

NON-NEURONAL SNARE-CLEAVING BOTULINUM NEUROTOXINS

INTRODUCTION

- 5 The present invention relates to a modified botulinum neurotoxin protease having the ability to cleave a non-neuronal SNARE protein, and to the use thereof for suppressing undesirable secretion from a mammalian cell by cleavage of said non-neuronal SNARE protein in said mammalian cell.
- 10 Toxins typically fall into one of two classes, namely cytotoxic toxins (e.g. plant toxin such as ricin) which kill their natural target cells, and non-cytotoxic toxins (e.g. botulinum neurotoxins) which do not kill their natural target cells. Non-cytotoxic toxins exert their effects on a target cell by inhibiting a cellular process other than protein synthesis.
- 15 Botulinum neurotoxin proteases act by proteolytically-cleaving intracellular transport proteins known as SNARE proteins (e.g. SNAP-25, VAMP, or Syntaxin). The acronym SNARE derives from the term Soluble NSF Attachment protein Receptor, where NSF means N-ethylmaleimide-Sensitive
- 20 Factor. SNARE proteins are a large superfamily of proteins. An important function of SNARE proteins is to mediate the exocytosis of neurotransmitter molecules to the post-synaptic junction. SNARE proteins are therefore integral to secretion of molecules via vesicle transport from a cell.
- 25 Clostridium botulinum produces seven (A to G) different neurotoxins (BoNTs) which are differentiated serologically by the lack of anti-serum cross serotype neutralization. BoNTs elicit neuronal-specific flaccid paralysis by targeting neurons and cleaving neuron-specific SNARE proteins.
- 30 BoNTs have a 150 kDa polypeptide chain comprising a 100kDa heavy chain and a 50kDa light chain linked by a disulfide bond. BoNTs are organized into three functional domains: an N-terminal proteolytic light chain (L-chain); and a C-terminal heavy chain (H-chain), the latter consisting of a translocation domain (H_N) and a C-terminal neuron-binding domain (H_C). The toxic effect of

- BoNTs (nerve intoxication) follows a 3-step mechanism of action. First, the H_C portion binds to a cholinergic nerve cell and becomes internalised via receptor-mediated endocytosis. Secondly, the H_N portion translocates the L-chain across the endosomal membrane and into the cytosol of the nerve cell.
- 5 Thirdly, the L-chain binds to and cleaves a neuronal SNARE protein within the cytosol, thereby suppressing neurotransmitter release from the nerve cell and resulting in nerve cell intoxication.

- The seven BoNT serotypes cleave specific residues on one of three SNARE proteins:
- 10 serotypes B, D, F, and G cleave VAMP-2;
serotypes A and E cleave SNAP-25; and
serotype C cleaves SNAP-25 and syntaxin 1a.

- 15 Whilst native BoNTs are able to target and cleave neuronal SNARE isoforms such as VAMP-2, SNAP-25 and syntaxin 1a said proteases have little or no cleavage effect on the majority of non-neuronal SNARE proteins. This neuronal SNARE substrate specificity is consistent with and understood to be reflective of the natural neuronal cell binding specificity demonstrated by
- 20 BoNTs. For example, BoNT/A cleaves human SNAP-25, but not human non-neuronal isoforms.

- As early as 1989, BoNT/A was approved by the FDA to treat strabismus, blepharospasm, and hemifacial spasm and then for cervical dystonia,
- 25 cosmetic use, glabellar facial lines and axillary hyperhidrosis. BoNT/A efficacy in dystonia and other disorders related to involuntary skeletal muscle activity, coupled with a satisfactory safety profile, has prompted empirical/ off-label use in a variety of secretions and pain and cosmetic disorders.

- 30 Clinical applications of BoNTs have focussed on targeting disorders associated with neuromuscular activity. More recently, pioneering research lead by Syntaxin Ltd has allowed the design of re-targeted BoNTs that bind to unique subset of neurons (e.g. nociceptive afferents – see WO96/33273, which is hereby incorporated in its entirety) and/ or to non-neuronal cells (e.g.

airway epithelium cells – see WO00/10598, which is hereby incorporated in its entirety). This technology, known as Targeted Secretion Inhibitor (TSI) technology, involves replacement of the native BoNT binding domain by a different targeting moiety (e.g. a growth factor or other signalling molecule),
5 and has opened the door for new BoNT-based therapeutics and therapies.

However, the selective cleavage of neuronal specific SNARE proteins by BoNTs has limited development of novel therapies in these non-neuronal systems. Neuronal and non-neuronal SNARE proteins are believed to be of
10 equal importance to the process of intracellular vesicle fusion, and thus to the secretion of molecules via vesicle transport from a cell. Accordingly, the use of conventional BoNT-based therapeutics to inactivate neuronal SNARE protein driven secretion will not address any corresponding non-neuronal SNARE driven cellular secretion.

15 Accordingly, a need exists for an engineered BoNT L-chain protease that cleaves efficiently a non-neuronal SNARE protein.

The present invention solves one or more of the above problems by providing
20 an engineered BoNT/A L-chain protease that cleaves a SNARE protein isoform that is mainly expressed in non-neuronal cells, namely human SNAP-23 (hSNAP-23). The present invention therefore provides a new class of non-cytotoxic therapeutic agent.

25 DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless
30 otherwise required by context, nomenclatures used herein, and techniques of cell and tissue culture are those well-known and commonly used in the art.

Nevertheless, with respect to the use of different terms throughout the current specification, the following definitions more particularly apply.

The singular terms “a”, “an” or “the” encompass meaning plural meaning, such as “one or more”, or “at least one”, unless the context dictates otherwise.

- 5 The term “protease” means herein an enzyme, which is capable to hydrolytically cleave proteins and/or peptides. In the context of the present invention, said protease is more particularly a botulinum neurotoxin (BoNT) light-chain (L-chain) protease, i.e., a protease (also described as proteolytic domain) derived from botulinum neurotoxin, in particular from botulinum neurotoxin A (BoNT/A). As is well-known to the skilled practitioner, the light-chain of a botulinum neurotoxin provides a protease function (also known as non-cytotoxic protease function), and commonly has a molecular weight of about 50 kDa. Such non-cytotoxic proteases typically 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 naturally-occurring (i.e. wild-type) BoNT/A L-chain is more particularly capable to efficiently cleave SNAP-25, but is only *de minimis* capable of cleaving hSNAP-23 as further explained below. In contrast, the BoNT/A L-chain protease of the present invention, as described in more detail below, differs from the naturally-occurring BoNT/A L-chain in that it has an improved capacity to cleave hSNAP-23, and is referred herein as a “modified BoNT/A L-chain that cleaves hSNAP-23”.

- 25 The capacity of cleaving hSNAP23 can be confirmed via a conventional assay, such as the assay described in Example 2 below. “Cleavage of hSNAP-23” more particularly means herein that the modified BoNT/A L-chain of the present invention demonstrates improved hSNAP-23 cleavage relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1). Any simple comparative assay may be employed, such as the hSNAP-23 assay illustrated in Example 2. Wild-type BoNT/A L-chain is capable of only *de minimis* (i.e. background) hSNAP-23 cleavage.

Thus, a modified BoNT/A L-chain of the present invention demonstrates one or more (preferably both) of:

- 5 a) greater than 0.2% (for example greater than 0.5%, preferably greater than 1%, more preferably greater than 5%) hSNAP-23 cleavage in a cell-free assay (1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) – see Example 2, Figure 1; and/ or
- b) less than a K_m of 225 (for example less than 200, preferably less than 150) micromolar in a cell-free assay (1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) – see Example 2, Figure 1; and/or
- 10 c) less than a K_{cat} (1/min) of 0.2 (for example, less than 0.18, preferably less than 0.1) in a cell-free assay (1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) – see Example 2, Figure 1.
- 15 The modified BoNT/A L-chain protease of the invention may optionally not only cleave hSNAP-23 but also SNAP-25. Cleavage of SNAP-25 can be confirmed via a conventional assay, such as the assay described in Example 3 below. According to this optional embodiment, “cleavage of SNAP-25” means herein that the modified BoNT/A L-chain of the present invention
- 20 preferably demonstrates at least 0.5%, at least 1%, at least 2% preferably at least 3%, still preferably at least 10% SNAP-25 cleavage relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1). Any simple comparative assay may be employed, such as the SNAP-25 assay illustrated in Example 3.
- 25 In a preferred embodiment, the modified BoNT/A L-chain protease of the invention cleaves hSNAP-23 but not SNAP-25, such as hSNAP-25.

The term “SNAP-23” (synaptosomal-associated protein 23) designates herein a SNARE protein which is capable of binding to various other SNARE proteins

30 and of forming a high affinity complex with these proteins in a cell, preferably in a non-neuronal cell, thereby regulating intracellular cell membrane fusion in said cell. “hSNAP-23” refers more particularly to human SNAP-23, and preferably to the protein of sequence SEQ ID NO: 2.

The term "SNAP-25" (synaptosomal-associated protein 25) designates herein a SNARE protein which is capable of binding to various other SNARE proteins and of forming a high affinity complex in a cell, preferably in a neuronal cell, thereby regulating intracellular cell membrane fusion in said cell. "hSNAP-25" refers more particularly to human SNAP-25, and preferably to the protein of sequence SEQ ID NO: 3.

The term "modification", "change" or "mutation" can be used herein interchangeably, and refer to the alteration in the amino acid sequence compared to that of a protein of reference, i.e. herein relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1). The amino acid sequence illustrated herein as SEQ ID NO: 1 is 438 amino acid residues in length and ends with K438. It is understood that K438 is the first lysine amino acid residue of the activation loop and most likely represents the C-terminal end of the L-chain after proteolytic cleavage of the activation loop. Thus, SEQ ID NO: 1 represents the (naturally) activated form of a wild-type BoNT/A L-chain. In this regard, prior to proteolytic activation, a wild-type BoNT/A L-chain is typically ~448 amino acid residues in length, which includes a short C-terminal extension of activation loop amino acid residues.

As further explained below, the present invention reveals the identification of critical amino acid positions within a wild-type BoNT/A L-chain that require rational change to a different amino acid residue in order to render a BoNT/A L-chain capable of hSNAP-23 cleavage. In this regard, introduction of an amino acid change (i.e. a mutation), may be effected by means of an amino acid insertion, a deletion or a substitution, and preferably by means of an amino acid substitution. Methods allowing introduction of such mutation are known to the skilled person in the art. For example, it is possible to introduce a mutation by random or directed mutagenesis, by PCR using degenerate primers, e.g. in the nucleotide sequence coding for the protein of reference. Said techniques are notably described by Sambrook et al. in "Molecular Cloning: A laboratory Manual", 4th edition, Cold Spring Harbor Laboratory Press, (2012, and updates from 2014), and by Ausubel et al. in "Current Protocols in Molecular Biology", John Wiley & Sons (2012).

