sensing nerves or to non-neuronal cells that secrete inflammatory mediators and contribute to pain.

[00039] Fig. 5A shows schematic diagrammatical structures of recombinant proteins LC.H_N/A-PT-1 and LC.H_N/HC_N/A-PT-1; both proteins contain a C-terminal portion comprising a His_{6} tag (SEQ ID NO: 19) for use in affinity purification, and a purotoxin-1 (PT-1) fragment for use as a targeting ligand (TL) of the toxin to the P2X purinoreceptor 3 receptor of purinergic neurons. The LC.H_N/A-PT-1 protein comprises recombinant BoNT/A lacking the entire He region; the LC.H_N/HC_N/A-PT-1 protein comprises the LC.H_N/A-PT-1 plus the BoNT/A \text{34}_N region (but not the Hcc region) linked to the C terminus thereof, and thence to the TL-His_{6} portion ("Hise" disclosed as SEQ ID NO: 19). A single thrombin cleavage site separates LC/A from the remainder of the chain, although it remains linked by the disulfide bond.

[00040] Fig. 5B shows reducing and non-reducing Coomassie blue-stained SDS-PAGE gels of lysate (1), flow-through (2), wash (3) and eluate (4-8) fractions of IMAC chromatography of E. coli cells expressing LC.H_N/HC_N/A-PT-1.

[00041] Fig. 5C shows reducing and non-reducing Coomassie blue-stained SDS-PAGE gels of lysate (1), flow-through (2), wash (3) and eluate (4-7) fractions of IMAC of E. coli cells expressing LC.H_N/A-PT-1.

[00042] Fig. 6A shows the results of a Coomassie blue-stained SDS-PAGE gel (left) of purified LC.H_N/HC_N/A-PT-1 under reducing and non-reducing conditions, and Western blots of the same sample under reducing and non-reducing conditions developed using anti-His_{6} antibody (middle) ("Hise" disclosed as SEQ ID NO: 19), or anti-LC/A antibody (right). The gels show that under non-reducing conditions the protein exists as a
disulfide-linked dichain; when reduced, the dissociated LC/A can be visualized with anti-
LC/A antibody, and the remainder of the recombinant toxin can be seen with the anti-His$_6$
antibody ("His$_6$" disclosed as SEQ ID NO: 19).

Fig. 6B shows the results of a Coomassie blue-stained SDS-PAGE gel
(left) of purified LC.H$_N$/A-PT-1 under reducing and non-reducing conditions, and Western
blots of the same sample developed using anti-His6 antibody (middle) ("Hise" disclosed as
SEQ ID NO: 19), or anti-LC/A antibody (right). The gels show that under non-reducing
conditions the protein exists as a disulfide-linked dichain; when reduced, the dissociated
LC/A can be visualized with anti-LC/A antibody, and the remainder of the recombinant toxin
can be seen with the anti-His6 antibody ("Hise" disclosed as SEQ ID NO: 19).

Fig. 6C shows Western blots of SDS-PAGE gels in which rat
trigeminal ganglionic neurons (TGNs) were pre-treated with 1.6 nM of either toxin derivative
LC.H$_N$/A-PT -1 (left) or LC.H$_N$HC$_N$/A-PT -1, then lysed and electrophoresed. The gels show
protein bands comprising the SNARE proteins SNAP-25 (upper band), or the LC/A cleavage
product SNAP-25A (lower band on right Western blot). The results show that SNAP-25 is
only digested by LC.H$_N$HC$_N$/A-PT -1, (and not by LC.H$_N$/A-PT-1). The results are consistent
with the ability of LC.H$_N$HC$_N$/A-PT-1 to effectively bind to enter the TGN cells.

Fig. 6D is a graph showing the results of the assay shown visually in
Fig. 6C. The percentage of SNAP-25 cleaved by 1.6 nM of LC.H$_N$HC$_N$/A-PT -1 is about
25%, and the percentage of SNAP-25 cleaved by LC.H$_N$/A-PT-1 is approximately 0%.

Fig. 7 shows Western blots of SDS-PAGE gels from lysates of: human
synovial cell line (hSC) (which contains SNAP-23, VAMP3, syntaxin 2, 3 and 4 as found in
mouse macrophase cells line RAW264.9 (mMC) and neural cells such as rat cerebellar
ganglia neurons (rCGNs), which contain the SNARE proteins SNAP-25, syntaxin 1, and
VAMP 2. Western blots were developed using antibodies directed against in the indicated
SNARE proteins.

Fig. 8A is a Western blot showing the results of an experiment in
which hSC cells were incubated for 7-10 days with shRNA (small hairpin RNA) lentivirus carrying nucleotide sequences specifically targeting SNAP-23. The cells were then incubated overnight with IL-1β (100 ng/ml) in complete culture medium to induce secretion of TNF-α and IL-6. After collecting the supernatant, lysates from these cells were subjected to SDS-PAGE and proteins detected using antibodies directed to SNAP-23 or the untargeted control protein β-tubulin. KD stands for shRNA-induced i knock down! or inhibition of expression.

[00048] Fig 8B is a graphical representation of the inhibition of expression (expressed as percentage "knock down" or KD) of SNAP-23 in the experiment shown in Fig. 8A. Also shown is the percent inhibition of secretion of TNF-α and IL-6 from these cells before lysis, relative to supernatant from a control cell culture not treated with the lentivirus vector. Note, the quantification of secreted TNF-α and IL-6 was performed using enzyme-linked immunosorbent assay (ELISA) according to a protocol provided by Mabtech AB (Sweden).

[00049] Fig. 8C is a Western blot showing the results of an experiment in which hSC cells were incubated for 7-10 days with shRNA (small hairpin RNA) lentivirus carrying nucleotide sequences specifically targeting VAMP 3. The cells were then incubated overnight with IL-1β (100 ng/ml) in complete culture medium to induce secretion of TNF-α and IL-6. After collecting the supernatant, lysates from these cells were subjected to SDS-PAGE and proteins detected using antibodies directed to VAMP 3 or the untargeted control protein β-tubulin. KD stands for shRNA-induced i knock down! or inhibition of expression.

[00050] Fig 8D is a graphical representation of the inhibition of expression (expressed as percentage i knock down! or KD) of VAMP 3 in the experiment shown in Fig. 8C. Also shown is the percent inhibition of secretion of TNF-α and IL-6 (quantified by ELISA as described in Fig. 8B) from these cells before lysis, relative to supernatant from a control cell culture not treated with the lentivirus vector.
DESCRIPTION OF THE FIELD

[00051] Chronic pain is a major challenge for patients and health providers alike. Patients suffering from chronic pain represent approximately 20% of the adult population.

[00052] There are two general types of chronic pain: inflammatory nociceptive pain and neuropathic pain. Inflammatory nociceptive pain usually arises from an insult to tissue and the resultant activation of inflammatory cascades and chemoreceptors. On the other hand, neuropathic pain (for example, without limitation, chronic pain, such as cancer pain, post-operative pain, neuropathic pain, arthritis pain, allodynia, post-herpetic neuralgia, irritable bowel syndrome, and other visceral pain, bone pain, peripheral neuropathy, circulatory system-affiliated pain, and some types of headache pain) results from neuronal damage in the peripheral or central nervous systems and involves sensitization (such as allodynia), i.e. increased stimulation of peripheral nociceptors that amplifies pain signals relayed to the brain.

[00053] There remains an unmet need for effective treatment of chronic pain because non-steroidal anti-inflammatory drugs, traditionally used for inflammatory nociceptive pain, are short-acting and can have serious side effects. Similarly, while pain involving an inflammatory nociceptive mechanism usually is limited in duration to the period of tissue repair and generally is relieved by available analgesic agents or opioids (Myers, REGIONAL ANESTHESIA 20:173-184 (1995)), the side and deleterious effects of long-term treatment with opioids is well known.

[00054] Likewise, despite the fact that approximately 3% of the population suffers from neuropathic pain at any given time, there is no satisfactory existing treatment; available therapies work poorly, are not effective for a significant segment of patients, or cause unacceptable adverse effects.

[00055] Encouragingly, at least some sufferers of chronic pain respond to the long-acting botulinum neurotoxin (BoNT) type A (one of 7 toxin serotypes (/A-G) produced...
by *Clostridium botulinum*) due to specific and persistent inhibition of the release of transmitters from peripheral nerves. This blockade results from the proteolytic cleavage of the SNARE proteins; proteins essential for Ca\(^{2+}\)-stimulated exocytosis of neurotransmitters and other agents via membrane-vesicle fusion.

[00056] The unique profile of activities provided by Clostridial neurotoxins (detailed below) has been exploited successfully for treating numerous human disorders (-100 conditions) arising from over-activity of nerves innervating skeletal/smooth muscles or glands; reviewed in Ward, A.B. & Barnes, M.P., *CLINICAL USES OF BOTULINUM TOXINS*, Cambridge University Press (2007).

[00057] *In vivo*, Clostridial bacteria produce a toxin complex (the ihemagglutinin complex!) that comprises the approximately 150-kDa di-chain Clostridial toxin along with other proteins. These other, non-toxin proteins are collectively called nontoxic associated proteins (NAPs). Identified NAPs include proteins possessing hemaglutination activity, such, e.g., a hemagglutinin of approximately 17-kDa (HA-17), a hemagglutinin of approximately 33-kDa (HA-33) and a hemagglutinin of approximately 70-kDa (HA-70); as well as a non-toxic non-hemagglutinin (NTNH), a protein of approximately 130-kDa, see, e.g., Eric A. Johnson and Marite Bradshaw, *Clostridial botulinum and its Neurotoxins: A Metabolic and Cellular Perspective*, 39 ToxICON 1703-1722 (2001); Stephanie Raffestin *et al.*, *Organization and Regulation of the Neurotoxin Genes in Clostridium botulinum and Clostridium tetani*, 10 Anaerobe 93-100 (2004) and Gu *et al.*, Botulinum Neurotoxin is Shielded by NTNHA in an Interlocked Complex, 335 Science 977-81 (2012).

