CATIONIC NEUROTOXINS

The present invention relates to engineered clostridial toxins comprising at least one amino acid modification, and the use of such engineered clostridial toxins in medicine and therapy.

Bacteria in the genus *Clostridia* produce highly potent and specific protein toxins, which can poison neurons and other cells to which they are delivered. Examples of such clostridial toxins include the neurotoxins produced by *C. tetani* (TeNT) and by *C. botulinum* (BoNT) serotypes A-G, as well as those produced by *C. baratii* and *C. butyricum*.

Among the clostridial toxins are some of the most potent toxins known. By way of example, botulinum neurotoxins have median lethal dose (LD_{50}) values for mice ranging from 0.5 to 5 ng/kg, depending on the serotype. Both tetanus and botulinum toxins act by inhibiting the function of affected neurons, specifically the release of neurotransmitters. While botulinum toxin acts at the neuromuscular junction and inhibits cholinergic transmission in the peripheral nervous system, tetanus toxin acts in the central nervous system.

In nature, clostridial toxins are synthesised as a single-chain polypeptide that is modified post-translationally by a proteolytic cleavage event to form two polypeptide chains joined together by a disulphide bond. Cleavage occurs at a specific cleavage site, often referred to as the activation site, that is located between the cysteine residues that provide the inter-chain disulphide bond. It is this di-chain form that is the active form of the toxin. The two chains are termed the heavy chain (H-chain), which has a molecular mass of approximately 100 kDa, and the light chain (L-chain), which has a molecular mass of approximately 50 kDa. The H-chain comprises an N-terminal translocation component (H_N domain) and a C-terminal targeting component (H_C domain). The cleavage site is located between the L-chain and the translocation domain components. Following binding of the H_C domain to its target neuron and internalisation of the bound toxin into the cell via an endosome, the H_N domain translocates the L-chain across the endosomal membrane and into the cytosol, and the L-chain provides a protease function (also known as a non-cytotoxic protease).

Non-cytotoxic proteases act by proteolytically cleaving intracellular transport proteins known as SNARE proteins (e.g. SNAP-25, VAMP, or Syntaxin) – see Gerald K (2002) "Cell and Molecular Biology" (4th edition) *John Wiley & Sons, Inc.* The acronym SNARE derives from the term Soluble NSF Attachment Receptor, where NSF means N-ethylmaleimide-Sensitive Factor. SNARE proteins are integral to intracellular vesicle fusion, and thus to secretion of molecules via vesicle transport from a cell. The protease function is a zinc-dependent endopeptidase activity and exhibits a high substrate specificity for SNARE proteins. Accordingly, once delivered to a desired target cell, the non-cytotoxic protease is capable of inhibiting cellular secretion from the target cell. The L-chain proteases of clostridial toxins are non-cytotoxic proteases that cleave SNARE proteins.

In view of the ubiquitous nature of SNARE proteins, clostridial toxins such as botulinum toxin have been successfully employed in a wide range of therapies.

By way of example, we refer to William J. Lipham, Cosmetic and Clinical Applications of Botulinum Toxin (Slack, Inc., 2004), which describes the use of clostridial toxins, such as botulinum neurotoxins (BoNTs), BoNT/A, BoNT/B, BoNT/C_1, BoNT/D, BoNT/E, BoNT/F and BoNT/G, and tetanus neurotoxin (TeNT), to inhibit neuronal transmission in a wide variety of therapeutic and cosmetic applications - as an example, BOTOX^{TM} is currently approved as a therapeutic 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. In addition, clostridial toxin therapies are described for treating neuromuscular disorders (see US 6,872,397); for treating uterine disorders (see US 2004/0175399); for treating ulcers and gastroesophageal reflux disease (see US 2004/0086531); for treating dystonia (see US 6,319,505); for treating eye disorders (see US 2004/0234532); for treating blepharospasm (see US 2004/0151740); for treating strabismus (see US 2004/0126396); for treating pain (see US 6,869,610, US 6,641,820, US 6,464,986, and US 6,113,915); for treating fibromyalgia (see US 6,623,742, US 2004/0062776); for treating lower back pain (see US 2004/0037852); for treating muscle injuries (see

US 6,423,319); for treating sinus headache (see US 6,838,434); for treating tension headache (see US 6,776,992); for treating headache (see US 6,458,365); for reduction of migraine headache pain (see US 5,714,469); for treating cardiovascular diseases (see US 6,767,544); for treating neurological disorders such as Parkinson's disease (see US 6,620,415, US 6,306,403); for treating neuropsychiatric disorders (see US 2004/0180061, US 2003/0211121); for treating endocrine disorders (see US 6,827,931); for treating thyroid disorders (see US 6,740,321); for treating cholinergic influenced sweat gland disorders (see US 6,683,049); for treating diabetes (see US 6,337,075, US 6,416,765); for treating a pancreatic disorder (see US 6,261,572, US 6,143,306); for treating cancers such as bone tumors (see US 6,565,870, US 6,368,605, US 6,139,845, US 2005/0031648); for treating otic disorders (see US 6,358,926, US 6,265,379); for treating autonomic disorders such as gastrointestinal muscle disorders and other smooth muscle dysfunction (see US 5,437,291); for treatment of skin lesions associated with cutaneous cell-proliferative disorders (see US 5,670,484); for management of neurogenic inflammatory disorders (see US 6,063,768); for reducing hair loss and stimulating hair growth (see US 6,299,893); for treating downturned mouth (see US 6,358,917); for reducing appetite (see US 2004/40253274); for dental therapies and procedures (see US 2004/0115139); for treating neuromuscular disorders and conditions (see US 2002/0010138); for treating various disorders and conditions and associated pain (see US 2004/0013692); for treating conditions resulting from mucus hypersecretion such as asthma and COPD (see WO 00/10598); and for treating non-neuronal conditions such as inflammation, endocrine conditions, exocrine conditions, immunological conditions, cardiovascular conditions, bone conditions (see WO 01/21213). All of the above publications are hereby incorporated by reference in their entirety.

The use of non-cytotoxic proteases such as clostridial toxins (e.g. BoNTs and TeNT) in therapeutic and cosmetic treatments of humans and other mammals is anticipated to expand to an ever-widening range of diseases and ailments that can benefit from the properties of these toxins.

To avoid systemic neurological effects, many clostridial toxin therapies utilise direct administration of the clostridial toxin therapeutic to a given target site (such as a target tissue). A problem when administering clostridial toxin-based therapeutics in

this fashion is the spread of toxin away from the administration site and into surrounding tissue or systemic circulation. The diffusion of toxin away from the target tissue is believed to be responsible for undesirable side effects that in extreme cases may be life threatening. This can be a particular concern when using clostridial toxin therapeutics (such as BoNT therapeutics) at high doses, concentrations and injection volumes. Adverse effects associated with this problem that have been reported for commercial BoNT/A therapeutics include asthenia, generalised muscle weakness, diplopia, ptosis, dysphagia, dysphonia, dysarthria, urinary incontinence, and breathing difficulties. Swallowing and breathing difficulties can be life threatening and there have been reported deaths related to the spread of toxin effects.

There is therefore a need in the art for clostridial toxins which have properties of increased tissue retention at the site of administration, and which accordingly exhibit a reduction in diffusion away from the administration site, as compared to known clostridial toxins.

The present invention solves the above problem by providing engineered clostridial toxins, as specified in the claims.

In one aspect, the invention provides an engineered clostridial toxin comprising at least one amino acid modification, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification. In one embodiment, said at least one amino acid modification increases the pI of the engineered clostridial toxin to a value that is at least 0.4 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification. In one embodiment, said at least one amino acid modification increases the pI of the engineered clostridial toxin to a value that is at least 0.5 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification. In one embodiment, said at least one amino acid modification increases the pI of the engineered clostridial toxin to a value that is at least 0.6 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification. In one embodiment, said at

least one amino acid modification increases the pI of the engineered clostridial toxin to a value that is at least 0.8 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification. In one embodiment, said at least one amino acid modification increases the pI of the engineered clostridial toxin to a value that is at least 1 pI unit higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

In one aspect, the invention provides an engineered clostridial toxin comprising at least one amino acid modification, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least one pI unit higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

In certain embodiments, the engineered clostridial toxin comprises at least 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 or 80 amino acid modifications.

In certain embodiments, said at least one amino acid modification increases the pI of the engineered clostridial toxin to a value that is at least 2, 3, 4 or 5 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

The present inventors have found that by increasing the pI of a clostridial toxin, for example, by at least 0.2 pI units, or 0.5 pI units, or one pI unit (through the introduction into the clostridial toxin protein of at least one amino acid modification), the resultant engineered clostridial toxin advantageously demonstrates properties of increased tissue retention and reduced diffusion away from sites of administration, while retaining abilities of target cell binding, translocation, and cleavage of target SNARE protein(s). Thus, the spread of clostridial toxin from the site of administration is significantly reduced, as compared to an otherwise identical clostridial toxin lacking said at least one amino acid modification.

The engineered clostridial toxins of the invention are suitable for use in any of the therapies described above, and advantageously may demonstrate a reduction in, or absence of, side effects compared to the use of known clostridial toxin therapeutics.

The increased tissue retention properties of the engineered clostridial toxins of the invention also provide increased potency and/or duration of action, and can allow for reduced dosages to be used compared to known clostridial toxin therapeutics (or increased dosages without any additional adverse effects), thus providing further advantages.

As discussed below in more detail, the increase in pI provided by the at least one amino acid modification means that an engineered clostridial toxin of the invention has, at a given pH, a net charge that is more positive than the net charge on an otherwise identical clostridial toxin lacking said at least one amino acid modification. Without wishing to be bound by any one theory, the present inventors believe that this increased positive charge allows the engineered clostridial toxins of the present invention to display longer tissue retention times at the site of administration due to favourable electrostatic interactions between the engineered clostridial toxin and anionic extracellular components (such as cell membranes and heparin sulphate proteoglycans) at the site of administration. These improved electrostatic interactions serve to reduce the diffusion of the engineered clostridial toxin away from the site of administration, thus improving tissue retention.

By way of example, the improved tissue retention properties of an engineered clostridial toxin of the invention may allow for (i) higher doses into individual muscles, such as the sternocleidomastoid, without spreading into nearby muscles in the neck to cause difficult swallowing, and (ii) higher total doses (to all muscles) in a single treatment, without spreading into the circulation and causing systemic effects such as difficult breathing. Advantages to patients may include more effective treatment of large muscles such as the sternocleidomastoid muscle, increased opportunity to inject several different muscles during each treatment, and possible longer duration of effective treatment (longer before re-treatment is necessary) because of higher dosing.

In one embodiment, an engineered clostridial toxin of the invention has, in use, a positive net charge (for example, when the engineered clostridial toxin, in use, is located at a desired administration site in a tissue).

The isoelectric point (pI) is a specific property of a given protein. As is well known in the art, proteins are made from a specific sequence of amino acids (also referred to when in a protein as amino acid residues). Each amino acid of the standard set of twenty has a different side chain (or R group), meaning that each amino acid residue in a protein displays different chemical properties such as charge and hydrophobicity. These properties may be influenced by the surrounding chemical environment, such as the temperature and pH. The overall chemical characteristics of a protein will depend on the sum of these various factors.

Certain amino acid residues (detailed below) possess ionisable side chains that may display an electric charge depending on the surrounding pH. Whether such a side chain is charged or not at a given pH depends on the pKa of the relevant ionisable moiety, wherein pKa is the negative logarithm of the acid dissociation constant (Ka) for a specified proton from a conjugate base.

For example, acidic residues such as aspartic acid and glutamic acid have side chain carboxylic acid groups with pKa values of approximately 4.1 (precise pKa values may depend on temperature, ionic strength and the microenvironment of the ionisable group). Thus, these side chains exhibit a negative charge at a pH of 7.4 (often referred to as "physiological pH"). At low pH values, these side chains will become protonated and lose their charge.

Conversely, basic residues such as lysine and arginine have nitrogen-containing side chain groups with pKa values of approximately 10-12. These side chains therefore exhibit a positive charge at a pH of 7.4. These side chains will become de-protonated and lose their charge at high pH values.

The overall (net) charge of a protein molecule therefore depends on the number of acidic and basic residues present in the protein (and their degree of surface exposure) and on the surrounding pH. Changing the surrounding pH changes the overall charge on the protein. Accordingly, for every protein there is a given pH at which the number of positive and negative charges is equal and the protein displays no overall net charge. This point is known as the isoelectric point (pI). The isoelectric point is a

standard concept in protein biochemistry with which the skilled person would be familiar.

The isoelectric point (pI) is therefore defined as the pH value at which a protein displays a net charge of zero. An increase in pI means that a higher pH value is required for the protein to display a net charge of zero. Thus, an increase in pI represents an increase in the net positive charge of a protein at a given pH. Conversely, a decrease in pI means that a lower pH value is required for the protein to display a net charge of zero. Thus, a decrease in pI represents a decrease in the net positive charge of a protein at a given pH.

Methods of determining the pI of a protein are known in the art and would be familiar to a skilled person. By way of example, the pI of a protein can be calculated from the average pKa values of each amino acid present in the protein. Alternatively, the pI of a protein can be determined experimentally using the technique of isoelectric focusing. This technique uses electrophoresis to separate proteins according to their pI. Isoelectric focusing is typically performed using a gel that has an immobilised pH gradient. When an electric field is applied, the protein migrates through the pH gradient until it reaches the pH at which it has zero net charge, this point being the pI of the protein.

The pI of a protein may be increased or decreased by altering the number of basic and/or acidic groups displayed on its surface. This can be achieved by modifying one or more amino acids of the protein. For example, an increase in pI may be provided by reducing the number of acidic residues, or by increasing the number of basic residues. Such amino acid modifications are discussed in more detail below.

Native (unmodified) clostridial toxins have a pI of approximately 5-6. Thus, at a pH of 7.4, native botulinum toxins possess a negative net charge. By way of example, the pI of BoNT/A is 6.4, and a BoNT/A molecule has a net charge at pH 7.4 of -8. These pI values are calculated as described above.

TABLE 1:

CLOSTRIDIAL TOXIN	pI
BoNT/A	6.4
BoNT/B	5.3
BoNT/C_1	5.5
BoNT/D	5.5
BoNT/E	6.0
BoNT/F	5.6
BoNT/G	5.2
TeNT	5.8

As described above, in one embodiment, an engineered clostridial toxin of the present invention comprises at least one amino acid modification, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

Thus, in the context of the present invention, an increase in pI of 0.2 units in the context of an engineered BoNT/A clostridial toxin would be an increase in pI from 6.4 to 6.6.