- The amino acid change occurs within one or more of the L-chain “binding pockets” relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1). By “binding pocket”, it is meant herein a region of the BoNT/A L-chain which comprises one or more amino acids which are the contact points (e.g. via hydrogen-bond, salt bridge, and/or hydrophobic contact) for binding to the corresponding binding site of hSNAP-23, and/or which provide the space to accommodate other substrate amino acid residue(s) (e.g. by modification, such as by substitution) capable to bind hSNAP-23. The term “binding to” as used herein means “suitable for binding to” and forms part of Applicant’s rationale for the present invention – said rationale does not constitute an essential technical feature of the present invention. For example, the BoNT/A L-chain protease binding pocket defined by amino acid residues E148, T307, A308 and Y312 of SEQ ID NO: 1 refers to a region of the BoNT/A L-chain protease comprising amino acids E148, T307, A308 and/or Y312, and/or mutants thereof as described herein that Applicant believes cooperate to bind to a predicted binding site on hSNAP-23 (e.g. to the P182/D178 binding site of hSNAP-23).
- The term “binding site” refers herein to a region of hSNAP-23 which comprises one or more amino acids that can be bound by the corresponding BoNT/A L-chain binding pocket. For example, the “P182/D178” binding site of hSNAP-23 comprises the amino acids P182 and/or D178 of hSNAP-23. The term “binding site” as used herein simply means “predicted binding site” (as predicted by Applicant) and forms part of Applicant’s rationale for the present invention – said rationale does not constitute an essential technical feature of the present invention.
- “Sequence identity” between amino acid or nucleic acid sequences can be determined by comparing a position in each of the sequences which may be aligned for the purposes of comparison. When a position in the compared sequences is occupied by the same nucleotide or amino acid, then the sequences are identical at that position. A degree of identity between amino acid sequences is a function of the number of identical amino acid sequences

that are shared between these sequences. A degree of sequence identity between nucleic acids is a function of the number of identical nucleotides at positions shared by these sequences.

- 5 To determine the “percentage of sequence identity” between two amino acid sequences or two nucleic acid sequences, the sequences are aligned for optimal comparison. For example, gaps can be introduced in the sequence of a first amino acid sequence or a first nucleic acid sequence for optimal alignment with the second amino acid sequence or second nucleic acid
10 sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, the molecules are identical at that position.

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- The percentage (%) of identity between the two sequences is a function of the number of identical positions shared by the sequences. Hence, the percentage of identity can be calculated by multiplying the number of identical positions by 100 and dividing by the length of the aligned region (overlapping
20 positions), including gaps (only internal gaps, not the gaps at the sequence ends).

- In this comparison, the sequences can be of the same length, or may be of different lengths. Identity scoring only counts perfect matches, and does not
25 consider the degree of similarity of amino acids to one another.

- Optimal alignment of sequences may be herein preferably conducted by a global homology alignment algorithm should the alignment be performed using sequences of the same or similar length, such as by the algorithm
30 described by Needleman and Wunsch (Journal of Molecular Biology; 1970, 48 (3): 443–53), by computerized implementations of this algorithm (e.g., using the DNASTAR® Lasergene software), or by visual inspection. Alternatively, should the alignment be performed using sequences of distinct length (e.g. the amino acid sequence of the light-chain according to the invention versus

the entire amino acid sequence of a naturally-occurring botulinum neurotoxin), the optimal alignment of sequences can be herein preferably conducted by a local homology alignment algorithm, such as by the algorithm described by Smith and Waterson (Journal of Molecular Biology; 1981, 147: 195-197), by
5 computerized implementations of this algorithm (e.g., using the DNASTAR® Lasergene software), or by visual inspection. The best alignment (i.e., resulting in the highest percentage of identity between the compared sequences) generated by the various methods is selected. Examples of global and local homology alignment algorithms are well-known to the skilled practitioner, and
10 include, without limitation, ClustalV (global alignment), ClustalW (local alignment) and BLAST (local alignment).

The skilled practitioner would further readily understand that the present invention embraces modified BoNT/A L-chains that are substantially
15 homologous, and which retain the capacity to cleave hSNAP-23, i.e. functional variants or homologs. These functional variants or homologs can be characterized as having one or more amino acid mutations (such as an amino acid deletion, addition, and/or substitution) other than the ones disclosed thereafter with regard to hSNAP-23 cleavage, and which do not significantly
20 affect the folding or protease activity, in particular hSNAP-23 cleavage. For example, such mutations include, without limitation, conservative substitutions, small deletions (typically of 1 to about 30 amino acids), small amino- or carboxyl-terminal extensions (such as an amino-terminal methionine residue), addition of a small linker peptide of up to about 20-25
25 residues or of an affinity tag.

Functional variants or homologs according to the invention preferably comprise mutations of minor nature, such as conservative amino acid substitutions. Conservative amino acid substitutions are well-known to the
30 skilled practitioner, and include, without limitation:

Basic:	arginine, lysine, histidine
Acidic:	glutamic acid, aspartic acid
Polar:	glutamine, asparagine
Hydrophobic:	leucine, isoleucine, valine, methionine

Aromatic: phenylalanine, tryptophan, tyrosine
Small: glycine, alanine, serine, threonine

5 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
polypeptides, 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
10 acid residues. The polypeptides of the present invention may 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-
15 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
20 residues into proteins.

The amino acid substitution may comprise the substitution of an amino acid
comprising a certain physiochemical property (e.g. hydrophobicity) with an
amino acid having a similar or alternative property. Examples of such
25 substitutions are listed below:

- Acidic amino acid substituted for a neutral, polar amino acid;
- Polar amino acid substituted for a non-polar amino acid;
- Non-polar amino acid substituted for a non-polar amino acid;
- Non-polar amino acid substituted for a polar amino acid;
- 30 Polar amino acid substituted for a basic amino acid;
- Non-polar amino acid substituted for an acidic amino acid;
- Non-polar amino acid substituted for a polar amino acid.

Accordingly, the L-chain of all BoNT/A subtypes, such as any of BoNT/A1 to BoNT/A8 L-chain, which comprise one or more of the mutations as described herein for cleavage of hSNAP-23, are encompassed by the present invention. Said BoNT/A L-chain may additionally comprise further mutations to provide a
5 non-native activation cleavage site, such as the cleavage site of enterokinase (SEQ ID NO: 10), PreScission, Factor Xa, Thrombin, TEV protease, etc.

Additional definitions are provided throughout the specification.

10 The present invention can be described as follows.

In a first aspect, the present invention provides a modified botulinum neurotoxin A (BoNT/A) L-chain protease that cleaves human SNAP-23 (hSNAP-23), having a modified amino acid sequence relative to the wild-type
15 BoNT/A L-chain (SEQ ID NO: 1), that comprises:

- a) at least one amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23;
- b) wherein said first BoNT/A L-chain protease binding pocket is
20 defined by amino acid residues E148, T307, A308 and Y312 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said at least one amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting
25 of asparagine and tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - ii. an amino acid residue selected from the group consisting
30 of phenylalanine, isoleucine and leucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue T307 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or

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- iii. an amino acid residue selected from the group consisting of proline, asparagine, threonine and isoleucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue A308 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
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- iv. an amino acid residue selected from the group consisting of lysine, valine, methionine and leucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Y312 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

Without wishing to be bound by any theory, Applicant believes that the above-defined BoNT/A L-chain binding pocket effects an important enzyme-substrate association with the recognition sequence on hSNAP-23.

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Thus, the present invention is predicated on the surprising finding (e.g. unexpected technical effect) that targeted amino acid substitutions as claimed allow for the generation of BoNT/A L-chain(s) increased hSNAP-23 cleavage (relative to wild-type BoNT/A L-chain). The present inventors have not only

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successfully identified suitable amino acid positions of BoNT/A L-chain which can be altered (e.g. substituted) to increase hSNAP-23 cleavage, but have also identified precise amino acid substitutions which provide this effect.

In a first aspect, the present invention provides a modified botulinum neurotoxin A (BoNT/A) L-chain protease that cleaves human SNAP-23 (hSNAP-23), having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:

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- a) at least one amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23;
- b) wherein said first BoNT/AL-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1);

c) and wherein said at least one amino acid residue change comprises:

- 5 i. an amino acid residue selected from the group consisting of asparagine and tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

10 In one aspect, the present invention provides a modified botulinum neurotoxin A (BoNT/A) L-chain protease that cleaves human SNAP-23 (hSNAP-23), having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:

- 15 a) at least one amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23;
- b) wherein said first BoNT/AL-chain protease binding pocket is defined by amino acid residues E148, T307, A308 and Y312 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- 20 c) and wherein said at least one amino acid residue change comprises:
- 25 i. an amino acid residue selected from the group consisting of phenylalanine, isoleucine and leucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue T307 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
- 30 ii. an amino acid residue selected from the group consisting of proline, asparagine, threonine and isoleucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue A308 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
- iii. an amino acid residue selected from the group consisting of lysine, valine, methionine and leucine at the position on the modified L-chain protease amino acid sequence that

corresponds to amino acid residue Y312 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

5 A modified BoNT/A L-chain may comprise one amino acid substitution (relative to wildtype SEQ ID NO:1). A modified BoNT/A L-chain may comprise two amino acid substitutions (relative wildtype WT SEQ ID NO:1). A modified BoNT/A L-chain may comprise three amino acid substitutions (relative to wildtype SEQ ID NO:1). A modified BoNT/A L-chain may comprise four amino acid substitutions (relative to wildtype SEQ ID NO:1).

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Modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the P182/D178 binding site on hSNAP-23 typically demonstrate at least a 1.15-fold increased hSNAP-23 cleavage (versus wild-type BoNT/A L-chain) - see the 1st data column in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

- 15 a modified BoNT/A L-chain comprising the substitution E148Y or E148N;
- a modified BoNT/A L-chain comprising the substitution T307I, A308P, and Y312V;
- 20 a modified BoNT/A L-chain comprising the substitution T307F, A308N, and Y312L;
- a modified BoNT/A L-chain comprising the substitution E148N, T307I, and A308P, Y312V;
- a modified BoNT/A L-chain comprising the substitution E148Y, T307F, 25 A308N, and Y312L;
- a modified BoNT/A L-chain comprising the substitution E148Y, T307I, A308P, and Y312V;
- a modified BoNT/A L-chain comprising the substitution E148Y, T307L, A308T, and Y312M;
- 30 a modified BoNT/A L-chain comprising the substitution E148Y, T307L, A308I, and Y312M.

In one embodiment, modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the P182/D178 binding site on

- hSNAP-23 demonstrate at least a 1.35-fold increased hSNAP-23 cleavage (versus wild-type BoNT/A L-chain) - see the 1st data column in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- 5 a modified BoNT/A L-chain comprising the substitution E148Y or E148N;
 - a modified BoNT/A L-chain comprising the substitution T307I, A308P, and Y312V;
 - a modified BoNT/A L-chain comprising the substitution T307F, A308N, and Y312L;
 - 10 a modified BoNT/A L-chain comprising the substitution E148N, T307I, A308P, and Y312V;
 - a modified BoNT/A L-chain comprising the substitution E148Y, T307F, A308N, and Y312L;
 - 15 a modified BoNT/A L-chain comprising the substitution E148Y, T307I, A308P, and Y312V;
 - a modified BoNT/A L-chain comprising the substitution E148Y, T307L, A308T, and Y312M;
 - a modified BoNT/A L-chain comprising the substitution E148Y, T307L, A308I, and Y312M.
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- In another embodiment, modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the P182/D178 binding site on hSNAP-23 demonstrate at least a 1.7-fold increased hSNAP-23 cleavage (versus wild-type BoNT/A L-chain) - see the 1st data column in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- a modified BoNT/A L-chain comprising the substitution E148Y;
 - 30 a modified BoNT/A L-chain comprising the substitution T307F;
 - a modified BoNT/A L-chain comprising the substitution T307I;
 - a modified BoNT/A L-chain comprising the substitution Y312K;
 - a modified BoNT/A L-chain comprising the substitution Y312K, E148Y;

- a modified BoNT/A L-chain comprising the substitution T307I, A308P, and Y312V;
- a modified BoNT/A L-chain comprising the substitution E148N, T307I, A308P, and Y312V;
- 5 a modified BoNT/A L-chain comprising the substitution E148Y, T307F, A308N, and Y312L;
- a modified BoNT/A L-chain comprising the substitution E148Y, T307L, A308T, and Y312M.
- 10 In a further embodiment, modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the P182/D178 binding site on hSNAP-23 may demonstrate at least a 2.0-fold increased hSNAP-23 cleavage (versus wild-type BoNT/A L-chain) - see the 1st data column in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid
- 15 substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- a modified BoNT/A L-chain comprising the substitution E148Y;
- a modified BoNT/A L-chain comprising the substitution E148N, T307I, A308P, and Y312V;
- 20 a modified BoNT/A L-chain comprising the substitution E148Y, T307F, A308N, and Y312L;
- a modified BoNT/A L-chain comprising the substitution E148Y, T307L, A308T, and Y312M.
- 25 In a further embodiment, modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the P182/D178 binding site on hSNAP-23 may demonstrate at least a 4.0-fold increased hSNAP-23 cleavage (versus wild-type BoNT/A L-chain) - see the 1st data column in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid
- 30 substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- a modified BoNT/A L-chain comprising the substitution E148Y;
- a modified BoNT/A L-chain comprising the substitution E148N, T307I, A308P, and Y312V;

a modified BoNT/A L-chain comprising the substitution E148Y, T307F, A308N, and Y312L;

a modified BoNT/A L-chain comprising the substitution E148Y, T307L, A308T, and Y312M.