[00058] In nature, the toxin complex is believed to be important for the intoxication process at least in part because it appears to provide protection to the toxin molecule from adverse environmental conditions and resistance to protease digestion. Importantly, certain domains of the HA and NTNHa proteins appear to coordinate with toxin binding and bind to locations on the cell surface (and may bind to the natural Clostridial neurotoxin cell surface receptor at sites other than or additional to the toxin binding site), thus facilitating binding, internalization, and activation of the toxin.
BoNT/A (and, to a lesser degree, BoNT/B) hemagglutinin complexes are presently in clinical use for a variety of medical conditions. All 7 BoNT serotypes contain a light chain protease domain (LC), which is linked to a heavy chain cell-binding and transport domain (HC) through a single disulfide bond and non-covalent bonds. A C-terminal moiety of HC (He) binds to the specific acceptors expressed on various nerve types (including motor, autonomic and sensory neurons), whereas the N-terminal half of HC (¾) forms a channel that allows the attached LC to translocate from 'endosomal-like' membrane vesicles through the H₅ pore into the cytosol (Dolly et al., CURR. OPIN. PHARMACOL. 9:326-35, 2009). Thereafter, with a selectivity depending on the toxin serotype, the LC cleaves a specific SNARE substrate and negates its role in neurotransmitter release. For example, the LC of BONT/A (LC/A) removes 9 amino acids from the C-terminal of the SNARE protein SNAP-25, whereas the LC/E deletes a further 17 C-terminal residues from the same SNARE and, thus, gives a more disruptive blockade of neuro-exocytosis; Meng et al., J. NEUROSCI. 29:4981-4192 (2009) (hereinafter iMeng et al. 2009i). Other Clostridial toxins cleave other SNARE proteins: for example, and without limitation, BoNT/C cleaves the SNARE proteins SNAP-25 and syntaxin 1, and TeTx, BoNT/B, BoNT/D, BoNT/F, and BoNT/G cleave the SNARE protein synaptobrevin (also known as VAMP). An example of this selective disruption, the inhibition of transmitter release by LC/A can usually be reversed, at least transiently, by elevating Ca²⁺ influx, but not to such an extent in the case of LC/E (Dolly et al., FEBS J. 278:4454-66, 2011). However, the short transient paralysis induced by LC/E limits its usefulness in clinical applications.

BoNT/A hemagglutinin complex (iBoNT/A complex!) has been found to be effective in some, but not all, migraine sufferers; see e.g., Naumann et al., NEUROLOGY 70:1707-1714 (2008), Jackson et al., JAMA 307:1736-1745 (2012), and Dodick et al, Headache 50:921-936 (2010). Moreover, BoNT/A is unable to block the release of pain-mediating peptides (such as calcitonin gene-related peptide (CGRP) and substance P) from sensory neurons when elicited by activating TRPV1 (transient receptor potential vanilloid type 1), non-selective cation channels which are sensitive to capsaicin (Meng et al., 2009; Meng et al, J. Cell Sci. 120:2864-2874 (2007)(hereinafter Meng et al, 2007).
A chimeric Clostridial neurotoxin derivative comprising BoNT/E LC protease (LC/E) and translocation domain (H₃/E) synthetically retargeted via the He of type A (Wang et al, J. BIOL. CHEM. 283:16993-17002 (2008)(hereinafter Wang et al, 2008 )), potently blocks CGRP release from nociceptive neurons and attenuates their firing elicited by CGRP or TRPV1 -activation (Meng et al 2009). Furthermore, a synthetic variant of LC/E protease engineered to be long-acting by appending the more robust LC/E protease to a mutated inactive form of the long-lived protease in BoNT/A has proved to be a very effective therapeutic both in cultured sensory neurons and in an animal model of inflammatory pain (Wang et al, J. BIOL. CHEM. 286:6375-85, 2011) (hereinafter "Wang et al, 2011").

Additionally, another long-acting toxin serotype, BoNT/C 1, blocks CGRP release from sensory trigeminal ganglionic neurons (TGNs) establishing its anti-nociceptive potential (Meng et al 2007). Although BoNT/D (having a long-lasting protease activity) is also effective in blocking CGRP release, it cannot be used as therapy for human patients in its wild-type form because BoNT/D does not bind human muscle or block neurotransmission; Coffield et al, J. PHARMACOL. EXP. THER. 280:1489-1498 (1997) (hereinafter "Coffield et al, 1997"). This notable finding highlights some difference between the response to BoNT/D in human and rodent because one group has recently claimed that the natural BoNT/A receptor synaptic vesicle protein 2 (SV2), can act as a protein receptor for BoNT/D in rat and mouse (Peng et al, PLoS PATHOGENS 7:e1002008, 2011).

However, all these BoNT variants suffer from the disadvantage of non-selectivity; they block the release of transmitters and mediators from motor and autonomic nerves, as well as sensory neurons. This lack of specificity could lead to serious side effects in clinical use for treatment of pain.

DESCRIPTION AND EXAMPLES

The present invention is directed to methods and compositions having several aspects and embodiments which are encompassed by the claims. Thus, and without limitation, in one embodiment the present invention is directed to novel biotherapeutics for the treatment of chronic and/or inflammatory pain; such agents may be designed by ablating
the normal tropism of Clostridial neurotoxins and their derivatives. Specifically, the biologies may be retargeted by deleting or modifying Hcc which possesses binding sites for the protein and ganglioside receptors, and appending one of several possible targeting ligands (hereinafter referred to as "TL")s to the C terminus of the HC. The TLs are preferably chosen to restrict the action of Clostridial toxin derivatives selectively to pain-sensing neurons, thereby leaving other neuron types unaffected. Retention of the HCN second subdomain is a novel advance due to our discovery of its importance for internalization of the LC of the toxin into neurons (or non-neural cells), with subsequent cleavage of SNAP-25 and inhibition of exocytosis; see, e.g., Fig. 2, Fig. 3 and Example 1.

[00065] As shown in Fig. 1 and described herein, in certain embodiments of the invention both of the He subdomains (HCN and Hcc) are retained (including the ganglioside binding region(s) of Hcc), but the ability to bind the protein receptor, e.g. synaptotagmin, is ablated by mutating residues identified as being essential for the latter interaction. For example, mutating either Lys1192 to Glu or Ala1196 to Lys in BoNT/B Hcc decreased its potency by >300-fold on neuromuscular junction (Rummel et al., PROC. NAT. ACAD. SCI. USA 104:359-64, 2007; Jin et al., NATURE444:1092-95, 2006). Thus this strategy has the advantage of exploiting the neurotoxin's ability to bind a "dual receptor" for TL to more effectively bind its target; binding to the gangliosides through the mutated Hcc portion appears to increase the local concentration of the therapeutic at the cell surface near putative protein receptors and, thus, enhance interaction of each TL with its own requisite protein ectoreceptor on the target cell surface. Thus, the efficacy of the therapeutic is increased by this dual binding modality.

[00066] In a third non-limiting set of embodiments also shown in Fig. 1, additional novel therapeutics may be made by creating proteins recombinantly in which a second active or inactive light chain protease, such as the active light chain protease of BoNT/E (a robust inhibitor of neuro-exocytosis) is attached to BoNT/A or one or more of the constructs described above. This may exert a stabilizing influence to yield a long-lasting protease activity on target SNARE proteins. The ability to make and use such a selective and long-acting family of biotherapeutics represents a milestone advance that should revolutionize the development of future generations of effective and selective drugs for
chronic and inflammatory pain.

[00067] As used herein the term "specific", when used with regard to ligand:target interactions, means that the ligand preferentially binds and/or catalyzes the target with an avidity of at least 10^2:1, 10^3:1, 10^4:1, or at least about 10^5:1 or at least about 10^6:1 over non-target substances under substantially physiological conditions. The term "selective", when used with regard to ligand:target interactions, means that the ligand preferentially binds and/or catalyzes the target with an avidity of 10:1, or at least about 10^2:1, or at least about 10^3:1 or up to 10^4:1 over non-target substances under substantially physiological conditions.

[00068] Thus, in one aspect, the present invention concerns the design, preparation, and use of one or more gene constructs encoding polypeptides comprising analgesic core therapeutics that inhibit neurotransmission (SNARE-selective proteases) and possess an analgesic activity. These may include, consist of, or consist essentially of, without limitation, derivatives of TeTx or BoNT/X (serotypes A, B, CI, D, E, F or G), but either contain a mutated Hcc (substantially lacking the ability to bind the protein receptor but capable of interacting with gangliosides, see above) or are substantially devoid of the Hcc region (see Fig. 1).

[00069] In another, supplementary approach, a second active LC coding region, such as the LC/E coding region, which encodes a protease that acts as an effective inhibitor of CGRP release from sensory neurons (see, e.g., Want et al., 2011; Meng et al., 2009) may be joined to one or more of the above-mentioned oligonucleotide candidates, preferably prior to the next step.

[00070] In some embodiments, the oligonucleotide may comprise an LC/X that is mutated so as to substantially lack neuronal SNARE-selective protease activity compared to the expressed unmutated protein. For example, mutating residue Lys^{224} in LC/E moiety to Asp significantly increased its cleavage to human SNAP-23 with reduced activity towards neuronal SNAP-25 (Chen and Barbierio, Proc. Nat. Acad. Sci. USA, 106:9180-9184, 2009). In such a case, it will be understood that such an oligonucleotide can be referred to using the
nomenclature mLC.BoNT/X (where iXi is any toxin serotype) generally refers to a BoNT/X in which the light chain protease has been mutated to have substantially no proteolytic activity towards neuronal SNARE proteins, while substantially maintaining the steric structure of the original BoNT/X toxin.

[00071] In either event, attachment of a gene encoding the requisite TL to these molecules will endow the translated polypeptide with the ability to selectively target sensory neurons or cytokine-releasing cells (shown diagrammatically in Fig. 1). Clearly, one of the advantages of the present invention is that it provides an array of different gene constructs, from which may be designed or chosen to fit one or more construct expressing therapeutic proteins possessing the capacity to effectively inhibit the release of pain mediators from nociceptors or cells releasing inducers of inflammation.

[00072] The invention, thus, also concerns the therapeutic Clostridial proteins produced using the oligonucleotides, methods of making the oligonucleotides and proteins, methods for the in vivo and/or in vitro expression of proteins encoded by these constructs, the purification of such proteins, and assays for their activity and physiochemical characterization, as well as methods to treat a patient suffering from, or at risk of suffering from chronic or inflammatory pain employing such proteins.

[00073] In a preferred embodiment, the construction of gene constructs according to the invention entails the steps (not necessarily in this order) modifying a nucleic acid encoding a single chain BoNT/X (for example, serotype /A, /B, /CI, /D, /E, /F and /G, or chimeric toxins comprising fragments from a plurality of toxin subtypes), removing or mutating the heavy chain Hcc region (see above) and linking of a targeting ligand (TL) selective for sensory neurons or inflammation-mediating cells. Depending upon the identity of the toxin serotype, the active LC of a more robust BoNT serotype (such as LC/E) can be appended to the LC/A to extend its longevity. These approaches are shown diagrammatically in Fig. 1.

[00074] Targeting ligands used in the present invention act to selectively direct the therapeutic biologic molecules of the present invention to sensory neurons and/or cells
capable of secreting inflammation-mediating factors. Thus, for example, a nucleic acid encoding a TL is attached to the 3′ end of a nucleic acid encoding a modified Clostridial toxin, such as one of those constructs described above, in order to permit the expressed proteins to selectively bind protein receptors of the nociceptive C-fibers, which are involved in chronic pain pathways. Exemplary TLs exhibiting the required selectivity against sensory neurons may include, without limitation: purotoxin-1 (PT-1) (an antagonist of the P2X3 purinergic receptor) or active fragments thereof; and antibodies or antibody fragments (such as single-chain variable fragments (scFv) of antibodies) reactive with P2X3 or transient receptor potential vanililoid receptor 1 (TRPV1).