As described above, in one embodiment, an engineered clostridial toxin of the present invention comprises at least one amino acid modification, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least one pI unit higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

Thus, in the context of the present invention, an increase in pI of 1 unit in the context of an engineered BoNT/A clostridial toxin would be an increase in pI from 6.4 to 7.4.

In one embodiment, said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least two pI units

higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

In one embodiment, said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is between 2 and 5 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

In one embodiment, the engineered clostridial toxin has a pI of at least 6 (for example, at least 6, at least 7, at least 8, or at least 9).

In one embodiment, the engineered clostridial toxin has a pI of at least 7.

In one embodiment, the engineered clostridial toxin has a pI of between 6 and 10 (for example a pI of between 7 and 9, or a pI of between 8 and 9).

As discussed above, the engineered clostridial toxins of the present invention have increased tissue retention properties that also provide increased potency and/or duration of action, and can allow for reduced dosages to be used compared to known clostridial toxin therapeutics (or increased dosages without any additional effects). One way in which these advantageous properties (which represent an increase in the therapeutic index) may be defined is in terms of the Safety Ratio of the engineered clostridial toxin. In this regard, undesired effects of a clostridial toxin (caused by diffusion of the toxin away from the site of administration) can be assessed experimentally by measuring percentage bodyweight loss in a relevant animal model (e.g. a mouse, where loss of bodyweight is detected within seven days of administration). Conversely, desired on-target effects of a clostridial toxin can be assessed experimentally by Digital Abduction Score (DAS) assay, a measurement of muscle paralysis. The DAS assay may be performed by injection of 20µl of clostridial toxin, formulated in Gelatin Phosphate Buffer, into the mouse gastrocnemius/soleus complex, followed by assessment of Digital Abduction Score using the method of Aoki (Aoki KR, Toxicon 39: 1815-1820; 2001). In the DAS assay, mice are suspended briefly by the tail in order to elicit a characteristic startle response in which the mouse extends its hind limbs and abducts its hind digits. Following clostridial

toxin injection, the varying degrees of digit abduction are scored on a five-point scale (0=normal to 4=maximal reduction in digit abduction and leg extension).

The Safety Ratio of a clostridial toxin may then be expressed as the ratio between the amount of toxin required for a 10% drop in a bodyweight (measured at peak effect within the first seven days after dosing in a mouse) and the amount of toxin required for a DAS score of 2. High Safety Ratio scores are therefore desired, and indicate a toxin that is able to effectively paralyse a target muscle with little undesired off-target effects. An engineered toxin of the present invention has a Safety Ratio that is higher than the Safety Ratio of an equivalent unmodified (native) botulinum toxin.

Thus, in one embodiment, an engineered clostridial toxin of the present invention has a Safety Ratio of at least 8 (for example, at least 8, 9, 10, 15, 20, 25, 30, 35, 40, 45 or 50), wherein Safety Ratio is calculated as: dose of toxin required for -10% bodyweight change (pg/mouse) divided by DAS ED_{50} (pg/mouse) [ED_{50} = dose required to produce a DAS score of 2].

In one embodiment, an engineered clostridial toxin of the present invention has a Safety Ratio of at least 10. In one embodiment, an engineered clostridial toxin of the present invention has a Safety Ratio of at least 15.

An engineered clostridial toxin of the present invention comprises at least one amino acid modification. Said at least one amino acid modification increases the pI of the clostridial toxin, as discussed above. In the context of the present invention, an amino acid modification is a modification of the amino acid sequence of a clostridial toxin. Such a modification may be effected by replacing one amino acid in the sequence with another (i.e. a substitution), by inserting a new amino acid into the sequence, or by deleting an amino acid of the sequence. Amino acids incorporated into an amino acid sequence in a protein are also referred to as amino acid residues.

The 20 standard amino acids found in proteins are as follows:

TABLE 2:

AMINO ACID			SIDE CHAIN
Aspartic acid	Asp	D	Charged (acidic)
Glutamic acid	Glu	E	Charged (acidic)
Arginine	Arg	R	Charged (basic)
Lysine	Lys	K	Charged (basic)
Histidine	His	H	Uncharged (polar)
Asparagine	Asn	N	Uncharged (polar)
Glutamine	Gln	Q	Uncharged (polar)
Serine	Ser	S	Uncharged (polar)
Threonine	Thr	T	Uncharged (polar)
Tyrosine	Tyr	Y	Uncharged (polar)
Methionine	Met	M	Uncharged (polar)
Tryptophan	Trp	W	Uncharged (polar)
Cysteine	Cys	C	Uncharged (polar)
Alanine	Ala	A	Uncharged (hydrophobic)
Glycine	Gly	G	Uncharged (hydrophobic)
Valine	Val	V	Uncharged (hydrophobic)
Leucine	Leu	L	Uncharged (hydrophobic)
Isoleucine	Ile	I	Uncharged (hydrophobic)
Proline	Pro	P	Uncharged (hydrophobic)
Phenylalanine	Phe	F	Uncharged (hydrophobic)

The following amino acids are considered charged amino acids: aspartic acid (negative), glutamic acid (negative), arginine (positive), and lysine (positive).

At a pH of 7.4, the side chains of aspartic acid (pKa 3.1) and glutamic acid (pKa 4.1) have a negative charge, while the side chains of arginine (pKa 12.5) and lysine (pKa 10.8) have a positive charge. Aspartic acid and glutamic acid are referred to as acidic amino acid residues. Arginine and lysine are referred to as basic amino acid residues.

The following amino acids are considered uncharged, polar (meaning they can participate in hydrogen bonding) amino acids: asparagine, glutamine, histidine, serine, threonine, tyrosine, cysteine, methionine, tryptophan.

The following amino acids are considered uncharged, hydrophobic amino acids: alanine, valine, leucine, isoleucine, phenylalanine, proline, and glycine.

An increase in the pI of a clostridial toxin can be effected by introducing into the clostridial toxin one or more amino acid modifications that increases the ratio of positive to negative charges in the clostridial toxin.

In one embodiment, the at least one amino acid modification is selected from: an amino acid substitution, an amino acid insertion, and an amino acid deletion.

In an amino acid substitution, an amino acid residue that forms part of the clostridial toxin amino acid sequence is replaced with a different amino acid residue. The replacement amino acid residue may be one of the 20 standard amino acids, as described above.

Alternatively, the replacement amino acid in an amino acid substitution may be a non-standard amino acid (an amino acid that is not part of the standard set of 20 described above). By way of example, the replacement amino acid may be a basic non-standard amino acid, e.g. L-Ornithine, L-2-amino-3-guanidinopropionic acid, or D-isomers of Lysine, Arginine and Ornithine). Methods for introducing non-standard amino acids into proteins are known in the art, and include recombinant protein synthesis using *E. coli* auxotrophic expression hosts.

In an amino acid insertion, an additional amino acid residue (one that is not normally present) is incorporated into the clostridial toxin amino acid sequence, thus increasing the total number of amino acid residues in said sequence. In an amino acid deletion, an amino acid residue is removed from the clostridial toxin amino acid sequence, thus reducing the total number of amino acid residues in said sequence.

Methods for modifying proteins by substitution, insertion or deletion of amino acid residues are known in the art. By way of example, amino acid modifications may be introduced by modification of a DNA sequence encoding a clostridial toxin. This can be achieved using standard molecular cloning techniques, for example by site-directed mutagenesis where short strands of DNA (oligonucleotides) coding for the desired amino acid(s) are used to replace the original coding sequence using a polymerase enzyme, or by inserting/deleting parts of the gene with various enzymes (e.g., ligases and restriction endonucleases). Alternatively a modified gene sequence can be chemically synthesised.

In one embodiment, the at least one amino acid modification is selected from: substitution of an acidic amino acid residue with a basic amino acid residue; substitution of an acidic amino acid residue with an uncharged amino acid residue; substitution of an uncharged amino acid residue with a basic amino acid residue; insertion of a basic amino acid residue; and deletion of an acidic amino acid residue.

In a preferred embodiment, the at least one amino acid modification is a substitution, which advantageously maintains the same number of amino acid residues in the clostridial toxin. In one embodiment, the substitution is selected from: substitution of an acidic amino acid residue with a basic amino acid residue, substitution of an acidic amino acid residue with an uncharged amino acid residue, and substitution of an uncharged amino acid residue with a basic amino acid residue. In one embodiment, the basic amino acid residue is a lysine residue or an arginine residue. In one embodiment, the basic amino acid residue is a lysine residue. In one embodiment, the basic amino acid residue is an arginine residue. In one embodiment, wherein the substitution is a substitution of an acidic amino acid residue with an uncharged amino acid residue, the acidic amino acid residue is replaced with its corresponding uncharged amide amino acid residue (i.e. aspartic acid is replaced with asparagine, and glutamic acid is replaced with glutamine).

An engineered clostridial toxin of the invention may comprise more than one amino acid modification. Thus, in one embodiment, the engineered clostridial toxin (as described above) comprises between 1 and 80 amino acid modifications (for example, between 1 and 70, between 1 and 60, between 1 and 50, between 4 and 40, between 4

and 30, between 5 and 40, between 5 and 30, or between 10 and 25 amino acid modifications). In one embodiment, the engineered clostridial toxin (as described above) comprises between 4 and 40 amino acid modifications. In one embodiment, the engineered clostridial toxin comprises at least 2, at least 3, at least 4, at least 5, or at least 10 amino acid modifications. In one embodiment, the engineered clostridial toxin comprises at least 4 amino acid modifications (for example, at least 4 amino acid substitutions). Each of said amino acid modifications is an amino acid modification as described above. Thus, each of said amino acid modifications contributes to the increase in pI of the engineered clostridial toxin (as compared to the pI of an otherwise identical clostridial toxin lacking said amino acid modifications).

Any clostridial toxin amino acid (i.e. amino acid residue) can be modified as described above, as long as the outcome of said modification is an increase in the clostridial toxin pI (as described above). However, the present inventors have identified subsets of clostridial toxin amino acids that are particularly suitable targets for modification.

Preferred target amino acids may possess certain qualities. By way of example, a preferred target amino acid may be: (i) a surface exposed amino acid; (ii) located outside of a clostridial toxin protein secondary structure; (iii) located in a clostridial toxin protein region that is non-essential for protein function; (iv) an amino acid whose identity is not conserved between clostridial toxin types, subtypes, or serotypes; (iv) an amino acid whose modification does not create a predicted ubiquitination site; or (v) any combination of the foregoing.

As discussed above, clostridial toxins are formed from two polypeptide chains, the heavy chain (H-chain), which has a molecular mass of approximately 100 kDa, and the light chain (L-chain), which has a molecular mass of approximately 50 kDa. The H-chain comprises a C-terminal targeting component (receptor binding domain or H_C domain) and an N-terminal translocation component (H_N domain).

In one embodiment, the at least one amino acid modification (as described above) is located in the clostridial toxin receptor binding domain (H_C domain).

Examples of light chain reference sequences include:

Botulinum type A neurotoxin: amino acid residues 1-448

Botulinum type B neurotoxin: amino acid residues 1-440

Botulinum type C_1 neurotoxin: amino acid residues 1-441

Botulinum type D neurotoxin: amino acid residues 1-445

Botulinum type E neurotoxin: amino acid residues 1-422

Botulinum type F neurotoxin: amino acid residues 1-439

Botulinum type G neurotoxin: amino acid residues 1-441

Tetanus neurotoxin: amino acid residues 1-457

The above-identified reference sequences should be considered a guide, as slight variations may occur according to sub-serotypes. By way of example, US 2007/0166332 (hereby incorporated by reference in its entirety) cites slightly different clostridial sequences:

Botulinum type A neurotoxin: amino acid residues M1-K448

Botulinum type B neurotoxin: amino acid residues M1-K441

Botulinum type C_1 neurotoxin: amino acid residues M1-K449

Botulinum type D neurotoxin: amino acid residues M1-R445

Botulinum type E neurotoxin: amino acid residues M1-R422

Botulinum type F neurotoxin: amino acid residues M1-K439

Botulinum type G neurotoxin: amino acid residues M1-K446

Tetanus neurotoxin: amino acid residues M1-A457

Examples of clostridial toxin H_C domain reference sequences include:

BoNT/A - N872-L1296

BoNT/B - E859-E1291

BoNT/C_1 - N867-E1291

BoNT/D - S863-E1276

BoNT/E - R846-K1252

BoNT/F - K865-E1274

BoNT/G - N864-E1297

TeNT - I880-D1315

The H_C domain of a clostridial toxin (such as a BoNT) comprises two distinct structural features that are referred to as the H_{CC} and H_{CN} domains. Amino acid residues involved in receptor binding are believed to be primarily located in the H_{CC} domain.

In one embodiment, wherein the at least one amino acid modification (as described above) is located in the clostridial toxin receptor binding domain (H_C domain), said at least one amino acid modification is located in the clostridial toxin H_{CN} domain (also referred to as a translocation facilitating domain). In one embodiment, wherein the at least one amino acid modification (as described above) is located in the clostridial toxin receptor binding domain (H_C domain), said at least one amino acid modification is located in the clostridial toxin H_{CC} domain.

Examples of clostridial toxin H_{CN} domain reference sequences include:
Botulinum type A neurotoxin: amino acid residues 872-1110
Botulinum type B neurotoxin: amino acid residues 859-1097
Botulinum type C_1 neurotoxin: amino acid residues 867-1111
Botulinum type D neurotoxin: amino acid residues 863-1098
Botulinum type E neurotoxin: amino acid residues 846-1085
Botulinum type F neurotoxin: amino acid residues 865-1105
Botulinum type G neurotoxin: amino acid residues 864-1105
Tetanus neurotoxin: amino acid residues 880-1127

The above sequence positions may vary slightly according to serotype/sub-type, and further examples of suitable (reference) clostridial toxin H_{CN} domains include:
Botulinum type A neurotoxin: amino acid residues 874-1110
Botulinum type B neurotoxin: amino acid residues 861-1097
Botulinum type C_1 neurotoxin: amino acid residues 869-1111
Botulinum type D neurotoxin: amino acid residues 865-1098
Botulinum type E neurotoxin: amino acid residues 848-1085
Botulinum type F neurotoxin: amino acid residues 867-1105
Botulinum type G neurotoxin: amino acid residues 866-1105
Tetanus neurotoxin: amino acid residues 882-1127

In one embodiment, the at least one amino acid modification (as described above) is a modification of a surface exposed amino acid residue. Surface exposed amino acid residues are those present on the exterior of a folded protein and so accessible to the surrounding solvent, in contrast to those amino acid residues that are located in the interior of a folded protein. The degree of surface exposure of an amino acid residue and thus its exposure to the surrounding solvent depends on its position within the folded protein, and also on the conformation adopted by the protein. Modification of an amino acid residue with a high degree of surface exposure may therefore have a greater effect on the protein's isoelectric point than modification of an amino acid residue with a low degree of surface exposure. Methods for determining the degree of surface exposure of an amino acid residue are known in the art. By way of example, the computer program AreaIMol (part of the CCP4 suite of computer programs) can be used to calculate the degree of surface exposure of amino acid residues in a given protein. Surface exposed amino acid residues may also be identified by visual inspection of a protein crystal structure (such as provided by X-ray crystallography). In one embodiment, a surface exposed amino acid residue has a sum AreaIMol value of at least 40.