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In another embodiment, modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the P182/D178 binding site on hSNAP23 demonstrate at least a 6.0-fold, preferably at least a 7.0-fold, more preferably at least a 8.0-fold increased hSNAP-23 cleavage (versus wild-type

10 BoNT/A L-chain) - see the 1st data column in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
a modified BoNT/A L-chain comprising the substitution E148Y.

15 Modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the P182/D178 binding site on hSNAP-23 typically demonstrate greater than 1.5% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column in Figure 1A. For
20 reference, wild-type BoNT/A L-chain (e.g. SEQ ID NO: 1) demonstrates less than 0.5% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour). Examples of such modified L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID
25 NO:1) include:

a modified BoNT/A L-chain comprising the substitution E148Y;

a modified BoNT/A L-chain comprising the substitution T307F;

a modified BoNT/A L-chain comprising the substitution T307I;

a modified BoNT/A L-chain comprising the substitution Y312K;

30 a modified BoNT/A L-chain comprising the substitution Y312K, and E148Y.

In one embodiment, modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the P182/D178 binding site on hSNAP-23 typically demonstrate greater than 2% hSNAP-23 cleavage (% at 1

- micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column in Figure 1A. Examples of such modified L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- 5 a modified BoNT/A L-chain comprising the substitution E148Y;
 - a modified BoNT/A L-chain comprising the substitution T307I;
 - a modified BoNT/A L-chain comprising the substitution Y312K.
- 10 In a further embodiment, modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the P182/D178 binding site on hSNAP-23 demonstrate at least 9% increased hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column in Figure
- 15 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- a modified BoNT/A L-chain comprising the substitution E148Y.
- 20 Additional examples of modified BoNT/A L-chain mutants having a binding pocket mutation for the P182/D178 binding site on hSNAP-23 (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- a modified BoNT/A L-chain comprising the substitution A308L;
 - 25 a modified BoNT/A L-chain comprising the substitution A308V;
 - a modified BoNT/A L-chain comprising the substitution A308I;
 - a modified BoNT/A L-chain comprising the substitution A308P;
 - a modified BoNT/A L-chain comprising the substitution A308N;
 - a modified BoNT/A L-chain comprising the substitution A308T;
 - 30 a modified BoNT/A L-chain comprising the substitution Y312V;
 - a modified BoNT/A L-chain comprising the substitution Y312M;
 - a modified BoNT/A L-chain comprising the substitution Y312L.

- A modified BoNT/A L-chain of the present invention having a binding pocket mutation for the P182/D178 binding site on hSNAP-23 can comprise one or more amino acid residue changes relative to the wild-type BoNT/A L-chain, as herein before defined. By way of illustration, a modified BoNT/A L-chain of the present invention may have a single amino acid residue mutation (within the binding pocket for the P182/D178 binding site on hSNAP-23, as defined above), for example a mutation corresponding to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1). Similarly, a modified BoNT/A L-chain of the present invention may comprise more than one amino acid residue mutation (within the binding pocket for the P182/D178 binding site on hSNAP-23, as defined above), for example mutations corresponding to amino acid residues T307, A308 and Y312 of wild-type BoNT/A L-chain (SEQ ID NO: 1).
- In a preferred embodiment, a modified BoNT/A L-chain of the present invention having one or more binding pocket mutations for the P182/D178 binding site on hSNAP-23 further comprises one or more mutations within one or more different BoNT/A L-chain binding pockets for hSNAP-23 as further described below.
- Said one or more different BoNT/A L-chain binding pockets for hSNAP-23 include a second BoNT/A L-chain protease binding pocket for binding to the D189/D192 binding site of hSNAP-23; a third BoNT/A L-chain protease binding pocket for binding to the I198 binding site of hSNAP-23; a fourth BoNT/A L-chain protease binding pocket for binding to the K185 binding site of hSNAP-23; a fifth BoNT/A L-chain protease binding pocket for binding to the R186 binding site of hSNAP-23; a sixth BoNT/A L-chain protease binding pocket for binding to the K206 binding site of hSNAP-23; a seventh BoNT/A L-chain protease binding pocket for binding to the D210 binding site of hSNAP-23; an eighth BoNT/A L-chain protease binding pocket for binding to the D168 binding site of hSNAP-23.

Alternatively, according to distinct technical features of the invention, a modified BoNT/A L-chain can comprise one or more mutations within one or

more BoNT/A L-chain binding pockets other than within the binding pocket for the P182/D178 binding site on hSNAP23 as defined above.

5 Said BoNT/A L-chain binding pockets for hSNAP-23 and corresponding mutations of interest are further detailed hereunder.

Accordingly, as an additional technical feature, or as an alternative technical feature), the present invention includes BoNT/A L-chain mutants comprising one or more mutations within a herein defined pocket of the BoNT/A L-chain.
10 By way of example, a modified BoNT/A L-chain of the present invention that cleaves human SNAP-23 (hSNAP-23) has a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- 15 a) an amino acid residue change located within a fifth BoNT/A L-chain protease binding pocket for binding to the R186 binding site of hSNAP-23;
- b) wherein said fifth BoNT/A L-chain protease binding pocket is defined by amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said amino acid residue change comprises:
 - 20 i. an amino acid residue selected from the group consisting of glutamine, glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

25 Without wishing to be bound by any theory, Applicant believes that the above-defined BoNT/A L-chain binding pocket provides a stabilising salt bridge between R186 on hSNAP-23 and S143 on BoNT/A.

30 Modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the R186 binding site on hSNAP-23 typically demonstrate a K_M for hSNAP-23 of less than 100 micromolar, for example less than 95 micromolar - see the 3rd data column in Figure 1A. For reference, wild-type BoNT/A L-chain (e.g. SEQ ID NO: 1) demonstrates a K_M for hSNAP-23 that is

greater than 150 micromolar, for example greater than 200 micromolar. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

- 5 a modified BoNT/A L-chain comprising the substitution V304D;
- a modified BoNT/A L-chain comprising the substitution V304E;
- a modified BoNT/A L-chain comprising the substitution G305D;
- a modified BoNT/A L-chain comprising the substitution G305E;
- a modified BoNT/A L-chain comprising the substitution S143D;
- 10 a modified BoNT/A L-chain comprising the substitution S143E;
- a modified BoNT/A L-chain comprising the substitution S143Q;
- a modified BoNT/A L-chain comprising the substitution K166F.

- In a preferred embodiment, a modified BoNT/A L-chain of the present invention having a binding pocket mutation for the R186 binding site on hSNAP-23 can further comprise one or more mutations within one or more different BoNT/A L-chain binding pockets as herein described (e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, as defined above). Such mutants typically demonstrate at least a 0.5-fold decreased hSNAP-23 cleavage (versus E148Y modified BoNT/A L-chain), or, in other words at least a 4.0-fold increased hSNAP-23 cleavage (versus wild-type BoNT/A L-chain) - see the 1st data column for multi-pocket mutants in Figure 1B; or at least 5 % hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- a modified BoNT/A L-chain comprising the substitution E148Y, and S143E;
 - a modified BoNT/A L-chain comprising the substitution E148Y, and S143D;
 - 30 a modified BoNT/A L-chain comprising the substitution E148Y, and S143Q.

Thus, in one embodiment there is provided a modified BoNT/A L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified

amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- 5 a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 10 b) an amino acid residue change located within a fifth BoNT/A L-chain protease binding pocket for binding to the R186 binding site of hSNAP-23; wherein said fifth BoNT/A L-chain protease binding pocket is defined by amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- c) and wherein said amino acid residue change comprises:
 - 15 i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
 - 20 ii. an amino acid residue selected from the group consisting of glutamate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

25 In another embodiment there is provided a modified BoNT/A L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- 30 a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and

- b) an amino acid residue change located within a fifth BoNT/A L-chain protease binding pocket for binding to the R186 binding site of hSNAP-23; wherein said fifth BoNT/A L-chain protease binding pocket is defined by amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said amino acid residue change comprises:
- i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
 - ii. an amino acid residue selected from the group consisting of aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

In another embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the R186 binding site on hSNAP-23 further comprises one or more mutations within one or more different BoNT/A L-chain binding pockets as herein described (e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, as defined above). Such mutants typically demonstrate at least a 2.0-fold increased hSNAP-23 cleavage (versus E148Y modified BoNT/A L-chain) - see the 1st data column for multi-pocket mutants in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

a modified BoNT/A L-chain having the substitution E148Y, and S143E;

a modified BoNT/A L-chain having the substitution E148Y, and S143D.

In another embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the R186 binding site on hSNAP-23 further comprises one or more mutations within one or more different BoNT/A L-chain binding pockets as herein described (e.g. within the binding pocket for

the P182/D178 binding site on hSNAP-23, as defined above). Such mutants typically demonstrate at least a 3.0-fold increased hSNAP-23 cleavage (as a percentage versus E148Y modified BoNT/A L-chain) - see the 1st data column for multi-pocket mutants in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
a modified BoNT/A L-chain comprising the substitution E148Y, S143D.

As an additional technical feature, or as an alternative technical feature, the present invention includes BoNT/A L-chain mutants comprising one or more mutations within a herein defined pocket of the BoNT/A L-chain. By way of example, a modified BoNT/A L-chain of the present invention that cleaves human SNAP-23 (hSNAP-23) has a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:

- a) at least one amino acid residue change located within a fourth BoNT/A L-chain protease binding pocket for binding to the K185 binding site of hSNAP-23;
- b) wherein said fourth BoNT/A L-chain protease binding pocket is defined by amino acid residues V304 and G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said at least one amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue V304 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - ii. an amino acid residue selected from the group consisting of glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

Without wishing to be bound by any theory, Applicant believes that the above-defined BoNT/A L-chain binding pocket provides a stabilising salt bridge between K185 on hSNAP-23 and BoNT/A.