[00075] With respect to the purotoxin 1 receptor P2X3, this receptor is selectively expressed on sensory fibers of primary afferent neurons as both homo- and hetero-trimeric membrane channels, some of which are also sensitive to capsaicin (North RA, J. PHYSIOL. 554, 301-308, 2004). Peripheral nerve injury has been reported to alter the functional expression of P2X3 (Brederson et al., CURR. OPIN. INVESTIG. DRUGS 9, 716-725, 2008). Moreover, up-regulation of P2X3 receptor occurs during stretch of bladder urothelial cells (Sun and Chai. J. Urol. 171:448-452, 2004).

[00076] Importantly, inhibition of the P2X3 receptor's activity relieves the symptoms of inflammatory and neuropathic pain (North RA, J. PHYSIOL. 554, 301-308, 2004; Burnstock, G. PHARMACOL. THER. 110, 433-454, 2006). The beneficial anti-nociceptive effects of P2X3 antagonists, and characteristics of P2X3 knock-out mice, highlight a role in inflammatory and neuropathic pain for the neural fibers that express this receptor (Cockayne et al, NATURE 407, 101 1-1015, 2000). Hence, a TL for use as a targeting element of an embodiment of the present invention may comprise the specific affinity of a P2X3 antagonist, PT-1 or derivative thereof. This P2X3 antagonist is derived from the central Asian spider Geolycosa (Grishin et al., ANN. NEUROL. 67, 680-683, 2010). The nucleic acid encoding this TL may comprise, consist essentially of, or consist of nucleic acid encoding a 35 residue peptide (SEQ. ID. No: 10); the amino acid sequence of this 35-residue peptide is disclosed herein as SEQ. ID. No. 9.

[00077] As disclosed in Example 2 of this application, an embodiment of this
TL has been prepared by recombinant means in active form; and may be used to target the
therapeutic biological molecules of the present invention selectively to neurons carrying the
purotoxin 1 receptor P2X3. In this way a selective, long-lasting analgesic may be made and
used that lacks the adverse effects and addictive properties of conventional chronic pain
medications. These therapeutics also offer the major advantage that they do not affect, or do
not substantially affect the secretion of cholinergic neurotransmitters in neuromuscular and
autonomic nerves, unlike the unmodified BoNTs.

[00078] In other embodiments of the invention, a TL based upon the human
CGRP (calcitonin gene-related peptide) protein fragment CGRPs-37, an antagonist of the cell
surface receptor CGRP receptor 1, may be used as a targeting ligand to direct the therapeutic
of the present invention to sensory neurons and/or non-neuronal cells that secrete
inflammatory mediators. Also useful as a TL for targeting the biotherapeutics to the latter
cells, genes encoding the human interleukin-1 receptor antagonist (IL-1RA) (or a selectively
functional derivative thereof) may also be employed in constructing the expressible nucleic
acid.

[00079] Additional or alternative TLs may comprise, consist essentially of, or
consist of single-chain antibodies (or derivatives thereof), or other ligands capable of
selective binding to TRPV1 or P2X3 receptors; for example, a bivalent tarantula toxin by
targeting the outer pore domain of TRPV1 (Bohlen et al, Cell. 141:834-845, 2010). Thus,
targeting the biotherapeutic molecule to nociceptive neurons by exploiting the receptors'
presence on these neurons (e.g. in TGNs) (Meng et al, 2007) and their roles in signaling of
chronic inflammatory and neuropathic pain (North RA J. PHYSIOL. 554, 301-308, 2004). An
attractive feature of TRPV1 targeting is that the trafficking of this cation-channel protein to
the plasma membrane is partly SNARE-dependent, involving protein kinase C-controlled
exocytosis, and the receptor is up-regulated in response to chronic pain (Morenilla-Palao et
al, J BIOL CHEM 279, 25665-72, 2004; Szallasi et al, TRENDS MOL MED 12, 545-54, 2006).
Thus, selectively targeting toxin derivatives to TRPV1-positive neurons may not only block
release of pain neurotransmitters from these neurons but also down-regulate the expression of
TRPV1, thus decreasing the sensitivity to hyperalgesia.
An aCGRP antagonist (residues 8-37), truncated version of CGRP (37 residues), is effective in antagonizing the action of basally-released CGRP in vitro from neurons in brainstem slices (Meng et al., 2009). This antagonist also can alleviate pain in vivo (Bird et al., MOL PAIN 2, 31, 2006) by binding to the CGRP receptor present on sensory ganglion and nociceptive presynaptic nerve terminals (Hay et al., BR J PHARMACOL 140, 477-86, 2003; Sams-Nielsen et al., BR J PHARMACOL 132, 1145-53, 2001; Zhang et al., J NEUROSCI 27, 2693-703, 2007).

The use of TLs comprising, consisting essentially of, or consisting of aCGRPg-37 (or derivatives thereof) to achieve targeted delivery of BoNT-derived core therapeutics into sensory neurons offers multiple advantages. For example, the resultant inhibition of CGRP release negates the vasodilation and mast cell degranulation associated with the activity of CGRP. Notably, the prevention of mast cell degranulation decreases the release of inflammatory affectors such as cytokines, including, without limitation, TNFa and IL-1β, that can act on sensory neurons. These factors, which induce the up-regulation of CGRP synthesis via MAPKs (mitogen-activated protein kinases) (Durham, P. L.; Russo, A. F. J NEUROSCI 23, 807-15, 2003), are thus involved in a feedback-regulated signaling cascade. The use of inhibitors of CGRP secretion in the present invention thus interrupts this expression and release cascade.

CGRP has been shown to be taken up by perivascular nociceptive nerve terminals, and this is efficiently reduced by CGRPg-37, suggesting that receptor-mediated endocytosis of CGRP occurs (Sams-Nielsen et al., BR J PHARMACOL 132, 1145-53, 2001). The use of the biotherapeutics of the present invention, comprising an CGRP-binding TL should therefore successfully deliver the analgesic biologies of the invention into target cells (e.g., presynaptic nerve and mast cells). In Example 3 of this specification a synthetic DNA sequence encoding human CGRP8-37 has been ligated to the 3’ end of an embodiment of an expressible open coding region for the synthesis of a CGRP receptor-targeted analgesic biological therapeutic.

The interleukin-1 receptor antagonist (IL-1RA), a naturally-occurring receptor antagonist, binds to IL-1 receptor expressed on various cells e.g. macrophages,
monocytes, synoviocytes, mast cells and neutrophils (Pou et al., BioCHiM BIOPHYS ACTA. 181 1:556-63, 2011; Chin et al., J CLIN INVEST. 82:420-6, 1988; McColl et al., J EXP MED. 176:593-8, 1992). The antagonist IL-IRA competitively inhibits the binding of both IL-la and IL-1β to the IL-1 receptor without inducing any detectable intracellular responses (Arend et al, ANNU. REV. IMMUNOL. 16:27-55, 1998).

[00084] According to the present invention, the targeted delivery of BoNT/X-derived therapeutics into inflammatory-mediating cells via IL-IRA binding to its receptor are expected to reduce the secretion of cytokines by cleaving the SNAREs which are essential for the release of these cytokines. As noted above, SNAP-23 and VAMP 3 were found to be essential for TNF-a and 21-6 release from human synovial cells (Fig. 8).

[00085] Although not necessarily limiting for the broadest embodiments, all of the constructs exemplifying the invention shown herein contain short sequences encoding amino acid residues, such as a "loop" region, situated between HC and LC of BONT/X (and located within (between) the cysteine residues involved in the di-sulfide bond bridging the LC and HC). The loop region is altered to contain a protease recognition amino acid sequence selectively or specifically recognized by an exogenous so the single-chain (SC) proteins expressed can be easily converted in vitro to the activated di-chain (DC) form by reaction with such an exogenous protease (for example, thrombin), for example in solution, or by using a column or batch reagent in which the exogenous protease is immobilized. Those of skill in the art are aware that any suitable exogenous protease may be used so long as it does not cleave the protein at undesired positions within the heavy or light chain regions.

[00086] The nucleic acid constructs of the present invention are constructed recombinantly, so as to permit the incorporation of alternations of the naturally-occurring BoNT/X sequences to provide therapeutic proteins for the treatment of chronic pain or inflammation when expressed in a suitable vector and host cell system. Examples of host cells which can be used for the expression of exogenous genes include, without limitation, insect cells, mammalian cells and cell lines, yeast cells, and bacterial cells, particularly the Gram-positive bacterium Escherichia coli (E. coli). Currently the Applicants prefer to use E. coli as a host cell expression system.
The therapeutic proteins expressed and/or made from the gene constructs described above offer several major advantages over the use of previously described agents for the treatment of pain, including previous Clostridial neurotoxin-based therapeutics. These advantages include, (a) directed and selective targeting to sensory neurons and/or inflammatory cells via an attached TL; (b) intra-cellular delivery and subsequent inhibition of the exocytosis of pain-stimulating peptides or cytokines, without substantially affecting other cells, such as motor and autonomic neurons, and (c) highly desirable and greatly extended life-time of the biotherapeutics (comparable to BoNT/A), which is a huge advantage decreasing the frequency of treatment or necessity for repeated treatment of chronic pain and inflammatory conditions.

Although aspects of the present invention have been described with reference to the disclosed embodiments, one skilled in the art will readily appreciate that the specific examples disclosed are only illustrative of these aspects and in no way limit the present invention. Various modifications can be made without departing from the spirit of the present invention.

**Detailed Description of the Invention**

Example 1: Construction of rA`HC and Characterization of the Purified Recombinant Protein

Recombinant nucleic acid rA`HC was created using "rA", a single chain construct of the synthetic BoNT/A nucleotide sequence in which the codons are optimized for expression in *E. coli*. The rA sequence is also engineered to possess one thrombin cleavage site in the loop region between the putative heavy chain and light chain regions of the toxin (LC-HC loop), and additional amino acids comprising a second thrombin cleavage site engineered near the carboxy terminus of the single chain toxin between the toxin sequences and a C-terminal His₆ (SEQ ID NO :19) to permit cleavage of the His₆ (SEQ ID NO :19) region and nicking of the inter-disulfide loop following purification. The rA sequence is cloned into *E. coli* expression vector pET29a(+) for propagation; pET29a(+)
is a commercially available pBR322-derived plasmid vector containing a pBR322 plasmid origin, a bacteriophage f1 viral origin of replication, the T7 bacteriophage promoter, an N-terminal S-tag, a C-terminal His\_6 tag (SEQ ID NO :19) for purification of the gene product, a multiple cloning sequence (MCS) and the lacI repressor gene. The vector can be obtained from, e.g., EMD4 Biosciences, Inc.

The nucleic acid sequence region encoding the HC\_N region (that is, encoding amino acids: 1874-Q\_1091) is removed from the pET29a-rA vector (Wang et al 2008, J. BIOL. CHEM) by reverse PCR, using suitable primers complementary to each nucleic acid strand followed by self-ligation of the expression vector. As part of this portion of the engineering of the coding nucleic acid, two additional amino acid residues (Gly-Gly) were introduced in place of the HC\_N region between the H\_N and Hcc domains of the heavy chain. The resulting DNA construct inherits the two thrombin cleavage sites from rA. Fig. 2A shows schematic diagrams of the single chain rA and rA \( ^{\gamma}N \) proteins showing the thrombin cleavage sites and the location of the inter-chain disulfide linkage.