In one embodiment, the at least one amino acid modification comprises modification of an amino acid residue selected from: an aspartic acid residue, a glutamic acid residue, a histidine residue, a serine residue, a threonine residue, an asparagine residue, a glutamine residue, a cysteine residue, or a tyrosine residue. The present inventors have identified that amino acid residues from this group (negatively charged residues and polar residues) represent particularly suitable targets for modification according to the present invention. Without wishing to be bound by any one theory, the present inventors believe that amino acid residues in this group appear on the surface of a clostridial toxin with a greater frequency than the hydrophobic residues not listed.

In one embodiment, wherein the amino acid modification comprises modification of an amino acid residue selected from an aspartic acid residue, a glutamic acid residue, a histidine residue, a serine residue, a threonine residue, an asparagine residue, a glutamine residue, a cysteine residue, or a tyrosine residue (as described above), the amino acid residue is substituted with a lysine residue or an arginine residue. Thus, in

one embodiment, a negatively charged residue or a polar residue is substituted with a positively charged residue, thus increasing the ratio of positive to negative charges and increasing the pI of the clostridial toxin.

In one embodiment, the at least one amino acid modification (as described above) comprises modification of an asparagine amino acid residue or a glutamine amino acid residue (both uncharged, polar residues). In one embodiment, the asparagine or glutamine amino acid residue is substituted with a lysine residue or an arginine residue (both positively charged residues). In one embodiment, the asparagine or glutamine amino acid residue is substituted with a lysine residue. In one embodiment, the asparagine or glutamine amino acid residue is substituted with an arginine residue.

Asparagine and glutamine residues are advantageously suitable for modification as they are polar, form only weak dipole interactions with other residues, and constitute 14% of a typical clostridial toxin molecule (such as BoNT/A).

In one embodiment, the engineered clostridial toxin is a BoNT/A. A reference BoNT/A sequence has the UniProtKB Accession Number P10845.

The present inventors have identified certain amino acids that represent preferred targets for amino acid modification in a BoNT/A clostridial toxin.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20 or 25) amino acid(s) selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086, and GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the

amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, or 25) amino acid(s) selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086, and GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, or 25) amino acid(s) selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086, and GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1,

2, 3, 4, 5, 10, 15, or 20) amino acid(s) selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, and ASP 1086; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, 25, or 30) amino acid(s) selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086, ASN 1188, ASP 1213, GLY 1215, ASN 1216, GLN 1229, ASN 1242, ASN 1243, SER 1274, and THR 1277; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, 25, or 30) amino acid(s) selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086,

ASN 1188, ASP 1213, GLY 1215, ASN 1216, GLN 1229, ASN 1242, ASN 1243, SER 1274, and THR 1277; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, 25, or 30) amino acid(s) selected from: ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086, ASN 1188, ASP 1213, GLY 1215, ASN 1216, GLN 1229, ASN 1242, ASN 1243, SER 1274, and THR 1277; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 1 pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, or 6) amino acid(s) selected from: ASN 886, ASN 930, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine

residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, or 6) amino acid(s) selected from: ASN 886, ASN 930, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, or 6) amino acid(s) selected from: ASN 886, ASN 930, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following seven amino acids: ASN 886, ASN 930, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking

said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following seven amino acids: ASN 886, ASN 930, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following seven amino acids: ASN 886, ASN 930, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, or 6) amino acid(s) selected from: ASN 930, ASN 954, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for

example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, or 6) amino acid(s) selected from: ASN 930, ASN 954, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, or 6) amino acid(s) selected from: ASN 930, ASN 954, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following seven amino acids: ASN 930, ASN 954, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and

said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following seven amino acids: ASN 930, ASN 954, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following seven amino acids: ASN 930, ASN 954, SER 955, GLN 991, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1,

2, 3, 4, 5, or 6) amino acid(s) selected from: ASN 930, SER 955, GLN 991, ASN 1025, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, or 6) amino acid(s) selected from: ASN 930, SER 955, GLN 991, ASN 1025, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, or 6) amino acid(s) selected from: ASN 930, SER 955, GLN 991, ASN 1025, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following seven amino acids: ASN 930, SER 955, GLN 991, ASN 1025, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following seven amino acids: ASN 930, SER 955, GLN 991, ASN 1025, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following seven amino acids: ASN 930, SER 955, GLN 991, ASN 1025, ASN 1026, ASN 1052, or GLN 1229; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 6 or 7) amino acid(s) selected from: ASN 1188, ASP 1213, GLY 1215, ASN 1216, ASN 1242, ASN 1243, SER 1274, THR 1277; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 6 or 7) amino acid(s) selected from: ASN 1188, ASP 1213, GLY 1215, ASN 1216, ASN 1242, ASN 1243, SER 1274, THR 1277; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 6 or 7) amino acid(s) selected from: ASN 1188, ASP 1213, GLY 1215, ASN 1216, ASN 1242, ASN 1243, SER 1274, THR 1277; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue.

In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following eight amino acids: ASN 1188, ASP 1213, GLY 1215, ASN 1216, ASN 1242, ASN 1243, SER 1274, THR 1277; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following eight amino acids: ASN 1188, ASP 1213, GLY 1215, ASN 1216, ASN 1242, ASN 1243, SER 1274, THR 1277; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of the following eight amino acids: ASN 1188, ASP 1213, GLY 1215, ASN 1216, ASN 1242, ASN 1243, SER 1274, THR 1277; and said amino acid modifications increase the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modifications. In one

embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment, said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 6, or all 7) amino acid(s) selected from: ASN 930, SER 955, GLN 991, ASN 1026, ASN 1052, HIS 1064, and GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or all 11) amino acid(s) selected from: ASN 886, ASN 930, ASN 954, SER 955, GLN 991, ASN 1026, ASN 1052, HIS 1064, ASN 1080, ASN 1147, and GLN 1229; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/A, said engineered BoNT/A comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or all 13) amino acid(s) selected from: ASN 886, ASN 905, ASN 930, ASN 954, SER 955, GLN 991, ASN 1025, ASN 1026, ASN 1052, HIS 1064, ASN 1080, ASN 1147, and GLN 1229; and said amino acid

modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/A to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/A lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, the engineered clostridial toxin is a BoNT/B. A reference BoNT/B sequence has the UniProtKB Accession Number P10844.

The present inventors have identified certain amino acids that represent preferred targets for amino acid modification in a BoNT/B clostridial toxin.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/B, said engineered BoNT/B comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, or 25) amino acid selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, and ASN 1273; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/B to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/B lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/B, said engineered BoNT/B comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, or 25) amino acid selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP

1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, and ASN 1273; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/B to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/B lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/B, said engineered BoNT/B comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, or 25) amino acid selected from: ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, and ASN 1273; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/B to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/B lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, the engineered clostridial toxin is a BoNT/C_1. A reference BoNT/C_1 sequence has the UniProtKB Accession Number P18640.

The present inventors have identified certain amino acids that represent preferred targets for amino acid modification in a BoNT/C_1 clostridial toxin.

Thus, in one embodiment, wherein the engineered clostridial toxin is a BoNT/C_1, said engineered BoNT/C_1 comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, or 15) amino acid selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN

1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/C_1 to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/C_1 lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

Thus, in one embodiment, wherein the engineered clostridial toxin is a BoNT/C_1, said engineered BoNT/C_1 comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, or 15) amino acid selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/C_1 to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/C_1 lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/C_1, said engineered BoNT/C_1 comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, or 15) amino acid selected from: ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, and ASP 1081; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/C_1 to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/C_1 lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the

amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, the engineered clostridial toxin is a BoNT/D. A reference BoNT/D sequence has the UniProtKB Accession Number P19321.

The present inventors have identified certain amino acids that represent preferred targets for amino acid modification in a BoNT/D clostridial toxin.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/D, said engineered BoNT/D comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, or 20) amino acid selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, and GLN 1122; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/D to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/D lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/D, said engineered BoNT/D comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, or 20) amino acid selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, and GLN 1122; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/D to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/D lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the

amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/D, said engineered BoNT/D comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, or 20) amino acid selected from: ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, and GLN 1122; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/D to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/D lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, the engineered clostridial toxin is a BoNT/E. A reference BoNT/E sequence has the UniProtKB Accession Number Q00496.

The present inventors have identified certain amino acids that represent preferred targets for amino acid modification in a BoNT/E clostridial toxin.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/E, said engineered BoNT/E comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, or 25) amino acid selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/E to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/E lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one

embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/E, said engineered BoNT/E comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, or 25) amino acid selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/E to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/E lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/E, said engineered BoNT/E comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, 20, or 25) amino acid selected from: ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/E to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/E lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, the engineered clostridial toxin is a BoNT/F. A reference BoNT/F sequence has the UniProtKB Accession Number YP_001390123.

The present inventors have identified certain amino acids that represent preferred targets for amino acid modification in a BoNT/F clostridial toxin.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/F, said engineered BoNT/F comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, or 20) amino acid selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/F to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/F lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/F, said engineered BoNT/F comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, or 20) amino acid selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/F to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI unit higher than the pI of an otherwise identical BoNT/F lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/F, said engineered BoNT/F comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, 15, or 20) amino acid selected from: ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, and ASP 1056; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/F to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/F lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, the engineered clostridial toxin is a BoNT/G. A reference BoNT/G sequence has the UniProtKB Accession Number Q60393.

The present inventors have identified certain amino acids that represent preferred targets for amino acid modification in a BoNT/G clostridial toxin.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/G, said engineered BoNT/G comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, or 15) amino acid selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/G to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/G lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/G, said engineered BoNT/G comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, or 15) amino acid selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/G to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI units higher than the pI of an otherwise identical BoNT/G lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a BoNT/G, said engineered BoNT/G comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, or 15) amino acid selected from: ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, and ASN 1090; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered BoNT/G to a value that is at least one pI unit higher than the pI of an otherwise identical BoNT/G lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, the engineered clostridial toxin is a TeNT. A reference TeNT sequence has the UniProtKB Accession Number P04958.

The present inventors have identified certain amino acids that represent preferred targets for amino acid modification in a TeNT clostridial toxin.

In one embodiment, wherein the engineered clostridial toxin is a TeNT, said engineered TeNT comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, or 15) amino acid selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered TeNT to a value that is at least 0.2 (for example, at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI unit higher than the pI of an otherwise identical TeNT lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a TeNT, said engineered TeNT comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, or 15) amino acid selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered TeNT to a value that is at least 0.5 (for example, at least 0.5, 0.6, 0.7, 0.8, 0.9 or 1) pI unit higher than the pI of an otherwise identical TeNT lacking said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

In one embodiment, wherein the engineered clostridial toxin is a TeNT, said engineered TeNT comprises a modification of at least one (for example, at least 1, 2, 3, 4, 5, 10, or 15) amino acid selected from: ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, and ASN 1097; and said amino acid modification(s) increase(s) the isoelectric point (pI) of the engineered TeNT to a value that is at least one pI unit higher than the pI of an otherwise identical TeNT lacking

said amino acid modification(s). In one embodiment said modification comprises substitution of the amino acid with a lysine residue or an arginine residue. In one embodiment, said modification comprises substitution of the amino acid with a lysine residue. In one embodiment said modification comprises substitution of the amino acid with an arginine residue.

The present invention is suitable for application to many different varieties of clostridial toxin. Thus, in the context of the present invention, the term "clostridial toxin" embraces toxins produced by *C. botulinum* (botulinum neurotoxin serotypes A, B, C_1, D, E, F and G), *C. tetani* (tetanus neurotoxin), *C. butyricum* (botulinum neurotoxin serotype E), and *C. baratii* (botulinum neurotoxin serotype F), as well as modified clostridial toxins or derivatives derived from any of the foregoing. The term "clostridial toxin" also embraces botulinum neurotoxin serotype H.

Botulinum neurotoxin (BoNT) is produced by *C. botulinum* in the form of a large protein complex, consisting of BoNT itself complexed to a number of accessory proteins. There are at present eight different classes of botulinum neurotoxin, namely: botulinum neurotoxin serotypes A, B, C_1, D, E, F, G, and H, all of which share similar structures and modes of action. Different BoNT serotypes can be distinguished based on inactivation by specific neutralising anti-sera, with such classification by serotype correlating with percentage sequence identity at the amino acid level. BoNT proteins of a given serotype are further divided into different subtypes on the basis of amino acid percentage sequence identity.

BoNTs are absorbed in the gastrointestinal tract, and, after entering the general circulation, bind to the presynaptic membrane of cholinergic nerve terminals and prevent the release of their neurotransmitter acetylcholine. BoNT/B, BoNT/D, BoNT/F and BoNT/G cleave synaptobrevin/vesicle-associated membrane protein (VAMP); BoNT/C_1, BoNT/A and BoNT/E cleave the synaptosomal-associated protein of 25 kDa (SNAP-25); and BoNT/C_1 cleaves syntaxin.

Tetanus toxin is produced in a single serotype by *C. tetani*. *C. butyricum* produces BoNT/E, while *C. baratii* produces BoNT/F.

The term "clostridial toxin" is also intended to embrace modified clostridial toxins and derivatives thereof, including but not limited to those described below. A modified clostridial toxin or derivative may contain one or more amino acids that has been modified as compared to the native (unmodified) form of the clostridial toxin, or may contain one or more inserted amino acids that are not present in the native (unmodified) form of the clostridial toxin. By way of example, a modified clostridial toxin may have modified amino acid sequences in one or more domains relative to the native (unmodified) clostridial toxin sequence. Such modifications may modify functional aspects of the toxin, for example biological activity or persistence. Thus, in one embodiment, the engineered clostridial toxin of the invention is an engineered modified clostridial toxin, or an engineered modified clostridial toxin derivative, or an engineered clostridial toxin derivative.