- 5 Modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the K185 binding site on hSNAP-23 typically demonstrate a K_M for hSNAP-23 of less than 100 micromolar - see the 3rd data column in Figure 1A. For reference, wild-type BoNT/A L-chain (e.g. SEQ ID NO: 1) demonstrates a K_M for hSNAP-23 that is greater than 150 micromolar, for
10 example greater than 200 micromolar. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- a modified BoNT/A L-chain comprising the substitution V304D;
 - a modified BoNT/A L-chain comprising the substitution V304E;
 - 15 a modified BoNT/A L-chain comprising the substitution G305D;
 - a modified BoNT/A L-chain comprising the substitution G305E.

A modified BoNT/A L-chain of the present invention having a binding pocket mutation for the K185 binding site on hSNAP-23 comprises one or more
20 amino acid residue changes relative to the wild-type BoNT/A L-chain, as herein before defined. By way of illustration, a modified BoNT/A L-chain of the present invention may have a single amino acid residue mutation (within the binding pocket for the K185 binding site on hSNAP-23, as defined above), for example a mutant corresponding to amino acid residue G305 of wild-type
25 BoNT/A L-chain (SEQ ID NO: 1). Similarly, a modified BoNT/A L-chain of the present invention may comprise more than one mutation (within the binding pocket for the K185 binding site on hSNAP-23 as defined above), for example corresponding to amino acid residues V304 and G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

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In a preferred embodiment, a modified BoNT/A L-chain of the present invention having a binding pocket mutation for the K185 binding site on hSNAP-23 can further comprise one or more mutations within one or more different BoNT/A L-chain binding pockets for hSNAP-23 as herein described

(e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, as defined above). Such mutants typically demonstrate at least a 2.0-fold, preferably at least a 2.5-fold increased hSNAP-23 cleavage (versus E148Y modified BoNT/A L-chain) - see the 1st data column for multi-pocket mutants in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

a modified BoNT/A L-chain having the substitution E148Y, and G305D.

Thus, in another embodiment there is provided a modified BoNT/A L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- b) an amino acid residue change located within a fourth BoNT/A L-chain protease binding pocket for binding to the K185 binding site of hSNAP-23; wherein said fourth BoNT/A L-chain protease binding pocket is defined by amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
 - ii. an amino acid residue selected from the group consisting of aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

Still, as an additional technical feature, or as an alternative technical feature, the present invention includes BoNT/A L-chain mutants comprising one or more mutations within a herein defined pocket of the BoNT/A L-chain. By way of example, a modified BoNT/A L-chain of the present invention that cleaves human SNAP-23 (hSNAP-23) has a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:

- a) an amino acid residue change located within a second BoNT/A L-chain protease binding pocket for binding to the D189/D192 binding site of hSNAP-23;
- b) wherein said second BoNT/A L-chain protease binding pocket is defined by amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said amino acid residue change comprises:
 - i. an amino acid residue alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

Without wishing to be bound by any theory, Applicant believes that the above-defined BoNT/A L-chain binding pocket provides a stabilising interaction between D189/D192 on hSNAP-23 and amino acid 29 of BoNT/A or close-by amino acids.

Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

a modified BoNT/A L-chain having the substitution Q29A.

In a preferred embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the D189/D192 binding site on hSNAP-23 further comprises one or more mutations within one or more different BoNT/A L-chain binding pockets as herein described (e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, as defined above). Such

mutants typically demonstrate at least a 1.50-fold% increased hSNAP-23 cleavage (versus E148Y modified BoNT/A L-chain) - see the 1st data column presented for multi-pocket mutants in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a
5 corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
a modified BoNT/A L-chain having the substitution E148Y, Q29A.

Thus, in another embodiment there is provided a modified BoNT/A L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified
10 amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148
15 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- b) an amino acid residue change located within a second BoNT/A L-chain protease binding pocket for binding to the D189/D192 binding site of hSNAP-23; wherein said second BoNT/A L-chain protease binding pocket is defined by amino acid residue Q29 of
20 wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to
25 amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
 - ii. an amino acid residue selected from the group consisting of alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid
30 residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

In another embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the D189/D192 binding site on hSNAP-

23 can further comprise one or more mutations within at least two different BoNT/A L-chain binding pockets as herein described (e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, and within the binding pocket for the R186 binding site on hSNAP-23, both as defined above). Such mutants typically demonstrate at least a 2.5-fold increased hSNAP-23 cleavage (versus E148Y modified BoNT/A L-chain - see the 1st data column presented in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

10 a modified BoNT/A L-chain comprising the substitution E148Y, Q29A, and S143D.

Thus, in another embodiment there is provided a modified BoNT/A L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

15

- a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 20 b) an amino acid residue change located within a second BoNT/A L-chain protease binding pocket for binding to the D189/D192 binding site of hSNAP-23; wherein said second BoNT/A L-chain protease binding pocket is defined by amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 25 c) an amino acid residue change located within a fifth BoNT/A L-chain protease binding pocket for binding to the R186 binding site of hSNAP-23; wherein said fifth BoNT/A L-chain protease binding pocket is defined by amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- 30 d) and wherein said amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain

protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and

- 5 ii. an amino acid residue selected from the group consisting of alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 10 iii. an amino acid residue selected from the group consisting of aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

15 In another embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the D189/D192 binding site on hSNAP-23 can further comprise one or more mutations within at least two different BoNT/A L-chain binding pockets (e.g. within the binding pocket for the P182 /D178 binding site on hSNAP-23, and within the binding pocket for the K185

20 binding site on hSNAP-23, as defined above). Such mutants typically demonstrate at least a 3.0-fold, preferably at least a 3.4-fold increased hSNAP-23 cleavage (versus E148Y modified BoNT/A L-chain) - see the 1st data column presented for multi-pocket mutants in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s)

25 relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include: a modified BoNT/A L-chain comprising the substitutions E148Y, Q29A, and G305D.

Thus, in another embodiment there is provided a modified BoNT/A L-chain

30 protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178

binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and

b) an amino acid residue change located within a second BoNT/A L-chain protease binding pocket for binding to the D189/D192 binding site of hSNAP-23; wherein said second BoNT/A L-chain protease binding pocket is defined by amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and

c) an amino acid residue change located within a fourth BoNT/A L-chain protease binding pocket for binding to the K185 binding site of hSNAP-23; wherein said fourth BoNT/A L-chain protease binding pocket is defined by amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1);

d) and wherein said amino acid residue change comprises:

i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and

ii. an amino acid residue selected from the group consisting of alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and

iii. an amino acid residue selected from the group consisting of aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

As an additional technical feature, or as an alternative technical feature, the present invention includes BoNT/A L-chain mutants comprising one or more mutations within a herein defined pocket of the BoNT/A L-chain. By way of example, a modified BoNT/A L-chain of the present invention that cleaves

human SNAP-23 (hSNAP-23) has a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- a) at least one amino acid residue change located within a sixth BoNT/A L-chain protease binding pocket for binding to the K206 binding site of hSNAP-23;
- b) wherein said sixth BoNT/A L-chain protease binding pocket is defined by amino acid residues Y251, L256, V258, L367 and F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said at least one amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - ii. an amino acid residue selected from the group consisting of glutamate, aspartate, glutamine, glycine, alanine and arginine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue L256 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - iii. an amino acid residue selected from the group consisting of serine, alanine, proline, leucine and glutamate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue V258 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - iv. an amino acid residue selected from the group consisting of alanine and glycine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue L367 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - v. an amino acid residue selected from the group consisting of glycine, serine and leucine at the position on the modified L-chain protease amino acid sequence that

corresponds to amino acid residue F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

As an additional technical feature, or as an alternative technical feature, the present invention includes BoNT/A L-chain mutants comprising one or more mutations within a herein defined pocket of the BoNT/A L-chain. By way of example, a modified BoNT/A L-chain of the present invention that cleaves human SNAP-23 (hSNAP-23) has a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- 10 a) at least one amino acid residue change located within a sixth BoNT/A L-chain protease binding pocket for binding to the K206 binding site of hSNAP-23;
- b) wherein said sixth BoNT/A L-chain protease binding pocket is defined by amino acid residues Y251, L256, V258, L367 and
15 F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said at least one amino acid residue change comprises:
 - 20 i. an amino acid residue selected from the group consisting of glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - 25 ii. an amino acid residue selected from the group consisting of glutamine, and arginine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue L256 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - 30 iii. an amino acid residue selected from the group consisting of serine, leucine and glutamate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue V258 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - iv. an amino acid residue selected from the group consisting of alanine and glycine at the position on the modified L-

chain protease amino acid sequence that corresponds to amino acid residue L367 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or

- 5 v. an amino acid residue selected from the group consisting of glycine, serine and leucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

- 10 Without wishing to be bound by any theory, Applicant believes that the above-defined BoNT/A L-chain binding pocket effects an important enzyme-substrate association with the (S3') position of the recognition sequence for hSNAP-23.

As an additional technical feature, or as an alternative technical feature, the present invention includes BoNT/A L-chain mutants comprising one or more mutations within a herein defined pocket of the BoNT/A L-chain. By way of example, a modified BoNT/A L-chain of the present invention that cleaves human SNAP-23 (hSNAP-23) has a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- 20 a) at least one amino acid residue change located within a sixth BoNT/A L-chain protease binding pocket for binding to the K206 binding site of hSNAP-23;
- b) wherein said sixth BoNT/A L-chain protease binding pocket is defined by amino acid residues Y251, L256, V258, L367 and F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- 25 c) and wherein said at least one amino acid residue change comprises:
- i. an amino acid residue selected from the group consisting of glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
- 30 ii. an amino acid residue selected from the group consisting of glutamine, and arginine at the position on the modified

- L-chain protease amino acid sequence that corresponds to amino acid residue L256 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
- 5 iii. an amino acid residue selected from the group consisting of serine, leucine and glutamate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue V258 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
- 10 iv. an amino acid residue selected from the group consisting of alanine and glycine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue L367 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
- 15 v. an amino acid residue selected from the group consisting of glycine, serine and leucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1).
- 20 A modified BoNT/A L-chain of the present invention having a binding pocket mutation for the K206 binding site on hSNAP-23 can comprise one or more amino acid residue changes relative to the wild-type BoNT/A L-chain as herein before defined. By way of illustration, a modified BoNT/A L-chain of the present invention may have a single amino acid residue mutation (within the
- 25 binding pocket for the K206 binding site on hSNAP-23), for example a mutant corresponding to amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1). Similarly, a modified BoNT/A L-chain of the present invention may comprise more than one mutation (within the binding pocket for the K206 binding site on hSNAP-23), for example mutants corresponding to amino acid
- 30 residues Y251 & L256 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

A modified BoNT/A L-chain of the present invention comprising a binding pocket mutation for the K206 binding site on hSNAP-23 typically demonstrate greater than 1.5% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A

L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column in Figure 1A. For reference, wild-type BoNT/A L-chain (e.g. SEQ ID NO: 1) demonstrates less than 0.5% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column presented in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

- a modified BoNT/A L-chain comprising the substitution Y251D;
- 10 a modified BoNT/A L-chain comprising the substitution Y251E;
- a modified BoNT/A L-chain comprising the substitution L256D.

In one embodiment, modified BoNT/A L-chains of the present invention demonstrate at least 3% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column presented in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

- 15 a modified BoNT/A L-chain comprising the substitution Y251E.