After verification of the DNA sequences of the resulting rA \( ^{\gamma}HC\_N \) insert, vector containing the single chain (SC) gene was transformed into E. coli strain BL21(DE3), and expression was elicited by auto-induction (Wang et al., 2008). Cells were then pelleted by centrifuge, washed, and lysed using lysozyme and several freeze/thaw cycles. Insoluble material was removed by centrifugation and the supernatant used for subsequent steps. The SC was separated from the remainder of the supernatant by IMAC on TALON resin, and eluted with 500 mM imidazole. Fig. 2B shows an SDS-PAGE gel in which the left lane shows molecular weight markers and, from left to right, the cleared lysate (1), the column flow-through (2), the column pre-elution wash (3), the eluted fractions (4-9). The eluted rA \( ^{\gamma}HC\_N \) protein is then buffer exchanged into storage buffer (20 mM Heps, 150mM NaCl, pH 7.4), and incubated with thrombin (1 unit/mg toxin) at 22°C for 1 hour for nicking the toxin. In some cases, the IMAC eluates were further purified by ion-exchange chromatography following the established protocol. For example, IMAC purified samples are buffer exchanged into 50 mM Tris-HCl buffer (pH 8.1) and loaded onto a resource Q column, and after washing with 30 mM NaCl, a stepwise gradient up to 1 M NaCl in 50 mM Tris-HCl buffer is applied. Pure samples are eluted by 70 mM NaCl (Wang et al 2008 and
2011).

[00093] Fig. 2C shows reducing and non-reducing SDS-PAGE gels of the purified toxin before (SC) and after nicking with thrombin (DC). Reduction was performed using dithiothreitol (DTT) to reduce and break the disulfide bonds linking the LC and HC. The DC lanes demonstrate that without reduction (-) the DC molecule migrates as a molecule of a single molecular weight (indicating that the disulfide bonds are intact); upon reduction (+) the gel demonstrates that the toxin derivative is nicked.

[00094] The IMAC-purified SC form of rA-H\textsubscript{CN} was converted to the double chain (DC) form by incubation with thrombin. The DC toxin derivative displayed the ability to bind a recombinant fragment of the intra-luminal loop of SV2C (the BoNT/A protein receptor), by a pull-down assay and Western blotting (see Fig. 3A).

[00095] An example of such an assay is conducted as follows: The IMAC-purified SC form of rA-H\textsubscript{CN} is converted to the double chain (DC) form by incubation with thrombin as above. Additionally, DC rA and rE proteins are also added to the assay as a control. GST-tagged, recombinantly expressed intra-luminal fragments of an acceptor for BoNT/A (GST-rat SV2C(454-579)) are expressed and purified as disclosed in Wang, et al., J. BIOL. CHEM. 283:16993-17002 (2008). About 100 µg of this protein is immobilized using 100 µl of a slurry of glutathione Sepharose-4B Fast Flow resin (GE Healthcare) and incubated with 100 nM of rA, rE or the rA-H\textsubscript{CN} toxin derivative in a total volume of 100 µl of binding buffer (50 mM Tris, 150 mM NaCl, 0.5% Triton X-100, pH 7.6). In each case the resin beads are then collected by centrifugation and washed five times with >10 bed volumes of the same buffer for 15 min at 4°C.

[00096] Bound proteins are eluted from the washed beads by adding SDS-PAGE non-reducing sample buffer. Toxins are detected by Western blotting as shown in Fig. 3A. The left two Western blots are samples of rA, rA-H\textsubscript{CN} and rE without performing the pull-down assay to confirm the specificity of antibodies used. This pair of Western blots show SDS-PAGE non-reducing gels with the lanes, from left to right, pre-stained molecular weight standards, rA, rA-H\textsubscript{CN} and rE. The far left hand Western blot is developed using
antibody selective to the light chain of BoNT/A (LC/A), and the right hand Western blot of
this pair is developed using an antibody to the light chain of BoNT/E (LC/E). As expected,
the anti-LC/A antibody detected both rA and rA\textsuperscript{¾}_N, while not detecting rE. Similarly, the
anti-LC/E antibody only detected rE, and not rA or rA\textsuperscript{¾}_N.

The right hand pair of Western blots shows the results of the binding assays against immobilized SV2C acceptor component, and are run on the final column eluate. The Western blots are developed in the same manner, and the SDS-PAGE run in the same manner with the same lane order as in the left hand pair described above. The results show that rE was not bound by the immobilized SV2C acceptor component (see last lane, far right hand Western blot). However, the left hand Western blot of the pair shows that both rA and rA\textsubscript{He}_N bound the acceptor component and were successfully eluted, with the molecular weights of these polypeptides being identical to that of the species detected in the positive control.

The DC toxin derivative displayed the ability to bind a recombinant fragment of the intra-luminal loop of SV2C acceptor component by a pull-down assay and Western blotting. As a control, a purified form of rE (recombinant BoNT/E toxin in double chain form), which naturally binds glycosylated forms of the related protein receptors SV2A and SV2B, was incubated with the SV2C fragment under identical conditions, and did not bind this fragment. Thus, the interaction of rA\textsuperscript{¾}_N with SV2C is selective and does not occur with rE.

When rA\textsuperscript{¾}_N was added in serial dilutions to cultures of rat cerebellar granule neurons (CGNs), the toxin derivative substantially failed to cleave SNAP-25. Fig 3B shows an experiment in which rat CGNs were incubated with ten-fold serial dilutions of rA or rA\textsubscript{He}_N (from 1000 pM to 0.01 pM, and with a negative control containing 0 pM) in culture medium at 37°C for 24 hours. The cells were harvested and washed, then lysed in SDS-PAGE sample buffer; Western blots were developed using an antibody selective for the intact SNAP-25 and reactive with the SNAP-25 cleavage product of digestion with BoNT/A. The results showed that upon treatment of rat CGNs with the rA toxin intracellular SNAP-25 is cleaved at toxin concentrations at and above about 0.01 pM of the toxin, while SNAP-25
remains largely intact upon treatment of the CGN cells with $\Gamma\alpha~\frac{3}{4}~$N of less than about 1000 pM. Since both proteins contain LC/A protease with similar activity towards recombinant substrates, the data suggest that the deletion of $\frac{3}{4}~$N from the $\Gamma\alpha~\frac{3}{4}~$N derivative deprives and/or attenuates the toxin derivative of the ability to undergo internalization and/or translocation of LC within the CGN cells. Additionally, as shown in Fig. 3B, neither rA nor rA HcN cleaved the SNARE protein syntaxin-1, which was added to the SDS-PAGE gels as a negative control.

[000100] Consistent with this hypothesis, intraperitoneal injection of each toxin into mice, in a mouse lethality assay, also showed the disproportional toxicity of the toxins. As shown in Fig. 3C, upon calculation of the mLDr/50/mg (the mLDr is defined as the lowest dose of toxin effective to kill 50% of a group of 4 mice within 4 days), the deleted $\Gamma\alpha~\frac{3}{4}~$N variant displayed approximately a 6.7x10^4-fold decrease in toxicity relative to rA.

[000101] These new findings suggested that the presence of the HcN portion of the heavy chain may be important for cell intoxication by BoNT/A (including rA) and its derivatives. Moreover, this experiment appears to dissect one or more elements of the multi-phasic intoxication mechanism of Clostridial neurotoxin (selective cell surface binding, internalization and translocation of LC to the cytosol and cleavage of the SNARE).

[000102] **Example 2: Construction of Toxin Derivatives**

**LC.HN.HN/A-PT-1 and LC.HN/A-PT-1**

[000103] The data disclosed in Example 1 show that it is possible to alter the specificity of BoNT/A (and, thus, of many or all other Clostridial neurotoxins) without altering the LC endopeptidase by removing the Hec region of the heavy chain binding region. Additionally, Applicants postulate that similar results would occur if the Hec region were mutated to rather than removed to eliminate the capability of the toxin to bind the protein receptor.

[000104] Applicants desired to investigate whether the altered toxin can be retargeted to selectively bind another cell type. As shown in Fig. 4, Applicants considered
that such Hcc-lacking or Hcc-inactive neurotoxin variants can be linked to carefully chosen targeting ligands (TLs), for example targeted to purinergic receptors. In preferred embodiments, the purinergic receptor may be P2X3, and the ligand may be selected from peptides such as PT-1 and receptor P2X3-binding derivatives thereof and scFv fragments selective for P2X3. Additionally, the TL may be targeted to TRPV1 or other sensory neuron-selective cell surface antigens.

[000105] For generating LC.H\textsubscript{N}.H\textsubscript{CN}/A-PT-1 (Fig. 5A), a PCR product encoding a synthetic LC.H\textsubscript{N}.H\textsubscript{CN}/A gene was obtained, using the pET29a-BoNT/A construct as template and a pair of primers (T7 forward primer and a specific reverse primer with a designed Sac I restriction site). This was digested using endonucleases Xba I and Sac I and cloned into the pET29a(+) vector using these two restriction sites. The resultant construct was digested by Sac I and Xho I before ligation with a nucleic acid comprising an endonuclease Sac I- and Sal I-digested synthetic purotoxin-1 gene fragment (abbreviated "PT-1": shown as nucleotide sequence SEQ ID NO: 11 and amino acid sequence SEQ ID NO: 12) to generate a targeted LC.H\textsubscript{N}.H\textsubscript{CN}/A-PT-1 construct. Similarly, LC.H\textsubscript{N}/A-PT-1 was created except the nucleotide sequence encoding LC.H\textsubscript{N} of BoNT/A was fused directly to PT-1 without the intervening H\textsubscript{CN} nucleotide sequences (Fig. 5A).

[000106] The nucleotide sequence of the resulting construct was verified by sequence analysis, then each of the above expression vector constructs were transformed into the Origami\textsuperscript{TM} 2(DE3) E. coli host strain; this strain is a K-12 derivative that has mutations in both the thioredoxin reductase (trxB) and glutathione reductase (gor) genes, which greatly enhance disulfide bond formation in the E. coli cytoplasm. Plasmid protein expression was induced using auto-induction medium (Wang et al., JBC, 2008).

[000107] The expressed proteins were purified by IMAC as above, followed by SDS-PAGE analysis of LC.H\textsubscript{N}.H\textsubscript{CN}/A-PT-1 (Fig. 5B) and LC.H\textsubscript{N}/A-PT-1 (Fig. 5C) on reducing and non-reducing gels, substantially as outlined in Example 1. Cells were then pelleted, washed, and lysed using lysozyme and several freeze/thaw cycles; insoluble material was removed by centrifugation. The proteins were trapped by IMAC on TALON resin, eluted with 500 mM imidazole, IN each of Fig. 5B and 5C, lane 1 corresponds to the
cleared lysate, 2 to the flow-through fraction, 3 to the wash fraction, and fractions 4-8 to the eluate fractions. The unlabeled lane on each gel comprises molecular weight standards.