A modified clostridial toxin may have one or more modifications in the amino acid sequence of the heavy chain (such as a modified H_C domain), wherein said modified heavy chain binds to target nerve cells with a higher or lower affinity than the native (unmodified) clostridial toxin. Such modifications in the H_C domain can include modifying residues in the ganglioside binding site of the H_C domain or in the protein (SV2 or synaptotagmin) binding site that alter binding to the ganglioside receptor and/or the protein receptor of the target nerve cell. Examples of such modified clostridial toxins are described in WO 2006/027207 and WO 2006/114308, both of which are hereby incorporated by reference in their entirety.

A modified clostridial toxin may have one or more modifications in the amino acid sequence of the light chain, for example modifications in the substrate binding or catalytic domain which may alter or modify the SNARE protein specificity of the modified LC. Examples of such modified clostridial toxins are described in WO 2010/120766 and US 2011/0318385, both of which are hereby incorporated by reference in their entirety.

A modified clostridial toxin may comprise one or more modifications that increases or decreases the biological activity and/or the biological persistence of the modified clostridial toxin. For example, a modified clostridial toxin may comprise a leucine- or tyrosine-based motif, wherein said motif increases or decreases the biological activity

and/or the biological persistence of the modified clostridial toxin. Suitable leucine-based motifs include xDxxxLL, xExxxLL, xExxxIL, and xExxxLM (wherein x is any amino acid). Suitable tyrosine-based motifs include Y-x-x-Hy (wherein Hy is a hydrophobic amino acid). Examples of modified clostridial toxins comprising leucine- and tyrosine-based motifs are described in WO 2002/08268, which is hereby incorporated by reference in its entirety.

The term "clostridial toxin" is intended to embrace hybrid and chimeric clostridial toxins. A hybrid clostridial toxin comprises at least a portion of a light chain from one clostridial toxin or subtype thereof, and at least a portion of a heavy chain from another clostridial toxin or clostridial toxin subtype. In one embodiment the hybrid clostridial toxin may contain the entire light chain of a light chain from one clostridial toxin subtype and the heavy chain from another clostridial toxin subtype. In another embodiment, a chimeric clostridial toxin may contain a portion (e.g. the binding domain) of the heavy chain of one clostridial toxin subtype, with another portion of the heavy chain being from another clostridial toxin subtype. Similarly or alternatively, the therapeutic element may comprise light chain portions from different clostridial toxins. Such hybrid or chimeric clostridial toxins are useful, for example, as a means of delivering the therapeutic benefits of such clostridial toxins to patients who are immunologically resistant to a given clostridial toxin subtype, to patients who may have a lower than average concentration of receptors to a given clostridial toxin heavy chain binding domain, or to patients who may have a protease-resistant variant of the membrane or vesicle toxin substrate (e.g., SNAP-25, VAMP and syntaxin). Hybrid and chimeric clostridial toxins are described in US 8,071,110, which publication is hereby incorporated by reference in its entirety. Thus, in one embodiment, the engineered clostridial toxin of the invention is an engineered hybrid clostridial toxin, or an engineered chimeric clostridial toxin.

The term "clostridial toxin" is intended to embrace re-targeted clostridial toxins. In a re-targeted clostridial toxin, the clostridial toxin is modified to include an exogenous ligand known as a Targeting Moiety (TM). The TM is selected to provide binding specificity for a desired target cell, and as part of the re-targeting process the native binding portion of the clostridial toxin (e.g. the H_C domain, or the H_{CC} domain) may be removed. Re-targeting technology is described, for example, in: EP-B-0689459;

WO 1994/021300; EP-B-0939818; US 6,461,617; US 7,192,596; WO 1998/007864; EP-B-0826051; US 5,989,545; US 6,395,513; US 6,962,703; WO 1996/033273; EP-B-0996468; US 7,052,702; WO 1999/017806; EP-B-1107794; US 6,632,440; WO 2000/010598; WO 2001/21213; WO 2006/059093; WO 2000/62814; WO 2000/04926; WO 1993/15766; WO 2000/61192; and WO 1999/58571; all of which are hereby incorporated by reference in their entirety. Thus, in one embodiment, the engineered clostridial toxin of the invention is an engineered re-targeted clostridial toxin.

The present invention also embraces clostridial toxins that have a non-native protease cleavage site. In such clostridial toxins, the native protease cleavage site (also known as the activation site, as described above) is modified or replaced with a protease cleavage site that is not native to that clostridial toxin (i.e. an exogenous cleavage site). Such a site will require an exogenous protease for cleavage, which allows for improved control over the timing and location of cleavage events. Non-native protease cleavage sites that may be employed in clostridial toxins include:

Enterokinase	(DDDDK↓)
Factor Xa	(IEGR↓ / IDGR↓)
TEV(Tobacco Etch virus)	(ENLYFQ↓G)
Thrombin	(LVPR↓GS)
PreScission	(LEVLFQ↓GP).

Additional protease cleavage sites include recognition sequences that are cleaved by a non-cytotoxic protease, for example by the light chain of a clostridial neurotoxin. These include the SNARE (e.g. SNAP-25, syntaxin, VAMP) protein recognition sequences that are cleaved by non-cytotoxic proteases such as the light chain of a clostridial neurotoxin. Clostridial toxins comprising non-native protease cleavage sites are described in US 7,132,259, EP 1206554-B2 and US 2007/0166332, all of which are hereby incorporated by reference in their entirety. Also embraced by the term protease cleavage site is an intein, which is a self-cleaving sequence. The self-splicing reaction is controllable, for example by varying the concentration of reducing agent present.

The present invention also embraces clostridial toxins comprising a "destructive cleavage site". In said clostridial toxins, a non-native protease cleavage site is

incorporated into the clostridial toxin, at a location chosen such that cleavage at said site will decrease the activity of, or inactivate, the clostridial toxin. The destructive protease cleavage site can be susceptible to cleavage by a local protease, in the event that the clostridial toxin, following administration, migrates to a non-target location. Suitable non-native protease cleavage sites include those described above. Clostridial toxins comprising a destructive cleavage site are described in WO 2010/094905 and WO 2002/044199, both of which are hereby incorporated by reference in their entirety.

The engineered clostridial toxins of the present invention, especially the light chain component thereof, may be PEGylated – this may help to increase stability, for example duration of action of the light chain component. PEGylation is particularly preferred when the light chain comprises a BoNT/A, B or C_1 protease. PEGylation preferably includes the addition of PEG to the N-terminus of the light chain component. By way of example, the N-terminus of a light chain may be extended with one or more amino acid (e.g. cysteine) residues, which may be the same or different. One or more of said amino acid residues may have its own PEG molecule attached (e.g. covalently attached) thereto. An example of this technology is described in WO2007/104567, which is hereby incorporated by reference in its entirety.

The engineered clostridial toxins of the present invention may be free from the complexing proteins that are present in a naturally occurring clostridial toxin complex.

An engineered clostridial toxin of the present invention may also comprise a limited number of non-standard amino acids. Thus, in addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-N-methyl lysine, 2-aminoisobutyric acid, isovaline and α-methyl serine) may be substituted for amino acid residues of the engineered clostridial toxins of the present invention. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for clostridial polypeptide amino acid residues. The engineered clostridial toxins of the present invention can also comprise non-naturally occurring amino acid residues.

Non-naturally occurring amino acids include, without limitation, trans-3-methylproline, 2,4-methano-proline, cis-4-hydroxyproline, trans-4-hydroxy-proline, N-methylglycine, allo-threonine, methyl-threonine, hydroxy-ethylcysteine, hydroxyethylhomo-cysteine, nitro-glutamine, homoglutamine, pipecolic acid, tert-leucine, norvaline, 2-azaphenylalanine, 3-azaphenyl-alanine, 4-azaphenyl-alanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., J. Am. Chem. Soc. 113:2722, 1991; Ellman et al., Methods Enzymol. 202:301, 1991; Chung et al., Science 259:806-9, 1993; and Chung et al., Proc. Natl. Acad. Sci. USA 90:10145-9, 1993). In a second method, translation is carried out in Xenopus oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., J. Biol. Chem. 271:19991-8, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the polypeptide in place of its natural counterpart. See, Koide et al., Biochem. 33:7470-6, 1994.

The engineered clostridial toxins of the present invention can be produced using recombinant nucleic acid technologies. Thus, in one embodiment, an engineered clostridial toxin (as described above) is a recombinant engineered clostridial toxin.

In another aspect, the present invention provides a nucleic acid (for example, a DNA) comprising a nucleic acid sequence encoding an engineered clostridial toxin as described above. In one embodiment, the nucleic acid sequence is prepared as part of a DNA vector comprising a promoter and a terminator.

In a preferred embodiment, the vector has a promoter selected from:

Promoter	Induction Agent	Typical Induction Condition
Tac (hybrid)	IPTG	0.2 mM (0.05-2.0mM)
AraBAD	L-arabinose	0.2% (0.002-0.4%)
T7-lac operator	IPTG	0.2 mM (0.05-2.0mM)

In another preferred embodiment, the vector has a promoter selected from:

Promoter	Induction Agent	Typical Induction Condition
Tac (hybrid)	IPTG	0.2 mM (0.05-2.0mM)
AraBAD	L-arabinose	0.2% (0.002-0.4%)
T7-lac operator	IPTG	0.2 mM (0.05-2.0mM)
T5-lac operator	IPTG	0.2 mM (0.05-2.0mM)

The nucleic acid molecules of the invention may be made using any suitable process known in the art. Thus, the nucleic acid molecules may be made using chemical synthesis techniques. Alternatively, the nucleic acid molecules of the invention may be made using molecular biology techniques.

The DNA construct of the present invention is preferably designed *in silico*, and then synthesised by conventional DNA synthesis techniques.

The above-mentioned nucleic acid sequence information is optionally modified for codon-biasing according to the ultimate host cell (e.g. *E. coli*) expression system that is to be employed.

In one embodiment, the nucleic acid sequence encoding an engineered clostridial toxin as described above is a nucleic acid sequence having at least 70% (for example, at least 75, 80, 85, 90, 95, 97, 98 or 99%) sequence identity to a nucleic acid sequence selected from SEQ ID NOs: 3, 5, 7 and 9.

In one aspect, the present invention provides a nucleic acid sequence having at least 70% (for example, at least 75, 80, 85, 90, 95, 97, 98 or 99%) sequence identity to a

nucleic acid sequence selected from SEQ ID NOs: 3, 5, 7 and 9. In one embodiment, the nucleic acid sequence has at least 90% sequence identity to a nucleic acid sequence selected from SEQ ID NOs: 3, 5, 7 and 9.

The present invention also provides polypeptides encoded by nucleic acid sequences as described above. Thus, in one aspect, the present invention provides a polypeptide comprising an amino acid sequence having at least 70% (for example, at least 75, 80, 85, 90, 95, 97, 98 or 99%) sequence identity to an amino acid sequence selected from SEQ ID NOs: 4, 6, 8 and 10. In one embodiment, the amino acid sequence has at least 90% sequence identity to an amino acid sequence selected from SEQ ID NOs: 4, 6, 8 and 10.

In one embodiment, the engineered clostridial toxin of the invention is an engineered BoNT/A as described above, and said engineered BoNT/A comprises (or consists of) an amino acid sequence having at least 70% (for example, at least 75, 80, 85, 90, 95, 97, 98, 99, 99.5 or 99.9%) sequence identity to an amino acid sequence selected from SEQ ID NOs: 4, 6, 8 and 10.

In one embodiment, the engineered clostridial toxin of the invention is an engineered BoNT/A as described above, and said engineered BoNT/A comprises (or consists of) the amino acid sequence of SEQ ID NO: 4, 6, 8 or 10.

In one aspect, the invention provides a polypeptide comprising (or consisting of) the amino acid sequence of SEQ ID NO: 4, 6, 8 or 10.

In one aspect, the invention provides a nucleic acid encoding an engineered clostridial toxin as described above, wherein said nucleic acid comprises a nucleic acid sequence having at least 70% (for example, at least 75, 80, 85, 90, 95, 97, 98, 99, 99.5 or 99.9%) sequence identity to a nucleic acid sequence selected from SEQ ID NOs: 3, 5, 7 and 9. In one embodiment, the nucleic acid comprises (or consists of) the nucleic acid sequence of SEQ ID NO: 3, 5, 7 or 9.

In one aspect, the invention provides a nucleic acid comprising (or consisting of) the nucleic acid sequence of SEQ ID NO: 3, 5, 7 or 9.

The "percent sequence identity" between two or more nucleic acid or amino acid sequences is a function of the number of identical positions shared by the sequences. Thus, % identity may be calculated as the number of identical nucleotides / amino acids divided by the total number of nucleotides / amino acids, multiplied by 100. Calculations of % sequence identity may also take into account the number of gaps, and the length of each gap that needs to be introduced to optimize alignment of two or more sequences. Sequence comparisons and the determination of percent identity between two or more sequences can be carried out using specific mathematical algorithms, such as BLAST, which will be familiar to a skilled person.

In one aspect, the present invention provides a method of producing a single-chain engineered clostridial toxin protein having a light chain and a heavy chain, the method comprising expressing a nucleic acid (said nucleic acid being as described above) in a suitable host cell, lysing the host cell to provide a host cell homogenate containing the single-chain engineered clostridial toxin protein, and isolating the single-chain engineered clostridial toxin protein.

In another aspect, the present invention provides a method of activating an engineered clostridial toxin, the method comprising providing a single-chain engineered clostridial toxin protein obtainable by the method of producing a single-chain engineered clostridial toxin protein as described above, contacting the polypeptide with a protease that cleaves the polypeptide at a recognition site (cleavage site) located between the light chain and heavy chain, thereby converting the polypeptide into a di-chain polypeptide wherein the light chain and heavy chain are joined together by a disulphide bond.

The engineered clostridial toxins of the invention may be used to prevent or treat certain medical or cosmetic diseases and conditions. Thus, in a further aspect, the present invention provides an engineered clostridial toxin as described above, for use in medicine.

In a related aspect, the present invention provides an engineered clostridial toxin as described above, for use in the prevention or treatment of a disease or condition selected from: strabismus, blepharospasm, squint, dystonia (e.g. spasmodic dystonia,

oromandibular dystonia, focal dystonia, tardive dystonia, laryngeal dystonia, limb dystonia, cervical dystonia), torticollis (e.g. spasmodic torticollis), beauty therapy (cosmetic) applications benefiting from cell/muscle incapacitation (via SNARE down-regulation or inactivation), neuromuscular disorder or condition of ocular motility (e.g. concomitant strabismus, vertical strabismus, lateral rectus palsy, nystagmus, dysthyroid myopathy), writer's cramp, blepharospasm, bruxism, Wilson's disease, tremor, tics, segmental myoclonus, spasms, spasticity due to chronic multiple sclerosis, spasticity resulting in abnormal bladder control, animus, back spasm, charley horse, tension headaches, levator pelvic syndrome, spina bifida, tardive dyskinesia, Parkinson's disease, stuttering, hemifacial spasm, eyelid disorder, cerebral palsy, focal spasticity, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions, muscle pain (e.g. pain from muscle spasms), headache pain (e.g. tension headache), brow furrows, skin wrinkles, cancer, uterine disorders, uro-genital disorders, urogenital-neurological disorders, chronic neurogenic inflammation, and a smooth muscle disorder.