Additional examples of modified BoNT/A L-chain mutants having a binding pocket mutation for K206 on hSNAP-23 (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

- 25 a modified BoNT/A L-chain comprising the substitution V245D;
- a modified BoNT/A L-chain comprising the substitution L256E;
- a modified BoNT/A L-chain comprising the substitution L256G;
- a modified BoNT/A L-chain comprising the substitution L256Q;
- 30 a modified BoNT/A L-chain comprising the substitution L256A;
- a modified BoNT/A L-chain comprising the substitution V258A;
- a modified BoNT/A L-chain comprising the substitution V258P;
- a modified BoNT/A L-chain comprising the substitution V258L;
- a modified BoNT/A L-chain comprising the substitution L256E, and V258P;

a modified BoNT/A L-chain comprising the substitution L256Q, and V258P;
a modified BoNT/A L-chain comprising the substitution L256A, and V258L;
a modified BoNT/A L-chain comprising the substitution L256G, and V258L.

- 5 In a preferred embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the K206 binding site on hSNAP-23 can further comprise one or more mutations within one or more different BoNT/A L-chain binding pockets as herein described (e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, as defined above). Such mutants
- 10 typically demonstrate at least a 0.5-fold decreased hSNAP-23 cleavage (versus E148Y modified BoNT/A L-chain), or, in other words at least a 4.0-fold increased hSNAP23 cleavage (versus wild-type BoNT/A L-chain) - see the 1st data column presented for multi-pocket mutants in Figure 1B, or at least 5 % hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column in Figure 1B. Preferable examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
- 15 a modified BoNT/A L-chain comprising the substitutions E148Y, and Y251D;
20 a modified BoNT/A L-chain comprising the substitutions E148Y, and L256D;
a modified BoNT/A L-chain comprising the substitutions E148Y, and Y251E.

Thus, in another embodiment there is provided a modified BoNT/A L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified

25 amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 30 b) an amino acid residue change located within a fifth BoNT/A L-chain protease binding pocket for binding to the K206 binding site of hSNAP-23; wherein said fifth BoNT/A L-chain protease

binding pocket is defined by amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1);

c) and wherein said amino acid residue change comprises:

- 5 i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 10 ii. an amino acid residue selected from the group consisting of aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

15 In another embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the K206 binding site on hSNAP-23 can further comprise one or more mutations within at least three different BoNT/A L-chain binding pockets as herein described (e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, within the binding pocket for the
20 R186 binding site on hSNAP-23, and within the binding pocket for the D189/D192 binding site, as defined above). Such mutants typically demonstrate at least a 1.3-fold increased hSNAP-23 cleavage (versus E148Y modified BoNT/A L-chain) - see the 1st data column presented for multi-pocket mutants in Figure 1B. Examples of such modified BoNT/A L-chains (referred
25 to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

a modified BoNT/A L-chain comprising the substitutions E148Y, S143D, Q29A, and Y251E.

30 Thus, in another embodiment there is provided a modified BoNT/A L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- 5 a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 10 b) an amino acid residue change located within a fifth BoNT/A L-chain protease binding pocket for binding to the R186 binding site of hSNAP-23; wherein said fifth BoNT/A L-chain protease binding pocket is defined by amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 15 c) an amino acid residue change located within a second BoNT/A L-chain protease binding pocket for binding to the D189/D192 binding site of hSNAP-23; wherein said second BoNT/A L-chain protease binding pocket is defined by amino acid residue Q29A of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 20 d) an amino acid residue change located within a sixth BoNT/A L-chain protease binding pocket for binding to the K206 binding site of hSNAP-23; wherein said sixth BoNT/A L-chain protease binding pocket is defined by amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- e) and wherein said amino acid residue change comprises:
- 25 i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- 30 ii. an amino acid residue selected from the group consisting of aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- iii. an amino acid residue selected from the group consisting of alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and

- iv. an amino acid residue selected from the group consisting of glutamate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

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As an additional technical feature, or as an alternative technical feature, the present invention includes BoNT/A L-chain mutants comprising one or more mutations within a herein defined pocket of the BoNT/A L-chain. By way of example, a modified BoNT/A L-chain of the present invention that cleaves human SNAP-23 (hSNAP-23) has a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:

- a) an amino acid residue change located within a third BoNT/A L-chain protease binding pocket for binding to the I198 binding site of hSNAP-23;
- b) wherein said third BoNT/A L-chain protease binding pocket is defined by amino acid residue K166 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of valine, phenylalanine, leucine and isoleucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue K166 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

Without wishing to be bound by any theory, Applicant believes that the above-defined BoNT/A L-chain binding pocket can provide a stabilising hydrophobic interaction between I198 on hSNAP-23 and amino acid 166 of BoNT/A.

Modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the I198 binding site on hSNAP-23 typically demonstrate greater than 9% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column presented in Figure 1A. For reference, wild-type BoNT/A L-chain (e.g. SEQ ID NO: 1) demonstrates less than 0.5%

hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column presented in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

- a modified BoNT/A L-chain comprising the substitution K166V;
- a modified BoNT/A L-chain comprising the substitution K166F;
- a modified BoNT/A L-chain comprising the substitution K166L;
- a modified BoNT/A L-chain comprising the substitution K166I.

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In one embodiment, modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the I198 binding site on hSNAP-23 typically demonstrate greater than 40% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column presented in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

- a modified BoNT/A L-chain comprising the substitution K166F;
- a modified BoNT/A L-chain comprising the substitution K166L.

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In a further embodiment, modified BoNT/A L-chains of the present invention comprising a binding pocket mutation for the I198 binding site on hSNAP-23 typically demonstrate greater than 60% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column presented in Figure 1A. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

- a modified BoNT/A L-chain comprising the substitution K166F.

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In a preferred embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the I198 binding site on hSNAP-23 can further comprise one or more mutations within one or more different BoNT/A

L-chain binding pockets as herein described (for example, within at least two different BoNT/A L-chain binding pockets such as the binding pocket for the P182/D178 binding site on hSNAP-23, and the binding pocket for the K185 or R186 binding site on hSNAP-23). Such mutants typically demonstrate at least 5 40% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column presented in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

10 a modified BoNT/A L-chain comprising the substitutions E148Y, K166F, and G305D;
a modified BoNT/A L-chain comprising the substitutions E148Y, K166V, and G305D;
a modified BoNT/A L-chain comprising the substitutions E148Y, S143D, and
15 K166F.

Thus, in another embodiment there is provided a modified BoNT/A L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1)
20 that comprises:

- a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A
25 L-chain (SEQ ID NO: 1); and
- b) an amino acid residue change located within a third BoNT/A L-chain protease binding pocket for binding to the I198 binding site of hSNAP-23; wherein said third BoNT/A L-chain protease binding pocket is defined by amino acid residue K166 of wild-type BoNT/A
30 L-chain (SEQ ID NO: 1); and
- c) an amino acid residue change located within a fourth BoNT/A L-chain protease binding pocket for binding to the K185 binding site of hSNAP-23; wherein said fourth BoNT/A L-chain protease binding

pocket is defined by amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1);

d) and wherein said amino acid residue change comprises:

- i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- ii. an amino acid residue selected from the group consisting of valine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue K166 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
- iii. an amino acid residue selected from the group consisting of aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

In another embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the I198 binding site on hSNAP-23 can further comprise one or more mutation(s) within at least two different BoNT/A L-chain binding pockets (e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, and within the binding pocket for the K185 or R186 binding site on hSNAP-23). Such mutants typically demonstrate at least 15% hSNAP-23 cleavage (% at 10 nanomolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column ** presented in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

- a modified BoNT/A L-chain comprising the substitution E148Y, K166F, and G305D;
- a modified BoNT/A L-chain comprising the substitution E148Y, S143D, and K166F.

In one embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the I198 binding site on hSNAP-23 can further

comprise one or more mutations within at least three different BoNT/A L-chain binding pockets (e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, within the binding pocket for the D189/D192 binding site on hSNAP23, and within the binding pocket for the K185 binding site on hSNAP-
5 23, as defined above). Such mutants typically demonstrate at least 60% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column presented in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a
10 corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
a modified BoNT/A L-chain comprising the substitutions E148Y, Q29A, K166V, and G305D;
a modified BoNT/A L-chain comprising the substitutions E148Y, Q29A, K166F, and G305D.

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Thus, in another embodiment there is provided a modified BoNT/A L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1) that comprises:

- 20 a) an amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182/D178 binding site of hSNAP-23; wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
25 b) an amino acid residue change located within a third BoNT/A L-chain protease binding pocket for binding to the I198 binding site of hSNAP-23; wherein said third BoNT/A L-chain protease binding pocket is defined by amino acid residue K166 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
30 c) an amino acid residue change located within a second BoNT/A L-chain protease binding pocket for binding to the D189/D192 binding site of hSNAP-23; wherein said second BoNT/A L-chain protease binding pocket is defined by amino acid residue Q29A of wild-type BoNT/A L-chain (SEQ ID NO: 1); and

- 5 d) an amino acid residue change located within a fourth BoNT/A L-chain protease binding pocket for binding to the K185 binding site of hSNAP-23; wherein said fourth BoNT/A L-chain protease binding pocket is defined by amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- e) and wherein said amino acid residue change comprises:
- 10 i. an amino acid residue selected from the group consisting of tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
 - ii. an amino acid residue selected from the group consisting of valine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue K166 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
 - 15 iii. an amino acid residue selected from the group consisting of alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and
 - 20 iv. an amino acid residue selected from the group consisting of aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

25 In another embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the I198 binding site on hSNAP-23 can further comprise one or more mutations within at least three different BoNT/A L-chain binding pockets (e.g. within the binding pocket for the P182 /D178 binding site on hSNAP-23, within the binding pocket for the D189/D192 binding site on hSNAP23, and within the binding pocket for the K185 binding site on hSNAP-23). Such mutants typically demonstrate at least 10% hSNAP-23 cleavage (% at 10 nanomolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column ** presented in Figure 1B. Examples of such modified BoNT/A L-

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chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

a modified BoNT/A L-chain comprising the substitution E148Y, Q29A, K166F, and G305D.

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Additional examples of modified BoNT/A L-chain mutants having a binding pocket mutation for I198 on hSNAP-23 (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

10 a modified BoNT/A L-chain comprising the substitution E148Y, Q29A, K166F, Y251E, and G305D.

In one embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the I198 binding site on hSNAP-23 can further
15 comprise one or more mutations within one or more different BoNT/A L-chain binding pockets (e.g. within the binding pocket for the P182/D178 binding site on hSNAP-23, or within the binding pocket for the K185 binding site on hSNAP-23, as defined above). Such mutants typically demonstrate at least 3% hSNAP-23 cleavage (% at 10 nanomolar modified BoNT/A L-chain; 20
20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column ** presented in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

a modified BoNT/A L-chain comprising the substitution E148Y, and K166F;

25 a modified BoNT/A L-chain comprising the substitution G305D, and K166F.

In another embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the I198 binding site on hSNAP-23 can further comprise one or more mutation(s) within one or more different BoNT/A
30 L-chain binding pockets (e.g. within the binding pocket for the P182 /D178 binding site on hSNAP-23, or within the binding pocket for the K185 binding site on hSNAP-23). Such mutants typically demonstrate at least 5% hSNAP-23 cleavage (% at 10 nanomolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd

data column ** presented in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:
a modified BoNT/A L-chain comprising the substitution E148Y, and K166F.