As can be seen, unlike the experiments using expressed TA \( \equiv N \), both of LC.H \( N_HC_N/A-PT \) -1 and LC.H \( N_HC_N/A-PT \) -1 were expressed largely in the DC form, as reflected by the appearance under reducing conditions of LC and \( H_N.HC_N/A-PT \) -1 or \( H_N/A-PT \) -1 in SDS-PAGE (Fig. 5B and Fig. 5C). This fact suggests that in this experiment the intra-loop thrombin site in each construct was cleaved after expression or during purification without the need for an \textit{in vitro} cleavage step. The presents of discrete LC and iHCi species was confirmed using Western blotting; Fig. 6A shows that anti-LC/A antibody detects the disulfide-linked double chain LC.H \( N_HC_N/A-PT \) -1 in non-reducing gels, but only the LC in reducing gels. Similarly, anti-His6 antibody ("His6" disclosed as SEQ ID NO :19) detects the disulfide-linked double chain LC.H \( N_HC_N/A-PT \) -1 in non-reducing gels, but only the \( H_N.HC_N/A-PT \) -1 under reducing conditions. Coomassie blue staining of the gel shows both chains to be present under reducing conditions. Fig. 6B shows the same experiment using the purified LC.H \( N_HC_N/A-PT \) -1, with similar results.

Incubation of 1.6 nM LC.H \( N_HC_N/A-PT \) -1 or LC.H \( N_HC_N/A-PT \) -1 with sensory neurons from rat trigeminal ganglia was conducted as described above. As shown in Fig. 6C, only the cells incubated with LC.H \( N_HC_N/A-PT \) -1 showed detectable cleavage of SNAP-25 using this concentration of protein. In contrast, LC.H \( N_HC_N/A-PT \) -1 failed to cleave SNAP-25 within the rat trigeminal ganglia cells despite carrying the same LC. The result is shown quantitatively in Fig. 6D. These findings suggest that H\( C_N \) plays an important role in permitting the LC.H \( N_HC_N/A-PT \) -1 polypeptide to enter sensory neurons and cleave its intracellular target; the lack of H\( C_N \) results in a greatly attenuated or absent ability for the protein to enter the cell. Thus, Applicants have found the presence of H\( C_N \) to be very important for permitting Clostridial toxin-based therapeutics (even those like LC.H \( N_HC_N/A-PT \) -1 that bear a TL selective for a protein receptor displayed by the target cell) to enter the parent cell.

In this specification (unless indicated otherwise) all amino acid sequences are shown in the direction from the amino terminus to the carboxy terminus, and
the nucleotide sequences are shown in the direction 5’ to 3’.

[000111] **Synthetic purotoxin-l nucleic acid fragment (with stop codons) and its encoded amino acids.**

[000122] SEQ ID NO: 9 and 10 are the nucleotide sequence and the amino acid sequence, respectively, of the synthetic purotoxin-l nucleic acid fragment and its encoded amino acids, including additional linker regions. The following shows an alignment of these sequences with relevant restriction endonuclease sites shown, as follows: Nucleotides 1-18 comprise restriction sites for Sal I, Sac I and EcoRV; nucleotides 19-63: three iterations of nucleotides encoding the amino acid sequence Gly₄Ser (SEQ ID NO :20) (a non-structured linker); nucleotides 64-171 (shown underlined and in bold): the purotoxin-l fragment, including a stop codon (*); nucleotides 172-177: the restriction site for endonuclease Xho I.

[000133] Synthetic purotoxin-l nucleic acid fragment (without stop codons) and its encoded amino acids.

[000144] SEQ ID NO: 11 and 12 are the nucleotide sequence and the amino acid sequence, respectively, of the synthetic purotoxin-l nucleic acid fragment and its encoded amino acids, including additional linker regions. The following shows an alignment of these sequences with relevant restriction endonuclease sites shown, as follows: Nucleotides 1-18 comprise restriction sites for Sal I, Sac I and EcoRV; nucleotides 19-63: three iterations of nucleotides encoding the amino acid sequence Gly₄Ser (SEQ ID NO :20) (a non-structured linker); nucleotides 64-168 (shown underlined and in bold): the purotoxin-l fragment
(without a stop codon); nucleotides 169-174: the restriction site for endonuclease Sal I.

\[ \text{VDELDIGGGG...} \]

\[ \text{GIRCDDIHCCTGLKCKCNASGYNCV} \]

\[ \text{RKKVVD (SEQ ID NO :12)} \]

\[ \text{GCAAAAAG GTCGAC 174 (SEQ ID NO :11)} \]

[000117] **Example 3: Targeting BoNT-derived Inhibitors of Exocytosis to Cells Secreting Inflammatory Affectors**

[000118] The release of cytokines and other mediators of inflammation is associated with several types of chronic pain. The release of many of these mediators involves SNARE-dependent exocytosis (Stow et al, NATURE REVIEWS Immunol. 6, 919-29, 2006). In another embodiment of the present invention, Clostridial toxin-derived therapeutics may be targeted to cells involved in the release of these actors by attaching TLs having an selective affinity for such cells, such as the peptides IL-IRA or CGRP antagonist (see above), which bind to their requisite receptors on the surface of non-neuronal cells secreting pain and/or inflammatory mediators. Such cells may be neurons or non-neurons.

[000119] Towards this end, a human synovial cell line (hSC) was analyzed for SNARE protein species and found to predominantly contain SNAP-23, VAMP 3 and syntaxin 2, 3 and 4. As shown in Fig. 7, the SNAREs SNAP-25, syntaxin 1 and VAMP 2 were not detected to an appreciable degree in hSC cells. Similarly, in a macrophage cell line RAW264.9 (mMC) SNAP-23, VAMP 3, syntaxin 2, syntaxin 3 and syntaxin 4 are detected.

[000120] Additional cell types analyzed for SNARE proteins included rat cerebellar granule neurons (rCGNs). As shown in Fig. 7, which contained SNAP-25, VAMP 2 and syntaxin 1.
As shown in Fig. 8A-8D, hSC cells were incubated for 7-10 days with shRNA (small hairpin RNA) lentivirus carrying nucleotide sequences specifically targeting the down-regulation of SNAP-23 expression. The cells were then incubated overnight with IL-1β (100 ng/ml) in culture medium to induce secretion of TNF-α and IL-6. After collecting the supernatant, lysates from these cells were subjected to SDS-PAGE and proteins detected by Western blot analysis using antibodies directed to SNAP-23, VAMP 3 or the untargeted control protein β-tubulin. KD stands for shRNA-induced knock down! or inhibition of expression. The gel results (Fig. 8A and 8C) show that expression of SNAP-23 and VAMP 3 is diminished substantially in cells treated with the shRNA as compared to untreated cells (control). Additionally, in Fig. 8B and 8D the levels of tissue necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) quantified by ELISA in siRNA-treated cells are compared with the levels in untreated cells, and the reduction expressed as percentage inhibition relative to control; Fig. 8B also shows the percent knock-down) of SNAP-23 and Fig. 8D the percent knock-down of VAMP 3, relative to untreated cells. These observations provide evidence for VAMP 3 and SNAP-23 being required in exocytosis of both of these cytokines tested. The levels of SNAP-23 or VAMP 3 in siRNA-treated cells relative to an internal reference protein (β-Tubulin) were compared with that in untreated cells, and the reduction expressed as percentage KD relative to control.

Thus, as illustrated by these findings, certain embodiments of the present invention involves compositions and methods for inhibiting the release of cytokines using a BoNT-derived analgesic therapeutic targeted to these cells via an joined TL such as IL-IRA or a CGRP antagonist; see Fig. 4.

For example, the cleavage of VAMP 3 in cells secreting inflammatory factors may be accomplished by ligating a prepared synthetic nucleic acid segment encoding the VAMP 3-cleaving protease LC/D in a therapeutic construct such as BoNT/D(Hcc), to a synthetic nucleic acid encoding a binding-capable polypeptide (IL-IRA) having selective affinity for human IL-1 receptor (the entire sequence is shown herein as SEQ ID NO: 13 with the translated amino acid sequence shown as SEQ ID NO: 14).

As an alternative TL to prevent the secretion of inflammatory factors
from cells, use of an antagonist to the CGRP receptor 1 provides a similar means of targeting non-neuronal cells and sensory neurons. Disclosed herein, a synthetic nucleic acid encoding a binding portion of the coding sequence of human CGRP antagonist (CGRP<subliced</sub>\textsuperscript{37}); this sequence is shown as SEQ ID NO: 15, and its translated amino acid sequence is provided as SEQ ID NO: 16. The nucleic acid fragment was fused via restriction endonuclease digestion and ligation to BoNT/D(\textsubscript{Hc<sub>37</sub>}) to generate a fusion gene, BoNT/D(-Hc<sub>37</sub>-CGRP<subliced</sub>\textsuperscript{37}). The sequence of the (SEQ ID NO: 17, and its translated amino acid sequence is provided as SEQ ID NO: 18).

[000125] These two hybrid nucleic acids are separately cloned into expression vector pET29a(+) and expressed in \textit{E.coli} strain BL 21(DE3). An additional or alternative strategy for inhibiting cytokine release may rely on inactivating SNAP-23; in this approach, an LC/E moiety, capable of cleaving SNAP-23, may be attached to BoNT/A·Hcc-IL-IRA or BoNT/X(PrP<sup>-</sup>)-IL-IRA. For example, mutating residue of Lys224 in LC/E moiety to Asp significantly increased its cleavage of human SNAP-23 (Chen and Barbieri, Proc. Nat. Acad. Sci. USA, 106:9180-9184, 2009). This mutant LC/E may be attached to BoNT/B (Lys<sup>1192</sup> V Glu and/or Ala<sup>1196</sup> V Lys:PrP<sup>-</sup>)-IL-IRA (see earlier text about PrP<sup>-</sup>). These constructs may be used in conjunction with biotherapeutics having the ability to cleave other SNARE proteins to provide a stronger therapeutic effect. Furthermore, since the IL-1 receptor also reside on macrophages which also possess BoNT-susceptible SNAREs (see Fig. 7), a similar approach as outlined above for the hSC may be adopted to target these cells.

[000126] All of these constructs comprise BoNT/X-TL hybrids (or nucleic acids encoding such hybrids) either lacking the Hcc region, or having an inactive Hcc region. The polypeptides preferably are constructed to contain short loop inter-chain sequences possessing a protease-labile, selective cleavage site situated between HC and LC of BoNT so the expressed single-chain proteins can be converted \textit{in vitro} to the activated di-chain form as necessary.

[000127] In the following nucleotide sequences, SEQ ID NO: 13, SEQ ID NO: 15 and SEQ ID NO: 17 and their respective amino acid sequences, SEQ ID NO: 14, SEQ ID NO: 16 and SEQ ID NO: 18), the amino acids are identified using the single letter amino acid
designations, with the amino acid sequence shown in the direction from the amino terminus to the carboxy terminus, and the nucleotide sequence shown in the direction 5' to 3'.