In use, the present invention employs a pharmaceutical composition, comprising an engineered clostridial toxin, together with at least one component selected from a pharmaceutically acceptable carrier, excipient, adjuvant, propellant and/or salt.

The engineered clostridial toxins of the present invention may be formulated for oral, parenteral, continuous infusion, inhalation or topical application. Compositions suitable for injection may be in the form of solutions, suspensions or emulsions, or dry powders which are dissolved or suspended in a suitable vehicle prior to use.

In the case of an engineered clostridial toxin that is to be delivered locally, the engineered clostridial toxin may be formulated as a cream (e.g. for topical application), or for sub-dermal injection.

Local delivery means may include an aerosol, or other spray (e.g. a nebuliser). In this regard, an aerosol formulation of an engineered clostridial toxin enables delivery to the lungs and/or other nasal and/or bronchial or airway passages.

Engineered clostridial toxins of the invention may be administered to a patient by intrathecal or epidural injection in the spinal column at the level of the spinal segment involved in the innervation of an affected organ.

A preferred route of administration is via laproscopic and/ or localised, particularly intramuscular, injection.

The dosage ranges for administration of the engineered clostridial toxins of the present invention are those to produce the desired therapeutic effect. It will be appreciated that the dosage range required depends on the precise nature of the engineered clostridial toxin or composition, the route of administration, the nature of the formulation, the age of the patient, the nature, extent or severity of the patient's condition, contraindications, if any, and the judgement of the attending physician. Variations in these dosage levels can be adjusted using standard empirical routines for optimisation.

Suitable daily dosages (per kg weight of patient) are in the range 0.0001-1 ng/kg, preferably 0.0001-0.5 ng/kg, more preferably 0.002-0.5 ng/kg, and particularly preferably 0.004-0.5 ng/kg. The unit dosage can vary from less than 1 picogram to 30ng, but typically will be in the region of 0.01 to 1 ng per dose, which may be administered daily or preferably less frequently, such as weekly or six monthly.
A particularly preferred dosing regimen is based on 0.05 ng of engineered clostridial toxin as the 1X dose. In this regard, preferred dosages are in the range 1X–100X (i.e. 0.05-5 ng).

Fluid dosage forms are typically prepared utilising the engineered clostridial toxin and a pyrogen-free sterile vehicle. The engineered clostridial toxin, depending on the vehicle and concentration used, can be either dissolved or suspended in the vehicle. In preparing solutions the engineered clostridial toxin can be dissolved in the vehicle, the solution being made isotonic if necessary by addition of sodium chloride and sterilised by filtration through a sterile filter using aseptic techniques before filling into suitable sterile vials or ampoules and sealing. Alternatively, if solution stability is adequate, the solution in its sealed containers may be sterilised by autoclaving. Advantageously additives such as buffering, solubilising, stabilising, preservative or

bactericidal, suspending or emulsifying agents and or local anaesthetic agents may be dissolved in the vehicle.

Dry powders, which are dissolved or suspended in a suitable vehicle prior to use, may be prepared by filling pre-sterilised ingredients into a sterile container using aseptic technique in a sterile area. Alternatively the ingredients may be dissolved into suitable containers using aseptic technique in a sterile area. The product is then freeze dried and the containers are sealed aseptically.

Parenteral suspensions, suitable for intramuscular, subcutaneous or intradermal injection, are prepared in substantially the same manner, except that the sterile components are suspended in the sterile vehicle, instead of being dissolved and sterilisation cannot be accomplished by filtration. The components may be isolated in a sterile state or alternatively it may be sterilised after isolation, e.g. by gamma irradiation.

Advantageously, a suspending agent for example polyvinylpyrrolidone is included in the composition(s) to facilitate uniform distribution of the components.

Administration in accordance with the present invention may take advantage of a variety of delivery technologies including microparticle encapsulation, viral delivery systems or high-pressure aerosol impingement.

List of Figures

Figure 1

Isoelectric focusing (IEF) gel of cationic constructs.

Figure 2

Percentage SNAP-25 cleavage in rat embryonic spinal cord neurons (eSCN) for Cat5v2(K1064H/N954K) (A), Cat5v2(K1064H/N886K) (B) and Cat5v2(K1064H/ N1025K) (C), and summary of pEC50 relative to nBoNT/A1. (A, B, C) Rat embryonic spinal cord neurons were cultured for three weeks and treated with Cat5v4 for 24 h, before Western blotting with SNAP-25 specific antibody. Data is mean ±SEM from three independent experiments in triplicate. (D) Relative potency of Cat5v2(K1064H/N886K), Cat5v2(K1064H/N954K) and Cat5v2(K1064H/ N1025K) to nBoNT/A1 (List Biological Laboratories) in the rat eSCN SNAP-25 cleavage potency assay. Each point corresponds to an individual batch and is a mean of 3 independent pEC50 determinations based on an 8-point concentration response curve (CRC). Each concentration in the CRC was assessed in triplicate. Potency comparisons are made to a mean of List batches, pooled data n=24. Data are mean ±SEM of n=3 batches per Cat5v4.

Figure 3

The potency (t_{50}) of nBoNT/A1 and Cat5v4 in the mouse phrenic nerve hemi-diaphragm assay (mPNHD). Mouse phrenic nerve hemi-diaphragm tissue was incubated with Cat5v4 or native BoNT/A1 as indicated. Diaphragm contractile force was recorded until the contraction was no longer detectable or after 140 minutes. Each point corresponds to independent determinations. The t_{50} value is the time required to inhibit the contractile force of the mouse hemi-diaphragm by 50%.

Sequences

SEQ ID NO: 1. BoNT/A1 nucleic acid sequence.

ATGCCATTCGTCAACAAGCAATTCAACTACAAAGACCCAGTCAACGGCGTCGACATCGCATACATCAAG
ATTCCGAACGCCGGTCAAATGCAGCCGGTTAAGGCTTTTAAGATCCACAACAAGATTTGGGTTATCCCG
GAGCGTGACACCTTCACGAACCCGGAAGAAGGCGATCTGAACCCGCCACCGGAAGCGAAGCAAGTCCCT
GTCAGCTACTACGATTCGACGTACCTGAGCACGGATAACGAAAAAGATAACTACCTGAAAGGTGTGACC
AAGCTGTTCGAACGTATCTACAGCACGGATCTGGGTCGCATGCTGCTGACTAGCATTGTTCGCGGTATC
CCGTTCTGGGGTGGTAGCACGATTGACACCGAACTGAAGGTTATCGACACTAACTGCATTAACGTTATT
CAACCGGATGGTAGCTATCGTAGCGAAGAGCTGAATCTGGTCATCATTGGCCCGAGCGCAGACATTATC
CAATTCGAGTGCAAGAGCTTTGGTCACGAGGTTCTGAATCTGACCCGCAATGGCTATGGTAGCACCCAG
TACATTCGTTTTTCGCCGGATTTTACCTTCGGCTTTGAAGAGAGCCTGGAGGTTGATACCAATCCGTTG
CTGGGTGCGGGCAAATTCGCTACCGATCCGGCTGTCACGCTGGCCCATGAACTGATCCACGCAGGCCAC
CGCCTGTACGGCATTGCCATCAACCCAAACCGTGTGTTCAAGGTTAATACGAATGCATACTACGAGATG
AGCGGCCTGGAAGTCAGCTTCGAAGAACTGCGCACCTTCGGTGGCCATGACGCTAAATTCATTGACAGC
TTGCAAGAGAATGAGTTCCGTCTGTACTACTATAACAAATTCAAAGACATTGCAAGCACGTTGAACAAG
GCCAAAAGCATCGTTGGTACTACCGCGTCGTTGCAGTATATGAAGAATGTGTTTAAAGAGAAGTACCTG
CTGTCCGAGGATACCTCCGGCAAGTTTAGCGTTGATAAGCTGAAGTTTGACAAACTGTACAAGATGCTG
ACCGAGATTTACACCGAGGACAACTTTGTGAAATTCTTCAAAGTGTTGAATCGTAAAACCTATCTGAAT
TTTGACAAAGCGGTTTTCAAGATTAACATCGTGCCGAAGGTGAACTACACCATCTATGACGGTTTTAAC
CTGCGTAACACCAACCTGGCGGCGAACTTTAACGGTCAGAATACGGAAATCAACAACATGAATTTCACG
AAGTTGAAGAACTTCACGGGTCTGTTCGAGTTCTATAAGCTGCTGTGCGTGCGCGGTATCATCACCAGC
AAAACCAAAAGCCTGGACAAAGGCTACAACAAGGCGCTGAATGACCTGTGCATTAAGGTAAACAATTGG
GATCTGTTCTTTTCGCCATCCGAAGATAATTTTACCAACGACCTGAACAAGGGTGAAGAAATCACCAGC
GATACGAATATTGAAGCAGCGGAAGAGAATATCAGCCTGGATCTGATCCAGCAGTACTATCTGACCTTT
AACTTCGACAATGAACCGGAGAACATTAGCATTGAGAATCTGAGCAGCGACATTATCGGTCAGCTGGAA
CTGATGCCGAATATCGAACGTTTCCCGAACGGCAAAAAGTACGAGCTGGACAAGTACACTATGTTCCAT
TACCTGCGTGCACAGGAGTTTGAACACGGTAAAAGCCGTATCGCGCTGACCAACAGCGTTAACGAGGCC
CTGCTGAACCCGAGCCGTGTCTATACCTTCTTCAGCAGCGACTATGTTAAGAAAGTGAACAAAGCCACT
GAGGCCGCGATGTTCCTGGGCTGGGTGGAACAGCTGGTATATGACTTCACGGACGAGACGAGCGAAGTG
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ATTCCGGAGATTGCGATCCCGGTGTTGGGTACCTTCGCGCTGGTGTCCTACATCGCGAATAAGGTTCTG
ACGGTTCAGACCATCGATAACGCGCTGTCGAAACGTAATGAAAAATGGGACGAGGTTTACAAATACATT
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AATAACATTAACTTCAATATCGATGATTTGAGCAGCAAGCTGAATGAATCTATCAACAAAGCGATGATC
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CAACTGAGCAAGTATGTTGATAATCAACGTCTGTTGAGCACTTTCACCGAGTATATCAAAAACATCATC
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GTCGTGGTTATGAAGAGCAAGAACGACCAGGGTATCACTAACAAGTGCAAGATGAACCTGCAAGACAAC
AATGGTAACGACATCGGCTTTATTGGTTTCCACCAGTTCAACAATATTGCTAAACTGGTAGCGAGCAAT

TGGTACAATCGTCAGATTGAGCGCAGCAGCCGTACTTTGGGCTGTAGCTGGGAGTTTATCCCGGTCGAT
GATGGTTGGGGCGAACGTCCGCTG

SEQ ID NO: 2. BoNT/A1 amino acid sequence.

MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMQPVKAFKIHNKIWVIPERDTFTNPEEGDLNPPPEAKQVP
VSYYDSTYLSTDNEKDNYLKGVTKLFERIYSTDLGRMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVI
QPDGSYRSEELNLVIIGPSADIIQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPL
LGAGKFATDPAVTLAHELIHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDS
LQENEFRLYYYNKFKDIASTLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDKLKFDKLYKML
TEIYTEDNFVKFFKVLNRKTYLNFDKAVFKINIVPKVNYTIYDGFNLRNTNLAANFNGQNTEINNMNFT
KLKNFTGLFEFYKLLCVRGIITSKTKSLDKGYNKALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITS
DTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENLSSDIIGQLELMPNIERFPNGKKYELDKYTMFH
YLRAQEFEHGKSRIALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAAMFLGWVEQLVYDFTDETSEV
STTDKIADITIIIPYIGPALNIGNMLYKDDFVGALIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVL
TVQTIDNALSKRNEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIINYQYNQYTEEEK
NNINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRG
TLIGQVDRLKDKVNNTLSTDIPFQLSKYVDNQRLLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASK
INIGSKVNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN
CMENNSGWKVSLNYGEIIWTLQDTQEIKQRVVFKYSQMINISDYINRWIFVTITNNRLNNSKIYINGRL
IDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFWGDY
LQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNI
VRNNDRVYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDN
NGNDIGFIGFHQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWGERPL

SEQ ID NO: 3. Engineered BoNT/A1 "Cat-A" nucleic acid sequence.

ATGCCATTCGTCAACAAGCAATTCAACTACAAAGACCCAGTCAACGGCGTCGACATCGCATACATCAAG
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AAGCTGTTCGAACGTATCTACAGCACGGATCTGGGTCGCATGCTGCTGACTAGCATTGTTCGCGGTATC
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CAACCGGATGGTAGCTATCGTAGCGAAGAGCTGAATCTGGTCATCATTGGCCCGAGCGCAGACATTATC
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TACATTCGTTTTTCGCCGGATTTTACCTTCGGCTTTGAAGAGAGCCTGGAGGTTGATACCAATCCGTTG
CTGGGTGCGGGCAAATTCGCTACCGATCCGGCTGTCACGCTGGCCCATGAACTGATCCACGCAGGCCAC
CGCCTGTACGGCATTGCCATCAACCCAAACCGTGTGTTCAAGGTTAATACGAATGCATACTACGAGATG
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GCCAAAAGCATCGTTGGTACTACCGCGTCGTTGCAGTATATGAAGAATGTGTTTAAAGAGAAGTACCTG
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AAGTTGAAGAACTTCACGGGTCTGTTCGAGTTCTATAAGCTGCTGTGCGTGCGCGGTATCATCACCAGC
AAAACCAAAAGCCTGGACAAAGGCTACAACAAGGCGCTGAATGACCTGTGCATTAAGGTAAACAATTGG
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ACCAAAGAGATCAAGCAGCGCGTCGTGTTCAAGTACTCTCAAATGATCAACATTTCCGATTACATTAAT
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GTCGTGGTTATGAAGAGCAAGAACGACAAGGGTATCACTAACAAGTGCAAGATGAACCTGCAAGACAAC
AATGGTAACGACATCGGCTTTATTGGTTTCCACCAGTTCAACAATATTGCTAAACTGGTAGCGAGCAAT
TGGTACAATCGTCAGATTGAGCGCAGCAGCcGTACTTTGGGCTGTAGCTGGGAGTTTATCCCGGTCGAT
GATGGTTGGGGCGAACGTCCGCTG

SEQ ID NO: 4. Engineered BoNT/A1 “Cat-A” amino acid sequence.

MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMQPVKAFKIHNKIWVIPERDTFTNPEEGDLNPPPEAKQVP
VSYYDSTYLSTDNEKDNYLKGVTKLFERIYSTDLGRMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVI
QPDGSYRSEELNLVIIGPSADIIQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPL
LGAGKFATDPAVTLAHELIHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDS
LQENEFRLYYYNKFKDIASTLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDKLKFDKLYKML
TEIYTEDNFVKFFKVLNRKTYLNFDKAVFKINIVPKVNYTIYDGFNLRNTNLAANFNGQNTEINNMNFT
KLKNFTGLFEFYKLLCVRGIITSKTKSLDKGYNKALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITS
DTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENLSSDIIGQLELMPNIERFPNGKKYELDKYTMFH
YLRAQEFEHGKSRIALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAAMFLGWVEQLVYDFTDETSEV
STTDKIADITIIIPYIGPALNIGNMLYKDDFVGALIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVL
TVQTIDNALSKRNEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIINYQYNQYTEEEK
NNINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRG
TLIGQVDRLKDKVNNTLSTDIPFQLSKYVDNQRLLSTFTEYIKNIINTSILNLRYESKHLIDLSRYASK
INIGSKVNFDPIDKNQIQLFNLESSKIEVILKKAIVYNSMYENFSTSFWIRIPKYFNKISLNNEYTIIN
CMENNSGWKVSLNYGEIIWTLQDTKEIKQRVVFKYSQMINISDYINRWIFVTITNNRLNKSKIYINGRL
IDQKPISNLGNIHASNKIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFWGDY
LQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNI
VRNNDRVYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDKGITNKCKMNLQDN
NGNDIGFIGFHQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWGERPL

SEQ ID NO: 5. Engineered BoNT/A1 “Cat-B” nucleic acid sequence.

ATGCCATTCGTCAACAAGCAATTCAACTACAAAGACCCAGTCAACGGCGTCGACATCGCATACATCAAG
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GTCAGCTACTACGATTCGACGTACCTGAGCACGGATAACGAAAAAGATAACTACCTGAAAGGTGTGACC
AAGCTGTTCGAACGTATCTACAGCACGGATCTGGGTCGCATGCTGCTGACTAGCATTGTTCGCGGTATC
CCGTTCTGGGGTGGTAGCACGATTGACACCGAACTGAAGGTTATCGACACTAACTGCATTAACGTTATT
CAACCGGATGGTAGCTATCGTAGCGAAGAGCTGAATCTGGTCATCATTGGCCCGAGCGCAGACATTATC
CAATTCGAGTGCAAGAGCTTTGGTCACGAGGTTCTGAATCTGACCCGCAATGGCTATGGTAGCACCCAG
TACATTCGTTTTTCGCCGGATTTTACCTTCGGCTTTGAAGAGAGCCTGGAGGTTGATACCAATCCGTTG
CTGGGTGCGGGCAAATTCGCTACCGATCCGGCTGTCACGCTGGCCCATGAACTGATCCACGCAGGCCAC
CGCCTGTACGGCATTGCCATCAACCCAAACCGTGTGTTCAAGGTTAATACGAATGCATACTACGAGATG
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GCCAAAAGCATCGTTGGTACTACCGCGTCGTTGCAGTATATGAAGAATGTGTTTAAAGAGAAGTACCTG
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AATATCAACAAGTTTTTGAATCAGTGTAGCGTTTCGTACCTGATGAATAGCATGATTCCGTATGGCGTC
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TGTATGGAGAACAACAGCGGTTGGAAGGTGTCTCTGAACTATGGTGAGATCATTTGGACCTTGCAGGAC
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GTCGTGGTTATGAAGAGCAAGAACGACAAGGGTATCACTAACAAGTGCAAGATGAACCTGCAAGACAAC
AATGGTAACGACATCGGCTTTATTGGTTTCCACCAGTTCAACAATATTGCTAAACTGGTAGCGAGCAAT
TGGTACAATCGTCAGATTGAGCGCAGCAGCCGTACTTTGGGCTGTAGCTGGGAGTTTATCCCGGTCGAT
GATGGTTGGGGCGAACGTCCGCTG

SEQ ID NO: 6. Engineered BoNT/A1 “Cat-B” amino acid sequence .

MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMQPVKAFKIHNKIWVIPERDTFTNPEEGDLNPPPEAKQVP
VSYYDSTYLSTDNEKDNYLKGVTKLFERIYSTDLGRMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVI
QPDGSYRSEELNLVIIGPSADIIQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPL
LGAGKFATDPAVTLAHELIHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDS
LQENEFRLYYYNKFKDIASTLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDKLKFDKLYKML
TEIYTEDNFVKFFKVLNRKTYLNFDKAVFKINIVPKVNYTIYDGFNLRNTNLAANFNGQNTEINNMNFT
KLKNFTGLFEFYKLLCVRGIITSKTKSLDKGYNKALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITS
DTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENLSSDIIGQLELMPNIERFPNGKKYELDKYTMFH
YLRAQEFEHGKSRIALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAAMFLGWVEQLVYDFTDETSEV
STTDKIADITIIIPYIGPALNIGNMLYKDDFVGALIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVL
TVQTIDNALSKRNEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIINYQYNQYTEEEK
NNINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRG
TLIGQVDRLKDKVNNTLSTDIPFQLSKYVDNQRLLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASK
INIGSKVNFDPIDKNQIQLFNLESSKIEVILKKAIVYNSMYENFSTSFWIRIPKYFKKISLNNEYTIIN
CMENNSGWKVSLNYGEIIWTLQDTKEIKQRVVFKYSQMINISDYINRWIFVTITNNRLNKSKIYINGRL

IDQKPISNLGNIHASNKIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFWGDY
LQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNI
VRNNDRVYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDKGITNKCKMNLQDN
NGNDIGFIGFHQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWGERPL

SEQ ID NO: 7. Engineered BoNT/A1 "Cat-C" nucleic acid sequence .

ATGCCATTCGTCAACAAGCAATTCAACTACAAAGACCCAGTCAACGGCGTCGACATCGCATACATCAAG
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CTGTCCGAGGATACCTCCGGCAAGTTTAGCGTTGATAAGCTGAAGTTTGACAAACTGTACAAGATGCTG
ACCGAGATTTACACCGAGGACAACTTTGTGAAATTCTTCAAAGTGTTGAATCGTAAAACCTATCTGAAT
TTTGACAAAGCGGTTTTCAAGATTAACATCGTGCCGAAGGTGAACTACACCATCTATGACGGTTTTAAC
CTGCGTAACACCAACCTGGCGGCGAACTTTAACGGTCAGAATACGGAAATCAACAACATGAATTTCACG
AAGTTGAAGAACTTCACGGGTCTGTTCGAGTTCTATAAGCTGCTGTGCGTGCGCGGTATCATCACCAGC
AAAACCAAAAGCCTGGACAAAGGCTACAACAAGGCGCTGAATGACCTGTGCATTAAGGTAAACAATTGG
GATCTGTTCTTTTCGCCATCCGAAGATAATTTTACCAACGACCTGAACAAGGGTGAAGAAATCACCAGC
GATACGAATATTGAAGCAGCGGAAGAGAATATCAGCCTGGATCTGATCCAGCAGTACTATCTGACCTTT
AACTTCGACAATGAACCGGAGAACATTAGCATTGAGAATCTGAGCAGCGACATTATCGGTCAGCTGGAA
CTGATGCCGAATATCGAACGTTTCCCGAACGGCAAAAAGTACGAGCTGGACAAGTACACTATGTTCCAT
TACCTGCGTGCACAGGAGTTTGAACACGGTAAAAGCCGTATCGCGCTGACCAACAGCGTTAACGAGGCC
CTGCTGAACCCGAGCCGTGTCTATACCTTCTTCAGCAGCGACTATGTTAAGAAAGTGAACAAAGCCACT
GAGGCCGCGATGTTCCTGGGCTGGGTGGAACAGCTGGTATATGACTTCACGGACGAGACGAGCGAAGTG
AGCACTACCGACAAAATTGCTGATATTACCATCATTATCCCGTATATTGGTCCGGCACTGAACATTGGC
AACATGCTGTACAAAGACGATTTTGTGGGTGCCCTGATCTTCTCCGGTGCCGTGATTCTGCTGGAGTTC
ATTCCGGAGATTGCGATCCCGGTGTTGGGTACCTTCGCGCTGGTGTCCTACATCGCGAATAAGGTTCTG
ACGGTTCAGACCATCGATAACGCGCTGTCGAAACGTAATGAAAAATGGGACGAGGTTTACAAATACATT
GTTACGAATTGGCTGGCGAAAGTCAATACCCAGATCGACCTGATCCGTAAGAAAATGAAAGAGGCGCTG
GAGAATCAGGCGGAGGCCACCAAAGCAATTATCAACTACCAATACAACCAGTACACGGAAGAAGAGAAG
AATAACATTAACTTCAATATCGATGATTTGAGCAGCAAGCTGAATGAATCTATCAACAAAGCGATGATC
AATATCAACAAGTTTTTGAATCAGTGTAGCGTTTCGTACCTGATGAAATAGCATGATTCCGTATGGCGTC
AAACGTCTGGAGGACTTCGACGCCAGCCTGAAAGATGCGTTGCTGAAATACATTTACGACAATCGTGGT
ACGCTGATTGGCCAAGTTGACCGCTTGAAAGACAAAGTTAACAATACCCTGAGCACCGACATCCCATTT
CAACTGAGCAAGTATGTTGATAATCAACGTCTGTTGAGCACTTTCACCGAGTATATCAAAAACATCATC
AATACTAGCATTCTGAACCTGCGTTACGAGAGCAATCATCTGATTGATCTGAGCCGTTATGCTAGCAAG
ATCAACATCGGTAGCAAGGTCAATTTTGACCCGATCGATAAGAACCAGATCCAGCTGTTTAATCTGGAA
TCGAGCAAAATTGAGGTTATCCTGAAAAAGGCCATTGTCTACAACTCCATGTACGAGAATTTCTCCACC
AGCTTCTGGATTCGCATCCCGAAATACTTCAACAAGATTAGCCTGAACAACGAGTATACTATCATCAAC
TGTATGGAGAACAACAGCGGTTGGAAGGTGTCTCTGAACTATGGTGAGATCATTTGGACCTTGCAGGAC
ACCAAAGAGATCAAGCAGCGCGTCGTGTTCAAGTACTCTCAAATGATCAACATTTCCGATTACATTAAT
CGTTGGATCTTCGTGACCATTACGAATAACCGTCTGAAGAAGAGCAAGATTTACATCAATGGTCGCTTG
ATCGATCAGAAACCGATTAGCAACCTGGGTAATATCCACGCAAGCAACAAGATTATGTTCAAATTGGAC
GGTTGCCGCGATACCCATCGTTATATCTGGATCAAGTATTTCAACCTGTTTGATAAAGAACTGAATGAG
AAGGAGATCAAAGATTTGTATGACAACCAATCTAACAGCGGCATTTTGAAGGACTTCTGGGGCGATTAT
CTGCAATACGATAAGCCGTACTATATGCTGAACCTGTATGATCCGAACAAATATGTGGATGTCAATAAT
GTGGGTATTCGTGGTTACATGTATTTGAAGGGTCCGCGTGGCAGCGTTATGACGACCAACATTTACCTG
AACTCTAGCCTGTACCGTGGTACGAAATTCATCATTAAGAAATATGCCAGCGGCAACAAAGATAACATT
GTGCGTAATAACGATCGTGTCTACATCAACGTGGTCGTGAAGAATAAAGAGTACCGTCTGGCGACCAAC
GCTTCGCAGGCGGGTGTTGAGAAAATTCTGAGCGCGTTGGAGATCCCTGATGTCGGTAATCTGAGCCAA
GTCGTGGTTATGAAGAGCAAGAACGACAAGGGTATCACTAACAAGTGCAAGATGAACCTGCAAGACAAC
AATGGTAACGACATCGGCTTTATTGGTTTCCACCAGTTCAACAATATTGCTAAACTGGTAGCGAGCAAT

TGGTACAATCGTCAGATTGAGCGCAGCAGCCGTACTTTGGGCTGTAGCTGGGAGTTTATCCCGGTCGAT
GATGGTTGGGGCGAACGTCCGCTG

SEQ ID NO: 8. Engineered BoNT/A1 "Cat-C" amino acid sequence.

MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMQPVKAFKIHNKIWVIPERDTFTNPEEGDLNPPPEAKQVP
VSYYDSTYLSTDNEKDNYLKGVTKLFERIYSTDLGRMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVI
QPDGSYRSEELNLVIIGPSADIIQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPL
LGAGKFATDPAVTLAHELIHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDS
LQENEFRLYYYNKFKDIASTLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDKLKFDKLYKML
TEIYTEDNFVKFFKVLNRKTYLNFDKAVFKINIVPKVNYTIYDGFNLRNTNLAANFNGQNTEINNMNFT
KLKNFTGLFEFYKLLCVRGIITSKTKSLDKGYNKALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITS
DTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENLSSDIIGQLELMPNIERFPNGKKYELDKYTMFH
YLRAQEFEHGKSRIALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAAMFLGWVEQLVYDFTDETSEV
STTDKIADITIIIPYIGPALNIGNMLYKDDFVGALIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVL
TVQTIDNALSKRNEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIINYQYNQYTEEEK
NNINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRG
TLIGQVDRLKDKVNNTLSTDIPFQLSKYVDNQRLLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASK
INIGSKVNFDPIDKNQIQLFNLESSKIEVILKKAIVYNSMYENFSTSFWIRIPKYFNKISLNNEYTIIN
CMENNSGWKVSLNYGEIIWTLQDTKEIKQRVVFKYSQMINISDYINRWIFVTITNNRLKKSKIYINGRL
IDQKPISNLGNIHASNKIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFWGDY
LQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNI
VRNNDRVYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMKSKNDKGITNKCKMNLQDN
NGNDIGFIGFHQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGWGERPL

SEQ ID NO: 9. Engineered BoNT/A1 "Cat-D" nucleic acid sequence.