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As an additional technical feature, or as an alternative technical feature, the present invention includes BoNT/A L-chain mutants comprising one or more mutations within a herein defined pocket of the BoNT/A L-chain. By way of example, a modified BoNT/A L-chain of the present invention that cleaves
10 human SNAP-23 (hSNAP-23) has a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:

- a) an amino acid residue change located within a seventh BoNT/A L-chain protease binding pocket for binding to the D210 binding site of hSNAP-23;
- 15 b) wherein said seventh BoNT/A L-chain protease binding pocket is defined by amino acid residue S254 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said amino acid residue change comprises:
 - 20 i. an (e.g. the) amino acid residue alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue S254 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

Without wishing to be bound by any theory, Applicant believes that the above-
25 defined BoNT/A L-chain binding pocket can exhibit hydrogen bonding with the D210 binding site of hSNAP-23. Moreover, Applicant believes modification of said binding pocket (as herein defined) precludes a hydrogen bond proposed to be formed between D210 on hSNAP-23 and amino acid 254 of BoNT/A. This is, in turn, believed to generate a C-terminal hSNAP-23 cleavage product
30 in the form of a better “leaving group”, and thereby enhance the hSNAP-23 cleavage rate.

Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

a modified BoNT/A L-chain comprising the substitution S254A.

5

In one embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the D210 binding site on hSNAP-23 can further comprise one or more mutations within one or more different BoNT/A L-chain binding pockets as herein described (for example, within at least two or three different BoNT/A L-chain binding pockets such as the binding pocket for the P182/D178 binding site on hSNAP-23, and the binding pocket for the I198 binding site on hSNAP-23, and optionally the binding pocket for the K185 binding site on hSNAP23). Such mutants typically demonstrate at least 10% hSNAP-23 cleavage (% at 10 nanomolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column ** presented in Figure 1B. Examples of such modified BoNT/A L-chains include:

- 10 a modified BoNT/A L-chain comprising the substitution E148Y, K166F, and S254A;
- 15 a modified BoNT/A L-chain comprising the substitution E148Y, K166F, S254A, and G305D.

In a preferred embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the D210 binding site on hSNAP-23 can further comprise one or more mutations within one or more different BoNT/A L-chain binding pockets as herein described (for example, within at least three different BoNT/A L-chain binding pockets such as the binding pocket for the P182/D178 binding site on hSNAP-23, the binding pocket for the I198 binding site on hSNAP-23, and the binding pocket for the K185 binding site on hSNAP23). Such mutants typically demonstrate at least 25% hSNAP-23 cleavage (% at 10 nanomolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 37°C for about 1 hour) - see the 2nd data column ** presented in Figure 1B. Examples of such modified BoNT/A L-

chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

a modified BoNT/A L-chain comprising the substitution E148Y, K166F, S254A, and G305D.

5

As an additional technical feature, or as an alternative technical feature, the present invention includes BoNT/A L-chain mutants comprising one or more mutations within a herein defined pocket of the BoNT/A L-chain. By way of example, a modified BoNT/A L-chain of the present invention that cleaves human SNAP-23 (hSNAP-23) has a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:

10

a) an amino acid residue change located within an eighth BoNT/A L-chain protease binding pocket for binding to the D168 binding site of hSNAP-23;

15

b) wherein said eighth BoNT/A L-chain protease binding pocket is defined by amino acid residue K340 of wild-type BoNT/A L-chain (SEQ ID NO: 1);

c) and wherein said amino acid residue change comprises:

20

i. an (e.g. the) amino acid residue histidine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue K340 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

Without wishing to be bound by any theory, Applicant believes that the above-defined BoNT/A L-chain binding pocket can provide a salt bridge between D168 on hSNAP-23 and amino acid 340 of BoNT/A.

25

Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

30

a modified BoNT/A L-chain comprising the substitution K340H.

In one embodiment, a modified BoNT L-chain of the present invention having a binding pocket mutation for the P182/D178 binding site on hSNAP-23 can

further comprise one or more mutations within the BoNT/A L-chain binding pocket for the D168 binding site on hSNAP-23. Such mutants typically demonstrate at least 3% hSNAP-23 cleavage (% at 1 micromolar modified BoNT/A L-chain; 20 micromolar hSNAP-23; preferably incubated at about 5 37°C for about 1 hour) - see the 2nd data column presented in Figure 1B. Examples of such modified BoNT/A L-chains (referred to by the amino acid substitution(s) relative to a corresponding wild-type BoNT/A L-chain SEQ ID NO:1) include:

a modified BoNT/A L-chain comprising the substitution E148Y, and K340H.

10

The modified BoNT/A L-chain protease as described above can comprise an amino acid sequence having at least 70%, for example, at least 80% or at least 85% or at least 90% or at least 95% or at least 97% or at least 98% or at least 99%, sequence identity to the wild-type BoNT/A L-chain (SEQ ID NO: 1).

15

The modified BoNT/A L-chain amino acid sequence has less than 100% sequence identity to a wild-type BoNT/A L-chain (e.g. SEQ ID NO: 1). As previously indicated, reference through this specification to a modified BoNT/A L-chain protease embraces functional fragments thereof, that is fragments of said protease that cleave hSNAP-23. For example, a modified BoNT/A L-chain protease of the invention comprises at least 300 (for example, at least 20 350 or at least 400 or at least 410) amino acids. By way of example, the N-terminal eight amino acids and/ or the carboxyl-terminus (for example, the last 32 amino acids) of a botulinum neurotoxin L-chain protease are not required for proteolytic activity.

25

A modified BoNT/A L-chain of the present invention may be PEGylated to increase stability, for example duration of action of the protease component. PEGylation preferably includes the addition of PEG to the N-terminus of the L-chain. By way of example, the N-terminus of the L-chain may be extended 30 with one or more amino acid (e.g. cysteine) residues. 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 incorporated in its entirety by reference thereto.

A modified BoNT/A L-chain of the present invention may include the addition (or removal) of "secondary modification sites" - see WO2002/040506, US7223577 and WO2005/068494, each of which is incorporated in its entirety by reference thereto. The additional presence or absence (vis-à-vis wild-type BoNT L-chain) of such sites alters the biological persistence (e.g. biological half-life) of a modified L-chain of the invention.

A second aspect of the present invention provides a nucleic acid construct, comprising or consisting of a nucleic acid sequence that encodes a modified BoNT/A L-chain as herein described. Said nucleic acid sequence may preferably encode a TSI delivery vehicle as further described below. A nucleic acid construct of the invention may include conventional regulatory elements such as a promoter and/or a terminator.

In one embodiment, the nucleic acid construct is provided in the form of a bacterial plasmid or viral vector. Said nucleic acid construct can optionally be codon-biased for optimizing expression (e.g. recombinant expression) in a desired host cell (e.g. *E.coli*).

In one embodiment, a nucleic acid construct encoding a modified BoNT/A L-chain as herein described can be employed for administration to a target cell of interest, such as for therapeutic or cosmetic purpose. To this end, said nucleic acid construct can be typically optimised by way of conventional methodology for delivery into (followed by expression within) a target cell, preferably a human cell. The target cell is preferably a non-neuronal cell.

A third aspect of the present invention provides a delivery vehicle for the modified BoNT/A L-chain, thereby facilitating entry of the modified BoNT/A L-chain into a target cell of interest. According to this aspect, the invention more specifically relates to a delivery vehicle, comprising:

- a) a modified BoNT/A L chain protease according to the invention, or a nucleic acid construct comprising or consisting of a nucleic acid sequence encoding a modified BoNT/A L-chain according to the invention; and

b) means for delivering said modified BoNT/A L chain protease, or said nucleic acid construct, to a target cell.

Preferred means for such delivery include any conventional delivery vehicle technique known in the art, such as liposomes, biolistic particles, cell-penetrating peptides, gene transfer vectors, etc.

One particularly preferred delivery technique that is highly suited for use with the present invention is Applicant's proprietary Targeted Secretion Inhibitor (TSI) technology. The basic methods employed to generate a TSI are well documented and now considered conventional (see for example, WO98/07864, WO2006/059113, WO2009/150469, WO2010/020811, WO2009/150470, WO2010/094905, WO2012/156743, each of which is incorporated by reference in its entirety). TSI technology relies on a delivery mechanism that mimics the same basic steps employed by a clostridial neurotoxin when it intoxicates a host cell (i.e. binding to a target cell, endosome formation, translocation of L-chain into the cytosol, proteolytic cleavage of SNARE protein by the L-chain). TSI delivery vehicles are based on a simple clostridial neurotoxin backbone having three principal components:

- 1) a clostridial neurotoxin L-chain;
- 2) a Targeting Moiety (TM) to direct the delivery vehicle to a target cell of choice. Typically, the native clostridial neurotoxin binding domain (H_{CC}) can be replaced by a ligand to provide selective binding of the delivery vehicle to a desired target cell other than the native target cell of said H_{CC} . In a preferred embodiment, more than one TM can be used (optionally including clostridial neurotoxin binding domain);
- 3) a translocation peptide (e.g. a clostridial neurotoxin H_N translocation domain) to ensure delivery of the clostridial neurotoxin L-chain into a target cell where it can then exert its proteolytic effect (i.e. cleavage of a SNARE protein).

Thus, in a preferred embodiment, the means b) of the delivery vehicle of the invention may comprise:

- i) a Targeting Moiety (TM) that binds the delivery vehicle to a target cell. Said Targeting Moiety can either be a native clostridial neurotoxin binding domain (H_{CC}) or, more preferably, a ligand providing binding to a target cell other than the native target cell of said H_{CC} ; and
- 5 - ii) a translocation peptide that translocates the modified BoNT/A L chain protease of the invention into the target cell, preferably into the cytosol of said cell.

A delivery vehicle of the present invention typically includes one or more
10 Targeting Moiety (TM). Reference to a TM embraces any structure (typically a peptide) that functionally interacts with a Site (e.g. a receptor or acceptor) to cause a physical association between the modified BoNT/A L-chain protease of the invention and the surface of a mammalian target cell (e.g. a human cell). The Site is preferably one that is capable of internalisation (eg.
15 endosome formation) - also referred to as receptor-mediated endocytosis. The TM may possess an endosomal membrane translocation function, in which case separate TM and Translocation Domain components need not be employed.

20 The TM of the present invention binds (e.g. specifically binds) to a target cell of choice. The term "specifically binds" preferably means that a given TM binds to the target cell with a binding affinity (K_a) of $10^6 M^{-1}$ or greater, for example $10^7 M^{-1}$ or greater, $10^8 M^{-1}$ or greater, or $10^9 M^{-1}$ or greater.

25 It is routine to confirm that a TM binds to the selected target cell. For example, a simple radioactive displacement experiment may be employed in which tissue or cells representative of a target cell of interest are exposed to labelled (eg. tritiated) TM in the presence of an excess of unlabelled TM. In such an experiment, the relative proportions of non-specific and specific binding may
30 be assessed, thereby allowing confirmation that the TM binds to the target cell. Optionally, the assay may include one or more binding antagonists, and the assay may further comprise observing a loss of TM binding. Examples of this type of experiment can be found in Hulme, E.C. (1990), Receptor-binding

studies, a brief outline, pp. 303-311, In Receptor biochemistry, A Practical Approach, Ed. E.C. Hulme, Oxford University Press.