![Sequence and its encoded amino acids](image-url)

**Synthetic BoNT/D (~Hc) - human IL-1RA gene sequence and its encoded amino acids (SEQ ID NO: 13 AND 14)**

<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>Amino Acid Sequence</th>
<th>Nucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AVAACCTGCGGCGGTAAGAAGCTTAAAAATAGGATCTCCGGAACACATAGTTTATGGTACCTGGAGAGCTCCGGAAGA</td>
<td>5' - TGGCCAAAAGACATTTTTGGATGATCTGCGATGATCAGCTGACCCCGATGACCTGACCTGAGAGTGGCGGAAGAAGC - 3'</td>
</tr>
<tr>
<td>10</td>
<td>CAAAAGAAGATACCTCTGTAAAAGCCTATCATAACTCTTTTAAAGGACCAAGGGAATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - CTGAGCCCGAACGAGGCTTGAGAGGGAAGAAGC - 3'</td>
</tr>
<tr>
<td>20</td>
<td>GACAAATGTCCTGAGGAGGAGGTATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - AATGTGGAGGTATGGGAGATGGAACACGTGGGTAAC - 3'</td>
</tr>
<tr>
<td>30</td>
<td>GACAAATGTCCTGAGGAGGAGGTATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - AATGTGGAGGTATGGGAGATGGAACACGTGGGTAAC - 3'</td>
</tr>
<tr>
<td>40</td>
<td>GACAAATGTCCTGAGGAGGAGGTATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - AATGTGGAGGTATGGGAGATGGAACACGTGGGTAAC - 3'</td>
</tr>
<tr>
<td>50</td>
<td>GACAAATGTCCTGAGGAGGAGGTATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - AATGTGGAGGTATGGGAGATGGAACACGTGGGTAAC - 3'</td>
</tr>
</tbody>
</table>

**Sequence and its encoded amino acids (SEQ ID NO: 13 AND 14)**

<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>Amino Acid Sequence</th>
<th>Nucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AVAACCTGCGGCGGTAAGAAGCTTAAAAATAGGATCTCCGGAACACATAGTTTATGGTACCTGGAGAGCTCCGGAAGA</td>
<td>5' - TGGCCAAAAGACATTTTTGGATGATCTGCGATGATCAGCTGACCCCGATGACCTGACCTGAGAGTGGCGGAAGAAGC - 3'</td>
</tr>
<tr>
<td>10</td>
<td>CAAAAGAAGATACCTCTGTAAAAGCCTATCATAACTCTTTTAAAGGACCAAGGGAATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - CTGAGCCCGAACGAGGCTTGAGAGGGAAGAAGC - 3'</td>
</tr>
<tr>
<td>20</td>
<td>GACAAATGTCCTGAGGAGGAGGTATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - AATGTGGAGGTATGGGAGATGGAACACGTGGGTAAC - 3'</td>
</tr>
<tr>
<td>30</td>
<td>GACAAATGTCCTGAGGAGGAGGTATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - AATGTGGAGGTATGGGAGATGGAACACGTGGGTAAC - 3'</td>
</tr>
<tr>
<td>40</td>
<td>GACAAATGTCCTGAGGAGGAGGTATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - AATGTGGAGGTATGGGAGATGGAACACGTGGGTAAC - 3'</td>
</tr>
<tr>
<td>50</td>
<td>GACAAATGTCCTGAGGAGGAGGTATGGGAGATGGAACACGTGGGTAAC</td>
<td>5' - AATGTGGAGGTATGGGAGATGGAACACGTGGGTAAC - 3'</td>
</tr>
</tbody>
</table>
The synthetic nucleotide sequence provided above contains the following regions, respectively (identified to the nucleotide residues and the peptides encoded therein) residues 1-3237: LC.HN.HCN/D; residues 3274-3729 (underlined), human IL-1RA. DNA sequences between these areas (for example, the sequence comprising nucleotides 3238-3273) are introduced as a linker between the TL and the remainder of the construct and ensures the proper reading frame. The amino acid sequences are displayed in [000129]...
alignment above the corresponding nucleotides. A thrombin protease recognition sequence is shown engineered into the loop between LC/D and H_{\text{N/D}}; similarly, another thrombin site was engineered to have a cleavage sequence to the carboxy site of the human IL-IRA gene for simultaneous nicking and removal of C-terminal His_{6} (SEQ ID NO:19); the arrows indicate cleavage sites.

[000130] Synthetic CGRP antagonist (CGRP_{8-37}) fragment and its encoded amino acids (SEQ ID NO: 15 and SEQ ID NO: 16)

<table>
<thead>
<tr>
<th>Ambiguous Sequence</th>
<th>Gene/Protein</th>
<th>ID No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L S R S G G V V K N N F V P T N V G S K A F * 81</td>
<td>156 Xho 1</td>
<td></td>
</tr>
</tbody>
</table>

[000131] The synthetic nucleotide sequence provided above contains the following regions, respectively (identified with respect to the nucleotide residues — residues 1-12: restriction sites for Sac I and EcoRV; residues 13-57: (Gly4Ser)x3 (SEQ ID NO :21) non-structured linker; residues 58-150 (underlined and bold): the CGRP_{8-37} binding fragment including a stop codon; residues 151-156: restriction site for Xho I. Deduced amino acid sequences are aligned above the corresponding nucleotides.

[000132] BoNT/D(Hcc)-CGRP_{8-37} gene sequence and its encoded amino acids (SEQ ID NO: 17 and 18)

<table>
<thead>
<tr>
<th>Ambiguous Sequence</th>
<th>Gene/Protein</th>
<th>ID No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>M T W P F V K D F N Y S D P V N D N D I L Y L R I P Q N 19</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>K L I T T F V K A F M I T Q N I W W I F E R F P S S D 30</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>T N F S L S K P F R P T S K Y Q S Y Y D P S Y L S T D 40</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>N Y L V V G S F P F M G D S S T F E D T F D F R H T 321</td>
<td>490</td>
<td></td>
</tr>
<tr>
<td>T N I A V E K F E N G S W K V T N I I T P S V L I P G 401</td>
<td>480</td>
<td></td>
</tr>
</tbody>
</table>

The synthetic nucleotide sequence provided above contains the following regions, respectively (identified with respect to the nucleotide residues — residues 1-12: restriction sites for Sac I and EcoRV; residues 13-57: (Gly4Ser)x3 (SEQ ID NO :21) non-structured linker; residues 58-150 (underlined and bold): the CGRP_{8-37} binding fragment including a stop codon; residues 151-156: restriction site for Xho I. Deduced amino acid sequences are aligned above the corresponding nucleotides.

BoNT/D(Hcc)-CGRP_{8-37} gene sequence and its encoded amino acids (SEQ ID NO: 17 and 18)
The synthetic nucleotide sequence provided above contains the following regions, respectively (identified with respect to the nucleotide residues - residues 1-3237 LC.HN.HCN/D; residues 3289-3381 (underlined and in bold), CGRP antagonist (CGRP8-37). The DNA sequence comprising nucleotides 3238-3288 is introduced as a linker and ensures the proper reading frame. The aligned amino acid sequences are displayed above the corresponding nucleotides. A thrombin recognition sequence is engineered into the interchain loop region between LC/D and HN/D; the arrow indicates this cleavage site.

It will be understood that each and every nucleotide sequence (including SEQ ID Nos. 13 and 17) encoding the amino acid sequence (including SEQ ID NOs. 14 and 18) is, and is intended to be, specifically and individually described as part of this patent application. It will also be understood by those of ordinary skill in the art that specific nucleic acid constructs described in Sequence ID No. 13 and 17 and their encoded respective amino acid sequences in Sequence ID No. 14 and 18 of this specification are exemplary, and that conservatively modified variations from these nucleotide and amino acid sequences may be made without departing from the scope of the invention disclosed herein. Thus, a nucleic acid construct having 95% or more, or 90% or more, or 85% or more, or 80% or more, or 75% or more, or 70% or more, or 60% or more homology to, for example and without limitation, SEQ ID Nos: 13 and 17 having the selective therapeutic activity indicated herein are intended to fall within the spirit of the present invention. Moreover, all nucleic acid constructs encoding the amino acid sequences disclosed in this specification are included within the scope of this invention.

Likewise, it will also be understood by those of ordinary skill in the art that amino acid sequences having 95% or more, or 90% or more, or 85% or more, or 80% or more, or 75% or more, or 70% or more, or 60% or more homology to Sequence ID No. 14 and 18 fall within the spirit of this embodiment of the invention.

It will also be understood that other analgesic bio-therapeutics with particularly valuable application to chronic pain may be generated using gene constructs
similar to those described above, having one or more TL moiety encoding antibody-based single chain variable fragments (scFVs) or Fabs which bind membrane-exposed domains of antigens such as TRPV1 and/or P2X3. Such constructs may have these TL moieties, either in place of the CGRP antagonist, PT-1 or IL-1RA TL moieties such as those described above, or may be inserted in addition to such a TL. All of the nucleic acids encoding such hybrid biotherapeutic proteins may be expressed in E. coli, mammalian or insect cells (or another suitable host cell/vector pair selected and utilized), and the resultant recombinant proteins purified by any suitable means, such as affinity and ion-exchange chromatography. Their specificities and potencies can then be evaluated in various models such as in cultured neurons, animal models of chronic neuropathic (e.g. spinal nerve injury) and inflammatory pain, and in in vitro systems, including, for example, the models and systems described herein.

Example 4: Treatment of Chronic Pain Using a BoNT/D(Hcc)-TL Clostridial Neurotoxin Derivative

A 42-year-old woman presents complaining with chronic irritable bowel syndrome (IBS). Clinical examination reveals significant abdominal distention, and chronic frequent diarrhoea, accompanied by localized abdominal pain, scored by the patient as an 8 on a scale of 1 to 10.

The patient is injected directly in the intestinal sensory nerves with a therapeutic amount of the analgesic biotherapeutic BoNT/D(Hcc)-TL, in which the TL is PT-1.

The patient is observed one week later, and examination reveals that the acute, chronic pain associated with IBS has been alleviated to a substantial amount, from a pain score of 8 to a pain score of 3 on a scale of 1 to 10.

The patient is again observed three weeks after the injection, and the analgesic activity of the biotherapeutic BoNT/D(Hcc)-TL remains high, with the patient reporting a pain score of 4 after three weeks.
Example 5: Treatment of Chronic Pain Associated with Esophageal Cancer Using a BoNT/C1(-Hcc)-(TRPV1 scFv) TL Clostridial Neurotoxin Derivative

A 55 year-old man with a history of alcoholism presents with Stage 3 esophageal cancer, nausea, severe chronic pain in his throat radiating to the base of the skull, and the inability to take oral nourishment.

The patient is administered the Clostridial neurotoxin derivative BoNT/C1(-Hcc)-TPvPVl scFvs in an effective dose by injection directly into both the vagal nodose ganglion and the jugular ganglion. The gene construct is made similarly as reported above for construction of the BoNT/D(-Hcc)-CGRP8-37 construct, and is expressed in E. coli. The Clostridial toxin derivative is affinity purified using the His6 tag (SEQ ID NO :19), and by ion exchange chromatography before use.