ATGCCATTCGTCAACAAGCAATTCAACTACAAAGACCCAGTCAACGGCGTCGACATCGCATACATCAAG
ATTCCGAACGCCGGTCAAATGCAGCCGGTTAAGGCTTTTAAGATCCACAACAAGATTTGGGTTATCCCG
GAGCGTGACACCTTCACGAACCCGGAAGAAGGCGATCTGAACCCGCCACCGGAAGCGAAGCAAGTCCCT
GTCAGCTACTACGATTCGACGTACCTGAGCACGGATAACGAAAAAGATAACTACCTGAAAGGTGTGACC
AAGCTGTTCGAACGTATCTACAGCACGGATCTGGGTCGCATGCTGCTGACTAGCATTGTTCGCGGTATC
CCGTTCTGGGGTGGTAGCACGATTGACACCGAACTGAAGGTTATCGACACTAACTGCATTAACGTTATT
CAACCGGATGGTAGCTATCGTAGCGAAGAGCTGAATCTGGTCATCATTGGCCCGAGCGCAGACATTATC
CAATTCGAGTGCAAGAGCTTTGGTCACGAGGTTCTGAATCTGACCCGCAATGGCTATGGTAGCACCCAG
TACATTCGTTTTTCGCCGGATTTTACCTTCGGCTTTGAAGAGAGCCTGGAGGTTGATACCAATCCGTTG
CTGGGTGCGGGCAAATTCGCTACCGATCCGGCTGTCACGCTGGCCCATGAACTGATCCACGCAGGCCAC
CGCCTGTACGGCATTGCCATCAACCCAAACCGTGTGTTCAAGGTTAATACGAATGCATACTACGAGATG
AGCGGCCTgGAAGTCAGCTTCGAAGAACTGCGCACCTTCGGTGGCCATGACGCTAAATTCATTGACAGC
TTGCAAGAGAATGAGTTCCGTCTGTACTACTATAACAAATTCAAAGACATTGCAAGCACGTTGAACAAG
GCCAAAAGCATCGTTGGTACTACCGCGTCGTTGCAGTATATGAAGAATGTGTTTAAAGAGAAGTACCTG
CTGTCCGAGGATACCTCCGGCAAGTTTAGCGTTGATAAGCTGAAGTTTGACAAACTGTACAAGATGCTG
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TTTGACAAAGCGGTTTTCaAGATTAACATCGTGCCGAAGGTGAACTACACCATCTATGACGGTTTTAAC
CTGCGTAACACCAACCTGGCGGCGAACTTTAACGGTCAGAATACGGAAATCAACAACATGAATTTCACG
AAGTTGAAGAACTTCACGGGTCTGTTCGAGTTCTATAAGCTGCTGTGCGTGCGCGGTATCATCACCAGC
AAAACCAAAAGCCTGGACAAAGGCTACAACAAGGCGCTGAATGACCTGTGCATTAAGGTAAACAATTGG
GATCTGTTCTTTTCGCCATCCGAAGATAATTTTACCAACGACCTGAACAAGGGTGAAGAAATCACCAGC
GATACGAATATTGAAGCAGCGGAAGAGAATATCAGCCTGGATCTGATCCAGCAGTACTATCTGACCTTT
AACTTCGACAATGAACCGGAGAACATTAGCATTGAGAATCTGAGCAGCGACATTATCGGTCAGCTGGAA
CTGATGCCGAATATCGAACGTTTCCCGAACGGCAAAAAGTACGAGCTGGACAAGTACACTATGTTCCAT
TACCTGCGTGCACAGGAGTTTGAACACGGTAAAAGCCGTATCGCGCTGACCAACAGCGTTAACGAGGCC
CTGCTGAACCCGAGCCGTGTCTATACCTTCTTCAGCAGCGACTATGTTAAGAAAGTGAACAAAGCCACT
GAGGCCGCGATGTTCCTGGGCTGGGTGGAACAGCTGGTATATGACTTCACGGACGAGACGAGCGAAGTG
AGCACTACCGACAAAaTTGCTGATaTTACCATCATTATCCCGTATATTGGTCCGGCACTGAACATTGGC
AACATGCTGTACAAAGACGATTTTGTGGGTGCCCTGATCTTCTCCGGTGCCGTGATTCTGCTGGAGTTC
ATTCCGGAGATTGCGATCCCGGTGTTGGGTACCTTCGCGCTGGTGTCCTACATCGCGAATAAGGTTCTG
ACGGTTCAGACCATCGATAACGCGCTGTCGAAACGTAATGAAAAATGGGACGAGGTTTACAAATACATT
GTTACGAATTGGCTGGCGAAAGTCaATACCCAGATCGACCTGATCCGTAAGAAAATGAAAGAGGCGCTG
GAGAATCAGGCGGAGGCCACCAAAGCAATTATCAACTACCAATACAACCAGTACACGGAAGAAGAGAAG
AATAACATTAACTTCAATATCGATGATTTGAGCAGCAAGCTGAATGAATCTATCAACAAAGCGATGATC
AATATCAACAAGTTTTTGAATCAGTGTAGCGTTTCGTACCTGATGAATAGCATGATTCCGTATGGCGTC

AAACGTCTGGAGGACTTCGACGCCAGCCTGAAAGATGCGTTGCTGAAATACATTTACGACAATCGTGGT
ACGCTGATTGGCCAAGTTGACCGCTTGAAAGACAAAGTTAACAATACCCTGAGCACCGACATCCCATTT
CAACTGAGCAAGTATGTTGATAATCAACGTCTGTTGAGCACTTTCACCGAGTATATCAAAAACATCATC
AATACTAGCATTCTGAACCTGCGTTACGAGAGCAATCATCTGATtGATCTGAGCCGTTATGCAAGCAAG
ATCAACATCGGTAGCAAGGTCAATTTTGACCCGATCGATAAGAACCAGATCCAGCTGTTTAATCTGGAA
TCGAGCAAAATTGAGGTTATCCTGAAAAACGCCATTGTCTACAACTCCATGTACGAGAATTTCTCCACC
AGCTTCTGGATTCGCATCCCGAAATACTTCAACAGCATTAGCCTGAACAACGAGTATACTATCATCAAC
TGTATGGAGAACAACAGCGGTTGGAAGGTGTCTCTGAACTATGGTGAGATCATTTGGACCTTGCAGGAC
ACCCAAGAGATCAAGCAGCGCGTCGTGTTCAAGTACTCTCAAATGATCAACATTTCCGATTACATTAAT
CGTTGGATCTTCGTGACCATTACGAATAACCGTCTGAATAACAGCAAGATTTACATCAATGGTCGCTTG
ATCGATCAGAAACCGATTAGCAACCTGGGTAATATCCACGCAAGCAACAACATTATGTTCAAATTGGAC
GGTTGCCGCGATACCCATCGTTATATCTGGATCAAGTATTTCAACCTGTTTGATAAAGAACTGAATGAG
AAGGAGATCAAAGATTTGTATGACAACCAATCTAACAGCGGCATTTTGAAGGACTTCTGGGGCGATTAT
CTGCAATACGATAAGCCGTACTATATGCTGAACCTGTATGATCCGAACAAATATGTGGATGTCAATAAT
GTGGGTATTCGTGGTTACATGTATTTGAAGGGTCCGCGTGGCAGCGTTATGACGACCAACATTTACCTG
AACTCTAGCCTGTACCGTGGTACGAAATTCATCATTAAGAAATATGCCAGCGGCAACAAAGATAACATT
GTGCGTAATAACGATCGTGTCTACATCAACGTGGTCGTGAAGCGTAAAGAGTACCGTCTGGCGACCAAC
GCTTCGCAGGCGGGTGTTGAGAAAATTCTGAGCGCGTTGGAGATCCCTCGTGTCCGTCGTCTGAGCCAA
GTCGTGGTTATGAAGAGCAAGAACGACCAGGGTATCACTAACAAGTGCAAGATGAACCTGCAAGACCGT
CGTGGTAACGACATCGGCTTTATTGGTTTCCACCAGTTCAACAATATTGCTAAACTGGTAGCGAGCAAT
TGGTACAATCGTCAGATTGAGCGCCGTAGCCGTCGTTTGGGCTGTAGCTGGGAGTTTATCCCGGTCGAT
GATGGTTGGGGCGAACGTCCGCTG

SEQ ID NO: 10. Engineered BoNT/A1 “Cat-D” amino acid sequence.

MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMQPVKAFKIHNKIWVIPERDTFTNPEEGDLNPPPEAKQVP
VSYYDSTYLSTDNEKDNYLKGVTKLFERIYSTDLGRMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVI
QPDGSYRSEELNLVIIGPSADIIQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPL
LGAGKFATDPAVTLAHELIHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDS
LQENEFRLYYYNKFKDIASTLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDKLKFDKLYKML
TEIYTEDNFVKFFKVLNRKTYLNFDKAVFKINIVPKVNYTIYDGFNLRNTNLAANFNGQNTEINNMNFT
KLKNFTGLFEFYKLLCVRGIITSKTKSLDKGYNKALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITS
DTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENLSSDIIGQLELMPNIERFPNGKKYELDKYTMFH
YLRAQEFEHGKSRIALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAAMFLGWVEQLVYDFTDETSEV
STTDKIADITIIIPYIGPALNIGNMLYKDDFVGALIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVL
TVQTIDNALSKRNEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIINYQYNQYTEEEK
NNINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRG
TLIGQVDRLKDKVNNTLSTDIPFQLSKYVDNQRLLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASK
INIGSKVNFDPIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIIN
CMENNSGWKVSLNYGEIIWTLQDTQEIKQRVVFKYSQMINISDYINRWIFVTITNNRLNNSKIYINGRL
IDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFWGDY
LQYDKPYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNI
VRNNDRVYINVVVKRKEYRLATNASQAGVEKILSALEIPRVRRLSQVVVMKSKNDQGITNKCKMNLQDR
RGNDIGFIGFHQFNNIAKLVASNWYNRQIERRSRRLGCSWEFIPVDDGWGERPL

Examples

The following Examples serve to illustrate particular embodiments of the invention, and do not limit the scope of the invention defined in the claims in any way.

Example 1

Three different examples of an engineered BoNT/A1 molecule according to the present invention were produced.

The amino acids chosen for modification (mutation sites) were selected using a number of different criteria.

The criteria for residue replacement were as follows:

1. Type of residue;
2. Degree of surface exposure;
3. Location with regard to secondary/tertiary structure;
4. Location with regard to the known functional domains of BoNT;
5. Degree of sequence conservation across the BoNT/A or BoNT/E subtypes;
6. Probability of introducing an additional ubiquitination site.

In this example, asparagine (Asn, N) and glutamine (Gln, Q), were selected for mutation because they are polar, are similar in size to Lys, only form weaker dipole interactions with other residues, and 14% of the molecule is composed of these two residues.

Asn and Gln residues visible at the surface of the molecule were identified from the crystal structure of BoNT/A1 (PDB ID: 3BTA). This ensures that all replaced residues will be able to display their charge externally. From this list, it was possible to rule out those less suitable for replacement by applying points 3-5 of the selection criteria above (a reiterative process).

Non-conserved residues within BoNT/A1 were identified from alignments with other subtypes of BoNT/A and of the functionally similar BoNT/E serotype. Those that

appeared as a basic residue in the other sequences were selected as top candidates for replacement.

After successive iterative rounds through the selection criteria mentioned in the sections above, a final list of candidate residues was identified. These were screened for the potential generation of additional ubiquitination consensus sequences (using the CKSAAP_UbSite server). The few that were identified were removed by changing the replacement lysine to an arginine.

The final example cationic constructs synthesised for BoNT/A1 sequence are listed below, named Cat-A, Cat-B and Cat-C. Each construct had a molecular weight of 149,637 Daltons.

Cat-A: N930K, S955K, Q991K, N1026K, N1052K, Q1229K, N886K.
Cat-B: N930K, S955K, Q991K, N1026K, N1052K, Q1229K, N954K.
Cat-C: N930K, S955K, Q991K, N1026K, N1052K, Q1229K, N1025K.

Example 2

The BoNT/B, F and E amino acid sequences were assessed for potential residues that could be substituted with Lys or Arg. This initial assessment identified residues that could be substituted to yield a BoNT/B, E or F protein with an increased pI.

The primary sequence of BoNT/B (Ac: P10844), BoNT/E (Ac: Q00496), and BoNT/F (Ac: P30996) was analysed, and the amino acid composition summarised in the table below:

TABLE 3:

Serotype	Theoretical pI	Net charge at pH 7.4	No. Asn & Gln	No. Asp & Glu
BoNT/B	5.3	-23	179	156
BoNT/E	6.2	-7	160	132
BoNT/F	5.4	-22	169	161

From the table, a similarly large number of polar Asn/Gln residues were present in the amino acid sequence as observed for BoNT/A1. There were also a relatively large number of acidic (Asp/Glu) residues which could potentially be changed to either their corresponding neutral residues (Asn/Gln) or to basic residues (Lys or Arg).

Example 3

Identification of preferred clostridial toxin amino acids for modification.

Full-length structural data was available for BoNT/A, BoNT/B, and BoNT/E; however, for the remaining four serotypes, a theoretical model was generated based on sequence and associated structural homology using the LOOPP computer program.

Each structure was analysed by AreaIMol (CCP4 suite), and exposed residues were identified as having a sum value greater than 40. Residues with polar-side chains were selected from this list, and from this preference was placed on residues that were either acidic (Asp & Glu) or had an H-bond acceptor side chain (Asn & Gln). The final computational step involved selecting residues in between α-helices and β-strands based on analytical data from the Stride server. The structure of each molecule was visually inspected to identify residues located within interface regions – these were avoided.

For BoNT/A1, the list of preferred residues was supplemented with functionally non-conserved residues in at least 90% of all aligned sequences [large non-polar side chains (Met, Pro, Phe, Trp) were considered to be equivalent, small non-polar side chains (Gly, Ala, Val, Leu, Ile) were considered to be equivalent, acidic side chains (Asp, Glu) were considered to be equivalent, and basic side chains (Arg, Lys) were considered to be equivalent]. More specifically, those non-conserved residues that appeared as basic residues in at least 10% of the sequences and non-conserved Asn, Gln, Asp or Glu in the reference sequence, were selected as candidates.

For the remaining serotypes, multiple sequence alignments between subtypes was performed to identify functionally non-conserved residues that appeared as basic residues in at least 10% of the sequences.