- A TM of the present invention preferably binds to a non-neuronal target cell (e.g. a mast cell and/ or an epithelial cell – see, for example WO00/10598 and WO01/21213, each of which is incorporated in its entirety by reference thereto). In doing so, said TM is able to direct the delivery vehicle to a chosen non-neuronal target cell that is expressing an undesired hSNAP-23 phenotype (and optionally an undesired SNAP-25 phenotype). In parallel, a TM of the present invention may separately bind (e.g. via the same TM or via a second TM) to a second target cell of choice, for example to a second non-neuronal target cell or to a neuronal target cell expressing an undesired hSNAP-23 and/ or SNAP-25 phenotype.
- Suitable TMs include: ligands to mammalian cell Binding Sites receptors such as cytokines, growth factors, neuropeptides, lectins, and antibodies – this term includes monoclonal antibodies, single-chain antibodies, and antibody fragments such as Fab, F(ab)₂, Fv, ScFv, etc.
- By way of further example, TMs include a leptin peptide, a ghrelin receptor, a somatostatin peptide, an insulin growth factor peptide, an ErbB peptide (e.g. EGF), a VIP-glucagon-GRF-secretin peptide (e.g. a PACAP peptide), an interleukin peptide (e.g. IL-1, IL-2, IL-6 or IL-10 peptide), a NGF peptide, a VEGF peptide, a bombesin peptide, a urotensin peptide, a melanin-concentrating hormone peptide, a prolactin releasing hormone peptide, a KiSS-1 peptide, a CRF peptide, a GHRH peptide, a substance P peptide, a beta-2 adrenoreceptor peptide, a gastrin-releasing peptide, a calcitonin gene-related peptide, a platelet-derived growth factor peptide, a keratinocyte growth factor peptide, a hepatocyte growth factor peptide, a TGF-alpha peptide, a TGF-beta peptide, an atrial natriuretic peptide, and an integrin peptide.

A delivery vehicle of the invention typically lacks a (functional) clostridial neurotoxin binding domain (as a TM).

Alternatively, a delivery vehicle of the invention may include a (functional) clostridial neurotoxin binding domain (as a TM). Reference to a clostridial neurotoxin binding domain embraces the H_C (more precisely, the H_{CC}) part of a clostridial neurotoxin, as well as mutants thereof that retain a binding capability of the H_C domain (e.g. to bind rat synaptosomal membranes in conventional binding assays such as described in Shone et al. (1985) Eur. J. Biochem. 151, 75-82).

The H_C binding domain/peptide of a native clostridial neurotoxin comprises approximately 400-440 amino acid residues, and consists of two functionally distinct domains of approximately 25kDa each, namely the N-terminal region (commonly referred to as the H_{CN} peptide or domain) and the C-terminal region (commonly referred to as the H_{CC} peptide or domain). It is the C-terminal region (H_{CC}), which constitutes the C-terminal 160-200 amino acid residues, that is responsible for binding of a clostridial neurotoxin (to nerve terminals at the neuromuscular junction). Exemplary H_{CC} peptides include:

Botulinum type A neurotoxin - amino acid residues (Y1111-L1296)
Botulinum type B neurotoxin - amino acid residues (Y1098-E1291)
Botulinum type C neurotoxin - amino acid residues (Y1112-E1291)
Botulinum type D neurotoxin - amino acid residues (Y1099-E1276)
Botulinum type E neurotoxin - amino acid residues (Y1086-K1252)
Botulinum type F neurotoxin - amino acid residues (Y1106-E1274)
Botulinum type G neurotoxin - amino acid residues (Y1106-E1297)
Tetanus neurotoxin - amino acid residues (Y1128-D1315).

The above-identified reference sequences should be considered a guide, as slight variations may occur according to sub-serotypes.

A delivery vehicle of the present invention typically includes a translocation peptide, which enables translocation of the modified L-chain into the cytosol of a target. Whether a peptide possesses the requisite translocation function of the present invention may be confirmed by any one of a number of conventional assays. For example, Shone C. (1987) describes an *in vitro* assay employing liposomes, which are challenged with a test molecule.

Presence of the requisite translocation function is confirmed by release from the liposomes of K^+ and/or labelled NAD, which may be readily monitored [see Shone C. (1987) Eur. J. Biochem; vol. 167(1): pp. 175-180]. A further example is provided by Blaustein R. (1987), which describes a simple *in vitro* assay
 5 employing planar phospholipid bilayer membranes. The membranes are challenged with a test molecule and the requisite translocation function is confirmed by an increase in conductance across said membranes [see Blaustein (1987) FEBS Letts; vol. 226, no. 1: pp. 115-120]. Additional methodology to enable assessment of membrane fusion and thus
 10 identification of Translocation Domains suitable for use in the present invention are provided by Methods in Enzymology Vol 220 and 221, Membrane Fusion Techniques, Parts A and B, Academic Press 1993.

The Translocation Domain may be of a clostridial origin, such as the H_N
 15 domain/portion of a neurotoxin. Reference to a " H_N domain" means a fragment of the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain. A H_N domain of a clostridial neurotoxin lacks the natural binding function of the H_C component of the H-chain. Thus, a H_N domain is incapable of binding to the Binding Site on a target cell to which
 20 native clostridial neurotoxin (i.e. holotoxin) binds.

Examples of suitable (reference) Translocation Domains include:

	Botulinum type A neurotoxin	- amino acid residues (449-871)
	Botulinum type B neurotoxin	- amino acid residues (441-858)
25	Botulinum type C neurotoxin	- amino acid residues (442-866)
	Botulinum type D neurotoxin	- amino acid residues (446-862)
	Botulinum type E neurotoxin	- amino acid residues (423-845)
	Botulinum type F neurotoxin	- amino acid residues (440-864)
	Botulinum type G neurotoxin	- amino acid residues (442-863)
30	Tetanus neurotoxin	- amino acid residues (458-879)

Research has shown that the entire length of a H_N portion from a clostridial neurotoxin heavy chain is not necessary for translocation activity. Thus, aspects of this embodiment can include clostridial toxin H_N regions comprising

a translocation domain having a length of, for example, at least 350 amino acids, at least 375 amino acids, at least 400 amino acids and at least 425 amino acids. For further details on the genetic basis of toxin production in *Clostridium botulinum* and *C. tetani*, we refer to Henderson *et al* (1997) in *The Clostridia: Molecular Biology and Pathogenesis*, Academic press.

The term H_N embraces naturally-occurring neurotoxin H_N portions as well as variant H_N portions having amino acid sequences that do not occur in nature so long as the variant H_N portions still demonstrate the above-mentioned translocation function. For example, a clostridial neurotoxin H_N portion embraces variant amino acid sequences having at least 70% (for example, at least 80% or at least 85% or at least 90% or at least 95% or at least 97% or at least 98% or at least 99%) sequence identity to a wild-type clostridial neurotoxin H_N portion, though with the proviso that a translocation function is retained.

Alternatively, the translocation peptide may be of a non-clostridial origin, for example, the translocation domain of diphtheria toxin [O.Keefe *et al.*, Proc. Natl. Acad. Sci. USA (1992) 89, 6202-6206; Silverman *et al.*, J. Biol. Chem. (1993) 269, 22524-22532; and London, E. (1992) *Biochem. Biophys. Acta.*, 1112, pp.25-51], the translocation domain of *Pseudomonas* exotoxin type A [Prior *et al.* Biochemistry (1992) 31, 3555-3559], the translocation domains of anthrax toxin [Blanke *et al.* Proc. Natl. Acad. Sci. USA (1996) 93, 8437-8442], a variety of fusogenic or hydrophobic peptides of translocating function [Plank *et al.* J. Biol. Chem. (1994) 269, 12918-12924; and Wagner *et al* (1992) *PNAS*, 89, pp.7934-7938], and amphiphilic peptides [Murata *et al* (1992) *Biochem.*, 31, pp.1986-1992].

Reference to non-clostridial neurotoxin translocation peptides embraces fragment and variant amino acid sequences having at least 70% (for example, at least 80% or at least 85% or at least 90% or at least 95% or at least 97% or at least 98% or at least 99%) sequence identity to the corresponding non-clostridial wild-type translocation peptide sequence, though with proviso that the variant possesses the requisite.

Translocation Domain source	Amino acid residues	References
Diphtheria toxin	194-380	Silverman <i>et al.</i> , 1994, J. Biol. Chem. 269, 22524-22532 London E. , 1992, Biochem. Biophys. Acta., 1113, 25-51
Domain II of pseudomonas exotoxin	405-613	Prior <i>et al.</i> , 1992, Biochemistry 31, 3555-3559 Kihara & Pastan , 1994, Bioconj Chem. 5, 532-538
Influenza virus haemagglutinin	GLFGAIAGFIENGWE GMIDGWYG, and Variants thereof	Plank <i>et al.</i> , 1994, J. Biol. Chem. 269, 12918-12924 Wagner <i>et al.</i> , 1992, PNAS, 89, 7934-7938 Murata <i>et al.</i> , 1992, Biochemistry 31, 1986-1992
Semliki Forest virus fusogenic protein	Translocation domain	Kielian <i>et al.</i> , 1996, J Cell Biol. 134(4), 863-872
Vesicular Stomatitis virus glycoprotein G	118-139	Yao <i>et al.</i> , 2003, Virology 310(2), 319-332
SER virus F protein	Translocation domain	Seth <i>et al.</i> , 2003, J Virol 77(11) 6520-6527
Foamy virus envelope glycoprotein	Translocation domain	Picard-Maureau <i>et al.</i> , 2003, J Virol. 77(8), 4722-4730

The polypeptides of the present invention may further comprise a translocation facilitating domain. Said domain facilitates delivery of the non-cytotoxic protease into the cytosol of the target cell and are described, for example, in WO 08/008803 and WO 08/008805, each of which is herein incorporated by reference thereto.

By way of example, suitable translocation facilitating domains include an enveloped virus fusogenic peptide domain, for example, suitable fusogenic peptide domains include influenzavirus fusogenic peptide domain (eg. influenza A virus fusogenic peptide domain of 23 amino acids), alphavirus fusogenic peptide domain (eg. Semliki Forest virus fusogenic peptide domain of 26 amino acids), vesiculovirus fusogenic peptide domain (eg. vesicular stomatitis virus fusogenic peptide domain of 21 amino acids), respirovirus fusogenic peptide domain (eg. Sendai virus fusogenic peptide domain of 25 amino acids), morbillivirus fusogenic peptide domain (eg. Canine distemper virus fusogenic peptide domain of 25 amino acids), avulavirus fusogenic peptide domain (eg. Newcastle disease virus fusogenic peptide domain of 25 amino acids), henipavirus fusogenic peptide domain (eg. Hendra virus fusogenic peptide domain of 25 amino acids), metapneumovirus fusogenic peptide domain (eg. Human metapneumovirus fusogenic peptide domain of 25 amino acids) or spumavirus fusogenic peptide domain such as simian foamy virus fusogenic peptide domain; or fragments or variants thereof.

By way of further example, a translocation facilitating domain may comprise a clostridial neurotoxin H_{CN} domain or a fragment or variant (having at least 70% sequence identity to the corresponding wild-type sequence), though with the proviso that an enhanced translocation function is retained. In more detail, a Clostridial toxin H_{CN} translocation facilitating domain may have a length of at least 200 amino acids, at least 225 amino acids, at least 250 amino acids, at least 275 amino acids. In this regard, a Clostridial toxin H_{CN} translocation facilitating domain preferably has a length of at most 200 amino acids, at most 225 amino acids, at most 250 amino acids, or at most 275 amino acids. Specific (reference) examples include:

	Botulinum type A neurotoxin	- amino acid residues (872-1110)
	Botulinum type B neurotoxin	- amino acid residues (859-1097)
30	Botulinum type C 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).

A fourth aspect of the present invention provides a method of cleaving hSNAP-23, said method comprising contacting hSNAP-23 with a modified BoNT/A L-chain protease, or with a nucleic acid construct, or with a delivery vehicle, as herein described, thereby allowing the modified BoNT/A L-chain to bind said hSNAP-23, followed by proteolytic cleavage of the hSNAP-23 by the modified BoNT/A L-chain protease. In one embodiment, said method is performed *in vitro*.