Within 48 hours, there is notable improvement in the extent and acuteness of pain, and within one week the patient is able to take oral nourishment. The patient is again observed three weeks after the injection, and the analgesic activity of the biotherapeutic BoNT/C1(-Hcc)-(TRPV1 scFv) remains high, with the patient reporting a pain score of i4i after three weeks.

Although aspects of the present invention have been described with reference to the disclosed embodiments, one skilled in the art will readily appreciate that the specific examples disclosed are only illustrative of these aspects and in no way limit the present invention. Various modifications can be made without departing from the spirit of the present invention.

Example 6: Treatment of Chronic Pain Associated with Rheumatoid Arthritis Using a BoNT/D-Hc-IL-1RA Clostridial Neurotoxin Derivative

A 42 year-old woman presents with severe chronic joint pain in the left
hip, and has difficulty walking. Following examination, the patient is diagnosed with rheumatoid arthritis of the acetabulofemoral (hip) joint.

The patient is administered the Clostridial neurotoxin derivative BoNT/D Hcc-IL-IRA in an effective dose by injection directly into both the femoral ganglion and the sciatic ganglion. The gene construct is made as described in Example 3 and expressed in E. coli. The Clostridial toxin derivative is affinity purified using the His$_6$ tag (SEQ ID NO:19), and by ion exchange chromatography before use.

Within 48 hours, there is notable improvement in the extent and acuteness of pain, and within one week the patient is able to walk.

Although aspects of the present invention have been described with reference to the disclosed embodiments, one skilled in the art will readily appreciate that the specific examples disclosed are only illustrative of these aspects and in no way limit the present invention. Various modifications can be made without departing from the spirit of the present invention.

The invention will now be described by the following numbered embodiments.

1) A composition comprising a Clostridial neurotoxin derivative, said composition comprising:
   a) a first active Clostridial toxin-derived endopeptidase domain effective to cleave a SNARE protein under physiological conditions;
   b) a Clostridial toxin-derived translocation domain effective to facilitate the movement of said first and second endopeptidase domains across a cellular membrane into the cytosol under physiological conditions; and
   c) a non-Clostridial toxin derived binding domain comprising a first targeting ligand (TL) selectively binding, under physiological conditions, to a first cell surface receptor displayed by a target cell selected from the group consisting of a sensory neuron and a cell that secretes at least one inflammatory cytokine, and substantially not displayed by a motor or autonomic neuron; wherein at least a light chain protease of said Clostridial neurotoxin
derivative is internalized by said target cell upon binding of said TL to the target cell, and
wherein the neurotoxin derivative possesses a functional Clostridial neurotoxin-derived HCN
domain and lacks a functional Clostridial neurotoxin-derived Hcc targeting domain activity.

2) The composition of embodiment 1 wherein the neurotoxin derivative lacks a
Clostridial neurotoxin-derived Hcc targeting domain.

3) The composition of embodiment 1 wherein the neurotoxin derivative contains a
Clostridial neurotoxin-derived Hcc domain mutated to impede binding of the Hcc domain to
its natural protein receptor.

4) The composition of embodiment 3 wherein said mutated Hcc domain comprises a
glutamic acid residue at a position corresponding to amino acid 1192 of BoNT/B.

5) The composition of embodiment 3 wherein said mutated HCC domain comprises
a lysine residue at a position corresponding to amino acid 1196 of BoNT/B.

6) The composition of embodiment 5 wherein said mutated Hcc domain also
comprises a glutamic acid residue at a position corresponding to amino acid 1192 of
BoNT/B.

7) The composition of embodiment 3 wherein said mutated Hcc domain is derived
from BoNT/B.

8) The composition of embodiment 4 wherein said mutated Hcc domain is derived
from BoNT/B.

9) The composition of embodiment 5 wherein said mutated Hcc domain is derived
from BoNT/B.

10) The composition of embodiment 6 wherein said mutated Hcc domain is derived
from BoNT/B.

11) The composition of embodiment 1 wherein said neurotoxin derivative comprises a
second Clostridial toxin-derived endopeptidase domain containing a mutation rendering it
substantially proteolytically inactive.

12) The composition of embodiment 1 wherein said neurotoxin derivative comprises a
second, different, active or inactive Clostridial toxin-derived endopeptidase domain.

13) The composition of claim 12 wherein the second Clostridial toxin-derived
endopeptidase domain is derived from BoNT/A.

14) The composition of embodiment 1 wherein the first Clostridial toxin-derived
endopeptidase domain comprises an endopeptidase derived from BoNT/E.
15) The composition of embodiment 1 wherein the Clostridial toxin-derived translocation domain is derived from a BoNT/X subtype selected from the group consisting of BoNT/A, BoNT/Cl, BoNT/D and BoNT/E.

16) The composition of embodiment 1 wherein the Clostridial toxin-derived translocation domain is derived from a BoNT/X subtype selected from the group consisting of BoNT/A, BoNT/Cl, BoNT/D and BoNT/E.

17) The composition of embodiment 1 wherein the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP-selective or CGRP-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof.

18) The composition of embodiment 17 wherein the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP-selective or CGRP-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof.

19) The composition of embodiment 1 comprising a polypeptide comprising an active Clostridial toxin-derived endopeptidase domain comprising an endopeptidase derived from BoNT/E, a Clostridial toxin-derived translocation domain is derived from BoNT/A, and a TL domain.

20) The composition of embodiment 17 wherein the TL comprises a targeting component comprising a CGRP antagonist.

21) The neurotoxin derivative of embodiment 1 in which said Clostridial toxin-derived endopeptidase domain comprises an endopeptidase having an enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological conditions.

22) The neurotoxin derivative of embodiment 11 in which said first or second Clostridial toxin-derived endopeptidase domain comprises an endopeptidase having an
enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological conditions.

23) The neurotoxin derivative of embodiment 1 comprising a BoNT/D light chain, a BoNT/D translocation domain, and a targeting ligand comprising CGRP8-37, and lacking a functional Hcc domain.

24) The neurotoxin derivative of embodiment 23 wherein the neurotoxin derivative contains a Clostridial neurotoxin-derived Hcc domain mutated to impede binding of the Hcc domain to its natural protein receptor.

25) The neurotoxin derivative of embodiment 23 comprising BoNT/D(Hcc)-CGRP8-37.

26) The neurotoxin derivative of embodiment 1 comprising a BoNT/D light chain, a BoNT/D translocation domain, and a targeting ligand comprising human IL-IRA, and lacking a functional Hcc domain.

27) The neurotoxin derivative of embodiment 26 wherein the neurotoxin derivative contains a Clostridial neurotoxin-derived Hcc domain mutated to impede binding of the Hcc domain to its natural protein receptor.

28) The neurotoxin derivative of embodiment 26 comprising BoNT/D(Hcc)-human IL-IRA.

29) The neurotoxin derivative of embodiment 1 comprising a BoNT/A light chain, a BoNT/A translocation domain, and a targeting ligand comprising a purinergic receptor ligand, and lacking a functional Hcc domain.

30) The neurotoxin derivative of embodiment 29 wherein the targeting ligand is a P2X3 receptor ligand.

31) The neurotoxin derivative of embodiment 30 wherein the targeting ligand comprises a purotoxin 1 or a selectively binding fragment thereof.

32) The neurotoxin derivative of embodiment 31 comprising L.C.H\textsubscript{N}.HC\textsubscript{N}/A-PT-1.

33) The neurotoxin derivative of embodiment 1 in which the TL specifically binds, under physiological conditions, to a first cell surface receptor displayed by a sensory neuron in preference to motor or autonomic neurons.

34) The neurotoxin derivative of embodiment 33 comprising at least two TL domains.

35) The neurotoxin derivative of embodiment 1 wherein the Clostridial neurotoxin translocation domain is selected from the group consisting of
a) a BoNT-A translocation domain;
b) a BoNT-B translocation domain;
c) a BoNT-C1 translocation domain;
d) a BoNT-D translocation domain;
e) a BoNT-E translocation domain;
f) a BoNT-F translocation domain;
g) a BoNT-G translocation domain, and
h) conservatively modified variants and isoforms of any of the above.

36) An analgesic Clostridial neurotoxin derivative comprising:
a) A first active Clostridial toxin-derived endopeptidase domain effective to cleave a SNARE protein under physiological conditions and having an enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological conditions;
b) a Clostridial toxin-derived translocation domain effective to facilitate the movement of said first endopeptidase domain across a celluarmembrane into the cytosol under physiological conditions; and
c) a binding domain comprising a first targeting ligand (TL) selectively binding, under physiological conditions, to a first cell surface receptor displayed by a target cell type selected from the group consisting of sensory neurons and cytokine secreting cells in preference to a non-target cell type selected from the group consisting of motor neurons and autonomic neurons; wherein said neurotoxin derivative lacks a functional Clostridial toxin Hcc domain and wherein a target cell internalizes at least the endopeptidase domain of said Clostridial neurotoxin derivative upon binding of said TL to the target cell.

37) The neurotoxin derivative of embodiment 36 wherein the neurotoxin derivative contains a Clostridial neurotoxin-derived Hcc domain mutated to impede binding of the HCC domain to its natural protein receptor.

38) The neurotoxin derivative of embodiment 37 comprising an active 3/4 N domain.

39) The neurotoxin derivative of embodiment 38 wherein said first active Clostridial toxin-derived endopeptidase domain is derived from a toxin serotype selected from the group consisting of BoNT/A, BoNT/E, BoNT/C1, and BoNT/D.

40) The neurotoxin derivative of embodiment 38 wherein the Clostridial neurotoxin translocation domain is selected from the group consisting of
a) a BoNT-A translocation domain;
b) a BoNT-B translocation domain;
c) a BoNT-Cl translocation domain;
d) a BoNT-D translocation domain;
e) a BoNT-E translocation domain;
f) a BoNT-F translocation domain;
g) a BoNT-G translocation domain, and
h) conservatively modified variants and isoforms of any of the above.

41) The neurotoxin derivative of embodiment 38 in which the endopeptidase domain and the first active endopeptidase domain are both derived from the same BoNT serotype.

42) The neurotoxin derivative of embodiment 37 comprising a second active or inactive Clostridial toxin-derived endopeptidase domain.

43) The neurotoxin derivative of embodiment 42 in which the second Clostridial toxin-derived endopeptidase domain lacks endopeptidase protease activity effective to substantially cleave a population of SNARE proteins under physiological conditions.

44) The neurotoxin derivative of embodiment 37 in which the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP-selective or CGRP-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof, and conservatively modified variants and isoforms of any of the above.

45) The neurotoxin derivative of embodiment 37 comprising at least two TL domains.

46) The neurotoxin derivative of embodiment 37 in which said first endopeptidase domain and said translocation domain are both derived from BoNT/D.

47) The neurotoxin derivative of embodiment 37 in which said first endopeptidase domain and said translocation domain are both derived from BoNT/A.

48) The neurotoxin derivative of embodiment 46 in which the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP-selective or CGRP-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist.
or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof, and conservatively modified variants and isoforms of any of the above.

49) The neurotoxin derivative of embodiment 47 in which the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP-selective or CGRP-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof, and conservatively modified variants and isoforms of any of the above.