Preferred clostridial toxin amino acids for modification:

BoNT/A:

ASN 886, ASN 905, GLN 915, ASN 918, GLU 920, ASN 930, ASN 954, SER 955, GLN 991, GLU 992, GLN 995, ASN 1006, ASN 1025, ASN 1026, ASN 1032, ASN 1043, ASN 1046, ASN 1052, ASP 1058, HIS 1064, ASN 1080, GLU 1081, GLU 1083, ASP 1086.

BoNT/B:

ASN 873, ASN 874, GLU 892, ASP 895, ASN 906, ASP 940, ASN 948, GLU 949, ASN 958, ASN 959, ASN 979, ASN 990, GLU 993, ASP 994, GLU 997, ASN 1012, ASN 1019, ASP 1030, ASP 1047, ASP 1049, GLU 1065, GLU 1072, GLN 1176, GLU 1189, GLU 1252, ASN 1273.

BoNT/C_1:

ASN 881, ASP 898, GLU 916, GLU 927, ASN 952, ASN 964, ASN 965, ASN 984, GLU 985, ASP 986, ASP 996, ASN 1000, GLU 1036, ASN 1041, ASP 1062, ASP 1064, GLU 1079, ASP 1081.

BoNT/D:

ASN 877, ASP 893, ASN 894, ASN 898, ASN 920, ASN 945, ASN 948, GLU 957, GLN 958, ASN 959, ASN 968, ASN 979, GLU 1030, ASP 1031, ASP 1033, GLU 1047, GLU 1051, ASN 1052, GLU 1066, GLN 1122.

BoNT/E:

ASN 859, ASP 860, ASN 892, ASP 893, ASP 904, ASP 909, ASN 928, ASN 932, ASN 934, ASN 935, GLU 936, ASP 945, ASN 946, ASN 947, ASN 966, ASN 976, ASN 979, ASN 981, ASP 985, GLN 1014, ASN 1019, ASN 1022, ASP 1027, ASN 1035, and ASN 1140.

BoNT/F:

ASN 879, ASP 896, ASN 922, ASN 923, ASN 928, ASN 947, ASN 950, ASN 952, ASN 953, GLU 954, ASN 963, ASN 964, ASN 965, ASN 987, GLN 997, ASN 1037, ASP 1040, ASP 1045, ASN 1055, ASP 1056.

BoNT/G:

ASP 900, ASN 909, ASN 910, GLU 912, ASN 913, ASN 945, ASN 947, GLU 956, ASN 965, ASP 966, ASN 986, ASN 1001, ASN 1038, ASP 1040, ASN 1046, ASP 1057, GLU 1073, ASN 1075, ASN 1090.

TeNT:

ASN 893, ASP 894, ASP 911, ASN 919, ASN 927, ASN 928, GLU 929, GLN 968, ASN 972, GLU 973, GLU 1010, ASP 1018, ASN 1079, ASN 1080, ASN 1081, ASN 1097.

Sequences used

Accession numbers:

BoNT/A: P10845

BoNT/B: P10844

BoNT/C_1: P18640

BoNT/D: P19321

BoNT/E: Q00496

BoNT/F: YP_001390123

BoNT/G: Q60393

TeNT: P04958

Structural data source

Crystal structures of BoNT/A (3BTA.pdb), BoNT/B (1EPW), and BoNT/E (3FFZ.pdb) obtained from RCSB.

Homology modelling of BoNT/C_1, BoNT/D, BoNT/F, BoNT/G, and TeNT performed using LOOPP and the following sequences, respectively: P18640, P19321, YP_001390123, Q60393, and P04958.

Structural analysis

Exposed residues determined using AreaIMol from the CCP4 suite.

Secondary structure assignments determined using Stride.

Interface residues determined by visual inspection with RasMol.

Sequence analysis

Full-length BoNT sequences obtained from NCBI.

Alignments performed with ClustalX.

Example 4

Cloning, Expression and Purification

DNA constructs encoding the engineered BoNT/A molecules described in Example 1 were synthesised, cloned into the pJ401 expression vector and then transformed into BL21 (DE3) *E. coli*. This allowed for soluble over-expression of the recombinant Cat-A, Cat-B and Cat-C proteins in BL21(DE3) *E. coli*.

The recombinant engineered BoNTs were purified using classical chromatography techniques from the *E. coli* lysates. An initial purification step using a cation-exchange resin was employed, followed by an intermediate purification step using a hydrophobic interaction resin. The recombinant engineered BoNT single-chain was then cleaved by proteolysis, resulting in the activated di-chain engineered BoNT. A final purification step was then employed to remove remaining contaminants.

Example 5

Characterization of purified engineered BoNTs

The engineered BoNTs described in Example 1 above were characterised experimentally as follows.

Measurement of the pI showed that the engineered BoNTs had an isoelectric point greater than that of unmodified (native) BoNT/A1 – see Figure 1 and Table below.

TABLE 4:

BoNT/A1 molecule	**pI (calculated)**	**pI (observed)**
Engineered, "Cat-A" [Cat5v2(K1064H/N886K]	6.9	~8.0
Engineered, "Cat-B" [Cat5v2(K1064/N954K)]	6.9	~8.0
Engineered, "Cat-C" [Cat5v2(K1064H/N1025K)]	6.9	7.8-8.0
Native BoNT/A1 [rBoNT/A1]	6.05	~7.4

The ability of the engineered BoNTs to enter neurons and cleave SNAP-25 (the target of BoNT/A1) was assessed using rat embryonic spinal cord neurons (eSCN). Figure 2 shows that the engineered BoNTs retained the same ability to enter the neuron and cleave SNAP-25 as native BoNT/A1.

Potency of the engineered BoNTs was further assessed using the mouse phrenic nerve hemi-diaphragm assay (mPNHD). Figure 3 shows that the engineered BoNTs retained the same ability to inhibit the contractile abilities of the mouse hemi-diaphragm as native BoNT/A1.

The *in vivo* mouse Digital Abduction Score (DAS) assay was used to assess potency as well as safety relative to native BoNT/A1. Both molecules displayed a higher safety ratio relative to native BoNT/A1 and were slightly more potent. These data are presented below (Table 4).

TABLE 4:

Molecule	DAS ED_{50} (pg/mouse)	Dose DAS 4 (pg/mouse)	Dose for -10% ΔBW (pg/mouse)	Safety Ratio
Native BoNT/A1 (n=5)	2	10-20	9.9-14.5	7
Engineered, "Cat-A"	1.16	10-20	27.4	24
Engineered, "Cat-B"	1.79	25	47.6	27

-DAS ED_{50}: Calculated dose inducing a DAS 2

-Dose DAS 4: Experimental dose inducing a DAS 4

-BW: Body weight

-Dose for -10% ΔBW: Calculated dose inducing a decrease of 10% on BW in comparison to BW at D0

-Safety Ratio: Dose for -10% ΔBW / DAS ED_{50}

The Safety Ratio is a measure of a negative effect of BoNT treatment (weight loss) with respect to potency (half maximal digital abduction score (DAS)). It is calculated as the ratio between -10% Body Weight (BW) and the DAS ED_{50}, where -10%BW refers to the amount of BoNT (pg/animal) required for a 10% decrease in body weight, and ED_{50} refers to the amount of BoNT (pg/animal) that will produce a DAS of 2.

The DAS assay is performed by injection of 20µl of engineered clostridial toxin, formulated in Gelatin Phosphate Buffer, into the mouse gastrocnemius/soleus complex, followed by assessment of Digit Abduction as previously reported by Aoki (Aoki KR, Toxicon 39: 1815-1820; 2001).

Example 6

A further engineered clostridial toxin according to the present invention was designed

using the criteria as set out in Example 1 above.

This cationic construct was also derived from BoNT/A1 and had a calculated pI of 7.4, and a molecular weight of 149,859. The construct was named Cat-D. While constructs Cat-A, Cat-B and Cat-C comprised residues mutated to lysine, Cat-D comprised residues mutated to arginine.

Cat-D: N1188R, D1213R, G1215R, N1216R, N1242R, N1243R, S1274R, T1277R.

Example 7

Treatment of a patient suffering from Cervical Dystonia

A 50 year old female suffering from spasmodic torticollis presents in the clinic, having previously been treated with a therapeutically effective amount of a standard BoNT/A preparation into the neck muscle; however, the patient experienced dysphagia due to spread of the toxin into the oropharynx. The patient is treated with an injection in the neck muscles of approximately 1.5ng (or more) of an engineered BoNT/A of the present invention. The patient's torticollis is significantly improved after 3-7 days, without the development of dysphagia, and the patient is able to hold her head and shoulder in a normal position for at least five months. Due to the engineered BoNT/A molecule's enhanced tissue retention and reduced spread, the physician is able to inject more product without fear of side effects; the enhanced dose leads to an extended duration of action.

Example 8

Treatment of a patient suffering from blepharospasm

A 47 year old male presents in the clinic with blepharospasm. The patient is treated by injection of between 5 pg and 25 pg of an engineered BoNT/A of the present invention into the lateral pre-tarsal orbicularis oculi muscle of the upper lid and the later pre-tarsal orbicularis oculi of the lower lid. Alleviation of the patient's symptoms

occur in about a week and last at least five months, without eye ptosis. The increased safety of the polypeptide of the present invention allows the physician to increase the dose and therefore extend the duration of the clinical effect.

Example 9

A 27 year old male suffering from cerebral palsy presents at the clinic with debilitating equinus foot and has difficulty walking. The patient had previously been treated with a therapeutically effective amount of BoNT/A, where alleviation improvement in his gait was accompanied by muscle weakness and pain in his extremities. The patient is treated by injection of about 20 pg/kg of an engineered BoNT/A of the present invention into each of two sites in the medial and lateral heads of the gastrocnemius muscle of the affected lower limb(s). Within a week, the patient's gait improves without previously seen side effects and the symptomatic relief lasts for at least four months. The ability to dose higher amounts of drug product leads to treatments which result in an extended duration of action.

Claims

1. An engineered clostridial toxin comprising at least one amino acid modification, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.2 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

2. The engineered clostridial toxin of claim 1, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least 0.5 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

3. The engineered clostridial toxin of claim 1, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least one pI unit higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

4. The engineered clostridial toxin of claim 1, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is at least two pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

5. The engineered clostridial toxin of claim 1, wherein said at least one amino acid modification increases the isoelectric point (pI) of the engineered clostridial toxin to a value that is between 2 and 5 pI units higher than the pI of an otherwise identical clostridial toxin lacking said at least one amino acid modification.

6. The engineered clostridial toxin of any preceding claim, wherein the engineered clostridial toxin has a pI of at least 6.

7. The engineered clostridial toxin of any preceding claim, wherein the engineered clostridial toxin has a pI of between 6 and 10.

8. The engineered clostridial toxin of claim 7, wherein the engineered clostridial toxin has a pI of between 7 and 9, preferably between 8 and 9.

9. The engineered clostridial toxin of any preceding claim, wherein the at least one amino acid modification is selected from: an amino acid substitution, an amino acid insertion, and an amino acid deletion.

10. The engineered clostridial toxin of claim 9, wherein the at least one amino acid substitution is selected from: substitution of an acidic amino acid residue with a basic amino acid residue, substitution of an acidic amino acid residue with an uncharged amino acid residue, and substitution of an uncharged amino acid residue with a basic amino acid residue.

11. The engineered clostridial toxin of any previous claim, wherein the engineered clostridial toxin comprises between 1 and 80 amino acid modifications.

12. The engineered clostridial toxin of claim 11, wherein the engineered clostridial toxin comprises between 4 and 40 amino acid modifications.

13. The engineered clostridial toxin of any previous claim, wherein the at least one amino acid modification is located in the clostridial toxin receptor binding domain (H_C domain).

14. The engineered clostridial toxin of claim 13, wherein the at least one amino acid modification is located in the clostridial toxin H_{CN} domain.

15. The engineered clostridial toxin of any previous claim, wherein the at least one amino acid modification is a modification of a surface exposed amino acid residue.

16. The engineered clostridial toxin of any previous claim, wherein the at least one amino acid modification comprises modification of an amino acid residue selected from: an aspartic acid residue, a glutamic acid residue, a histidine residue, a serine residue, a threonine residue, an asparagine residue, a glutamine residue, a cysteine residue, or a tyrosine residue.

17. The engineered clostridial toxin of claim 16, wherein the amino acid residue is substituted with a lysine residue or an arginine residue.

18. A nucleic acid comprising a nucleic acid sequence encoding an engineered clostridial toxin according to any one of claims 1-17.

19. A method of producing a single-chain engineered clostridial toxin protein having a light chain and a heavy chain, the method comprising expressing a nucleic acid according to claim 18 in a suitable host cell, lysing the host cell to provide a host cell homogenate containing the single-chain engineered clostridial toxin protein, and isolating the single-chain engineered clostridial toxin protein.

20. A method of activating an engineered clostridial toxin, the method comprising providing a single-chain engineered clostridial toxin protein obtainable by the method of claim 19, contacting the polypeptide with a protease that cleaves the polypeptide at a recognition site (cleavage site) located between the light chain and heavy chain, and converting the polypeptide into a di-chain polypeptide wherein the light chain and heavy chain are joined together by a disulphide bond.

21. An engineered clostridial toxin according to any one of claims 1-17, for use in medicine.

22. An engineered clostridial toxin according to any one of claims 1-17, for use in the prevention or treatment of a disease or condition selected from: strabismus, blepharospasm, squint, dystonia (e.g. spasmodic dystonia, oromandibular dystonia, focal dystonia, tardive dystonia, laryngeal dystonia, limb dystonia, cervical dystonia), torticollis (e.g. spasmodic torticollis), beauty therapy (cosmetic) applications benefiting from cell/muscle incapacitation (via SNARE down-regulation or inactivation), neuromuscular disorder or condition of ocular motility (e.g. concomitant strabismus, vertical strabismus, lateral rectus palsy, nystagmus, dysthyroid myopathy), writer's cramp, blepharospasm, bruxism, Wilson's disease, tremor, tics, segmental myoclonus, spasms, spasticity due to chronic multiple sclerosis, spasticity resulting in abnormal bladder control, animus, back spasm, charley horse, tension headaches, levator pelvic syndrome, spina bifida, tardive dyskinesia, Parkinson's

disease, stuttering, hemifacial spasm, eyelid disorder, cerebral palsy, focal spasticity, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions, muscle pain (e.g. pain from muscle spasms), headache pain (e.g. tension headache), brow furrows, skin wrinkles, cancer, uterine disorders, uro-genital disorders, urogenital-neurological disorders, chronic neurogenic inflammation, and a smooth muscle disorder.

23. An engineered clostridial toxin substantially as described herein and with reference to the accompanying drawings.