50) The neurotoxin derivative of embodiment 36 in which said first endopeptidase domain and said translocation domain are both derived from BoNT/C.

51) The neurotoxin derivative of embodiment 50 in which the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP-selective or CGRP-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof, and conservatively modified variants and isoforms of any of the above.

52) A nucleic acid encoding a polypeptide comprising the Clostridial neurotoxin derivative of embodiment 1.

53) A nucleic acid encoding a polypeptide comprising the Clostridial neurotoxin derivative of embodiment 36.

54) A host cell selected from the group consisting of a bacterial host cell, yeast, a mammalian cell, and an insect cell, wherein the host cell contains a nucleic acid vector comprising the nucleic acid of embodiment 52.

55) The host cell of embodiment 52 wherein the host cell is an E. coli cell.

56) A cleared E. coli cell lysate comprising the Clostridial neurotoxin derivative of embodiment 1.
57) A host cell selected from the group consisting of a bacterial host cell, yeast, a mammalian cell, and an insect cell, wherein the host cell contains a nucleic acid vector comprising the nucleic acid of embodiment 53.

58) The host cell of embodiment 53 wherein the host cell is an E. coli cell.

59) A cleared E. coli cell lysate comprising the Clostridial neurotoxin derivative of embodiment 36.

60) A method of treating a patient in need of treatment for pain or prophylaxis for pain, comprising administering to a patient a Clostridial neurotoxin derivative of embodiment 1.

61) The method of embodiment 60 in which said administration is by injection.

62) The method of embodiment 60 wherein said pain is chronic pain.

63) The method of embodiment 62 wherein said chronic pain is selected from the group consisting of inflammatory nociceptive pain and neuropathic pain.

64) The method of embodiment 62 wherein said chronic pain is neuropathic pain.

65) The method of embodiment 64 wherein said neuropathic pain is selected from the group consisting of cancer pain, post-operative pain, neuropathic pain, allodynia, post-herpetic neuralgia, irritable bowel syndrome, and other visceral pain, bone pain, peripheral neuropathy, circulatory system-affiliated pain, and headache pain.

66) A method of treating a patient in need of treatment for pain or prophylaxis for pain, comprising administering to a patient a Clostridial neurotoxin derivative of embodiment 36.

67) The method of embodiment 66 in which said administration is by injection.

68) The method of embodiment 66 wherein said pain is chronic pain.

69) The method of embodiment 68 wherein said chronic pain is selected from the group consisting of inflammatory nociceptive pain and neuropathic pain.

70) The method of embodiment 68 wherein said chronic pain is neuropathic pain.

71) The method of embodiment 70 wherein said neuropathic pain is selected from the group consisting of cancer pain, post-operative pain, neuropathic pain, arthritis pain, allodynia, post-herpetic neuralgia, irritable bowel syndrome, and other visceral pain, bone pain, peripheral neuropathy, circulatory system-affiliated pain, and headache pain.
Any and all patents, publications, patent applications, and nucleotide and/or amino acid sequences referred to by accession numbers cited in this specification are hereby incorporated by reference as part of this specification in its entirety. Each and every feature described herein, and each and every combination of two or more of such features, is included within the scope of the present invention provided that the features included in such a combination are not mutually inconsistent. These and other aspects of the present invention are set forth in the following claims.
CLAIMS:

1) A composition comprising a Clostridial neurotoxin derivative, said composition comprising:
   a) a first active Clostridial toxin-derived endopeptidase domain which cleaves a SNARE protein under physiological conditions;
   b) a Clostridial toxin-derived translocation domain which facilitates the movement of said first endopeptidase domain across a cellular membrane into the cytosol under physiological conditions;
   c) a Clostridial toxin-derived functional H\textsubscript{CN} domain; and
   d) a non-Clostridial toxin derived binding domain comprising a first targeting ligand (TL) selectively binding, under physiological conditions, to a first cell surface receptor displayed by a target cell, said target cell selected from the group consisting of
      i) a sensory neuron, and
      ii) a cell that secretes at least one inflammatory cytokine;

said cell surface receptor being substantially absent from motor or autonomic neurons; wherein said light chain protease of said Clostridial neurotoxin derivative is internalized by said target cell upon binding of said TL to the target cell, and wherein an \textbf{Hcc} targeting domain is either absent or is mutated to impede binding of the \textbf{Hcc} domain to its natural protein receptor.

2) The composition of claim 1 wherein said \textbf{Hcc} targeting domain is present as a mutated, inactive \textbf{Hcc} domain.

3) The composition of claim 2 wherein said \textbf{Hcc} targeting domain is mutated to comprise one or both of: a glutamic acid residue at a position corresponding to amino acid 1192 of BoNT/B, and a lysine residue at a position corresponding to amino acid 1196 of BoNT/B.
4) The composition of any of the preceding claims wherein said mutated Hcc domain is derived from BoNT/B.

5) The composition of any of the preceding claims wherein said neurotoxin derivative comprises a second Clostridial toxin-derived endopeptidase domain.

6) The composition of claim 5 wherein said second endopeptidase domain is proteolytically active.

7) The composition of claim 5 wherein said second endopeptidase domain is substantially proteolytically inactive.

8) The composition of any of the preceding claims wherein each of the first Clostridial toxin-derived endopeptidase domain and the translocation domain is individually derived from a BoNT/X subtype selected from the group consisting of BoNT/A, Bo/NT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/G, and BoNT/F.

9) The composition of any of the preceding claims wherein said target cell is a cell that secretes at least one inflammatory cytokine, wherein said cell is selected from the group consisting of a macrophage, a synoviocyte, and a mast cell.

10) The composition of any of the preceding claims in which the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP receptor-selective or CGRP receptor-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof, and conservatively modified variants and isoforms of any of the above.

11) An analgesic Clostridial neurotoxin derivative comprising:
a) A first active Clostridial toxin-derived endopeptidase domain which cleaves a SNARE protein under physiological conditions and has an enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological conditions;
b) a Clostridial toxin-derived translocation domain which facilitates the movement of said first endopeptidase domain across a cellular membrane into the cytosol under physiological conditions;
c) a Clostridial toxin-derived functional HcN domain; and
d) a binding domain comprising a first targeting ligand (TL) selectively binding, under physiological conditions, to a first cell surface receptor displayed by a target cell type selected from the group consisting of:
i) sensory neurons, and
ii) cytokine secreting cells
in preference to a non-target cell type selected from the group consisting of motor neurons and autonomic neurons;

wherein said neurotoxin derivative lacks a functional Clostridial toxin Hc domain and wherein a target cell internalizes said first endopeptidase domain of said Clostridial neurotoxin derivative upon binding of said TL to the target cell.

12) The neurotoxin derivative of claim 11 wherein said target cell is a cell that secretes at least one inflammatory cytokine, wherein said cell is selected from the group consisting of a macrophage, a synoviocyte, and a mast cell.

13) The composition of either of claims 11 or 12 wherein said Hc targeting domain is present as a mutated, inactive Hc domain.

14) The composition of claim 13 wherein said Hc targeting domain is mutated to comprise one or both of: a glutamic acid residue at a position corresponding to amino acid 1192 of BoNT/B, and a lysine residue at a position corresponding to amino acid 1196 of BoNT/B.
15) The composition of any of claims 11 to 14, further comprising a second Clostridial toxin-derived endopeptidase domain.

16) The neurotoxin derivative of any of the preceding claims in which the TL comprises a targeting component selected from the group consisting of a CGRP antagonist, a CGRP receptor-selective or CGRP receptor-specific antibody or selective fragment thereof, a TRPV1 antagonist, a TRPV1-selective or TRPV1-specific antibody or fragment thereof, an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof, or a P2X3 antagonist, or a P2X3-selective or P2X3-specific antibody or fragment thereof, and conservatively modified variants and isoforms of any of the above.

17) The neurotoxin derivative of any of the preceding claims in which said first endopeptidase domain and said translocation domain are both derived from BoNT/D.

18) The neurotoxin derivative of any of the preceding claims in which said first endopeptidase domain and said translocation domain are both derived from BoNT/A.

19) The neurotoxin derivative of any of the preceding claims in which said first endopeptidase domain and said translocation domain are both derived from BoNT/Cl.

20) A nucleic acid encoding a polypeptide comprising the Clostridial neurotoxin derivative of any of the preceding claims.

21) A host cell selected from the group consisting of a bacterial host cell, yeast, a mammalian cell, and an insect cell, wherein the host cell contains a nucleic acid vector comprising the nucleic acid of claim 20.
22) A cleared cell lysate comprising the Clostridial neurotoxin derivative of any of claims 1-19.


24) The compound for use according to claim 23, wherein said chronic pain is selected from the group consisting of cancer pain, post-operative pain, neuropathic pain, allodynia, post-herpetic neuralgia, irritable bowel syndrome, and other visceral pain, arthritis pain, bone pain, peripheral neuropathy, circulatory system-affiliated pain, and headache pain.

25) The compound for use according to claim 24 for the treatment of arthritis.

26) The compound for use according to claim 24 wherein the TL comprises an interleukin 1 agonist or an interleukin 1 receptor antagonist, an IL-1 receptor selective or IL-1 receptor specific antibody or fragment thereof.

27) A Clostridial neurotoxin derivative comprising:
   a) a first active Clostridial toxin-derived endopeptidase domain which cleaves a SNARE protein under physiological conditions and has an enzymatic half-life of 10 days or greater when injected into mouse gastrocnemius muscle under substantially physiological conditions;
   b) a second Clostridial toxin-derived endopeptidase domain;
   c) a Clostridial toxin-derived translocation domain which facilitates the movement of said first endopeptidase domain across a cellular membrane into the cytosol under physiological conditions; and
   d) a Clostridial toxin-derived functional H C N domain.
FIG. 4

Targeting to sensory neurons

Targeting ligand (TL)

(scFvs for P2X, TRPV1 or others)

Human IL-1RA

CGRPβ-37

Core therapeutic

BoNT domains as in Embodiment A, B or C
% Inhibition of TNF-α (□) and IL-6 (□)

**FIG. 8B**
KD SNAP-23 (□) (% of control)

**FIG. 8D**
KD VAMP-3 (□) (% of control)

**FIG. 8A**

**FIG. 8C**

**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K38/48 C12N9/64 A61P29/00

**ADD.**

According to International Patent Classification (IPC) onto both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K C12N A61P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>WO 2007/138336 A2 (SYNTAXIN LTD [GB])</td>
<td>1,2, 4-13, 15, 17-25, 27</td>
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<td>ALLERGAN INC [US]; FOSTER KEITH [GB]; CHADDOCK JOHN</td>
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<td>see cl.1-10, pages 23, 26-27, 33 and 66-69, examples 30-45</td>
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<td>STEWARD LANCE E [US]; FERNANDEZ-SALAS ESTER [US]; F</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

**A** document defining the general state of the art which is not considered to be of particular relevance.

**E** earlier application or patent but published on or after the international filing date.

**L** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified).

**O** document referring to an oral disclosure, use, exhibition or other means.

**P** document published prior to the international filing date but later than the priority date claimed.

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Name and mailing address of the ISA:

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer

Merckling-Rui z, V
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