10

In one embodiment, said method of cleaving hSNAP-23 includes the preliminary steps of:

- 1) binding of the delivery vehicle via its Targeting Moiety (TM) to a target cell; and
- 15 2) translocation of the modified BoNT/A L-chain into the target cell, preferably into the cytosol of said target cell, via the translocation peptide of the delivery vehicle.

Preferably, the TM binds to a site on the target cell (e.g. to a protein, sugar, and/or lipid molecule), said site being capable of receptor-mediated endocytosis, and the delivery vehicle is subsequently internalised within the target cell via endosome formation. Thereafter, the translocation peptide of the delivery vehicle can translocate the modified BoNT/A L-chain across the endosomal membrane and into the cytosol of the target cell.

25

In another aspect, the invention relates to a modified (BoNT/A) L-chain protease, or to a nucleic acid construct, or to a delivery vehicle, as herein described, for use in a method of cleaving hSNAP23, as described above.

- 30 In another aspect, the present invention embraces a modified (BoNT/A) L-chain protease described herein for use in a method of treatment, preferably a method of treating a secretory disorder.

Thus, one aspect provides a BoNT/A L-chain protease described herein, or a nucleic acid construct described herein, or a delivery vehicle described herein for use as a medicament.

- 5 In such aspects, said modified (BoNT/A) L-chain protease is preferably comprised within a BoNT additionally comprising a heavy chain i.e. a full length BoNT. Such full length BoNTs typically have a 150 kDa polypeptide chain comprising a 100kDa heavy chain and a 50kDa light chain linked by a disulfide bond, and are organized into three functional domains: an N-terminal proteolytic light chain (L-chain); and a C-terminal heavy chain (H-chain), the
10 latter consisting of a translocation domain (H_N) and a C-terminal neuron-binding domain (H_C).

- Preferred secretory disorders include muscle spasticity/overactive muscle
15 movement (including post-stroke spasticity, post-spinal cord injury spasticity, spasms of the head and neck, eyelid, vagina, limbs, jaw, and vocal cords), strabismus, hyperhidrosis, and severe primary axillary hyperhidrosis.

- The present invention will be better understood in the light of the following
20 detailed examples. Nevertheless, the skilled artisan will appreciate that this detailed description is not limitative and that various modifications, substitutions, omissions, and changes may be made without departing from the scope of the invention.

25 **DESCRIPTION OF THE DRAWINGS**

- Figure 1** - hSNAP-23 and hSNAP25 cleavage data for modified BoNT/A L-chain proteases of the present invention. **(A)** data for BoNT/A L-chain protease having mutations in a single binding pocket, **(B)** data for BoNT/A L-chain protease having mutations in multiple binding pockets.
30

Figure 2 – A 3D rendered image of the BoNT/A L-chain protease (countour) interaction with binding pockets of SNAP-25/23 (line rendered).

AMINO ACID SEQUENCES

SEQ ID NO: 1 - wild-type BoNT/A light chain (amino acid residues 1-438 of Uniprot A5HZZ9)

5 MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMOPVKAFKIHNKI WVIPERDTFTNP EEG
DLNPPPEAKQVPVSYDSTYLSTDNEKDNYLKGVTKL FERIYSTDLGRMLLTSIVRG
IPFWGGSTIDTELKVIDTNCINVIQPDGSYRSEELNLVI IGPSADIIQFECKSFGHE
VLNLTRNGYGSTQYIRFSPDFTFGFEESLEVD TNPLL GAGKFATDPAVTLAHELIHA
GHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDSLQENEFRLY
10 YYNKFKDIAS TLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDKLFKDKLY
KMLTEIYTEDNFVKFFKVLNRKTYLNFDAVF KINIVPKVNYTIYDGFNLRNTNLAA
NFNGQNT EINNMF TKLKNFTGLFEFYKLLCV RGIITSK

SEQ ID NO: 2 - human SNAP23

15 MDNLSSEEIQQRAHQITDESLESTRILGLAIESQDAGIKTITMLDEQKEQLNRIEE
GLDQINKDMRETEKTLTELNKCCGLCVCPCNRTKNFESGKAYKTTWGDGGENS PCNV
VSKQPGPVTNGQLQQPTTGAASGGYIKRITNDAREDEMEENLTQVGSILGNLKD MAL
NIGNEIDAQNPQIKRITDKADTNRDRIDIANARAKKLIDS

20 SEQ ID NO: 3 - human and rodent SNAP25

MAEDADMRNELEEMQRRADQLADESLESTRMLQLVEESKDAGIRTLVMLDEQGEQL
ERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNKLKSSDAYKKAWGNNQDGVVA
SQPARVVDEREQMAISGGFIRRVTDARENEMDENLEQVSGIIGNLRHMA LDMGNEI
DTQNRQIDRIMEKADS NKTRIDEANQRATKMLGSG

25

SEQ ID NO: 4 – IgA-protease site His6Tag (artificial)
PPTPGHHHHHH

SEQ ID NO: 5 - Twin Strep Tag (artificial)

30 MASWSHPQFEKGGSGGGSGGGSWSHPPQFEKGAGS

SEQ ID NO: 6 - His6 Tag (artificial)
GHHHHHHH

35 SEQ ID NO: 7 – V-IgA-protease site His6Tag (artificial)
VPPTPGHHHHHH

SEQ ID NO: 8 - wild-type BoNT/A1

40 MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMOPVKAFKIHNKI WVIPERDTFTNP EEG
DLNPPPEAKQVPVSYDSTYLSTDNEKDNYLKGVTKL FERIYSTDLGRMLLTSIVRG
IPFWGGSTIDTELKVIDTNCINVIQPDGSYRSEELNLVI IGPSADIIQFECKSFGHE
VLNLTRNGYGSTQYIRFSPDFTFGFEESLEVD TNPLL GAGKFATDPAVTLAHELIHA
GHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDSLQENEFRLY
45 YYNKFKDIAS TLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDKLFKDKLY
KMLTEIYTEDNFVKFFKVLNRKTYLNFDAVF KINIVPKVNYTIYDGFNLRNTNLAA
NFNGQNT EINNMF TKLKNFTGLFEFYKLLCV RGIITSKTKSLDKGYNKALNDLCIK
VNNWDLFFSPSEDNFTNDLNKGEEITS DTNIEAAEENISLDLIQQYYLT FNFDNEPE
NIS IENLSSDIIGQLELMPNIERFPNGK KYELDKYTMFHYLRAQEF EHGKSRIALTN
SVNEALLNPSRVYTFSSDYVKKVNKATEAAMFLGWVEQLVYDFTDETSEVSTTDKI
50 ADITIIIPYIGPALNIGNMLYKDDFVGALIFSGAVILLEFIPEIAIPVLGTFALVSY

IANKVLTQVQIDNALS KRNEKWDEVYKYIVTNWLAKVNTQIDLRKKMKEALENQAE
 ATKAIINYQYNQYTEEEKNNINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMN
 SMIPYGVKRLDFDASLKDALLKYIYDNRGTLIGQVDRKDKVNNLTSTDIPFQLSK
 YVDNQRLSTFTTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFDPIDKN
 5 QIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIINCME
 NNSGWKVS LNYGEIIWTLQDTQEIKQRVVFKYSQMINISDYINRWIFVTITNNRLNN
 SKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWI KYFNLFDKELNEKE
 IKDLYDNQSN SGILKDFWGDYLDKPYMYMLNLYDPNKYVDVNNVVGIRGYMYLKGPR
 GSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNA
 10 SQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQ
 FNNIAKLVASNWYNRQIERSSRTLGCSEWEIFPVDDGWGERPL

SEQ ID NO: 9 - LH_N linker (artificial)

VRGIITSKTKSLDKGYNKALNDL

15

SEQ ID NO: 10 - enterokinase activation site

DDDDK

SEQ ID NO: 11 - BoNT/A1 activation loop

20 VDGIIITSKTKSDDDDKNKALNLQ

EXAMPLES

25 **Example 1 – Manufacture of modified BoNT/A L-chains (BoNT/A LC) according to the invention**

Wild-type BoNT/A L-chain (amino acids 1-448, SEQ ID NO: 1) encoding
 plasmid, pBN3, was generated by PCR and suitable oligonucleotide primers
 30 using bacterial DNA of strain 62A as template. DNA encoding the amino acid
 sequence PPTPGHHHHHH (SEQ ID NO: 4) was inserted following the codon
 for amino acid Ala-449. The *E.coli* strain M15pREP4 (Qiagen, Hilden,
 Germany) was transfected with pBN3 containing the *wt* BoNT/A LC or with its
 mutants, i.e. with protease mutants of SEQ ID NO: 1 as described in the
 35 present application. For each transfected *E.coli* strain, a single bacterial colony
 grown overnight in 5 ml of 2YT medium was used to inoculate 500 ml 2YT
 medium.

After the culture reached an OD₆₀₀ of 0.7, BoNT/A L-chains were produced
 40 during 15 h of induction using 0.2 mM IPTG at 21°C. Bacteria were collected
 by centrifugation and frozen at -20°C over night. Bacteria were resuspended

in lysis buffer (300 mM NaCl, 50 mM phosphate, pH 8.0) supplemented with benzamidine, pepstatin A, and PMSF at final concentrations of 5 mM, 1 µg/ml, and 0.5 mM, respectively, lyzed by sonication, the lysate cleared by centrifugation for 30 minutes at 29.000 g, and BoNT/A L-chain bound to Ni²⁺-nitrilotri-acetic acid-agarose beads. Beads were washed with 20 bed volumes of lysis buffer containing 10 mM imidazole, and BoNT/A L-chain eluted by lysis buffer containing 100 mM imidazole. Fractions containing the desired protein were dialyzed against toxin assay buffer (150 mM potassium glutamate, 10 mM Hepes-KOH, pH 7.2), and the purified L-chain finally frozen in liquid nitrogen, and kept at -70 °C.

Example 2 - hSNAP-23 cell-free cleavage assay

hSNAP-23 (SEQ ID NO: 2) plasmid for *E. coli* expression and *in vitro* transcription/translation, pS3-hSNAP-23His6, was generated.

It encodes an N-terminally fused twin strep-tag (MASWSHPQFEKGGSGGGSGGGWSHPQFEKGAGS, SEQ ID NO: 5) and a C-terminally fused His6-tag (GHHHHHH, SEQ ID NO: 6) downstream of the codon for carboxyl-terminal serine-211.

20

For protein production and purification, pS3-hSNAP-23His6 was transfected into the *E. coli* strain BL21-DE3 (Stratagene Europe, Ebsdorfergrund, Germany) and the same protocol was applied as detailed for BoNT/A L-chain protease in Example 1. However, protein eluted from the Ni²⁺-nitrilotri-acetic acid-agarose beads was further purified on Strep-Tactin agarose beads (IBA Lifesciences, Göttingen, Germany) by washing with 20 bed volumes of 0.1 M Tris pH 8.0 and elution with 10 mM desthiobiotin in 0.1 M Tris pH 8.0. In addition, all buffers used for hSNAP-23 purification were supplemented with 10 mM β-mercaptoethanol.

30

Radiolabeled hSNAP-23 was subsequently generated by *in vitro* transcription/translation using pS3-hSNAP-23His6, the T7 coupled TNT reticulocyte lysate system (Promega), and [35S] methionine (370 KBq/µl, >37

TBq/mmol; Hartmann Analytic, Braunschweig, Germany) according to the manufacturer's instructions.

hSNAP-23 cell-free cleavage assay contained recombinant hSNAP-23 at a 20
5 micromolar final concentration plus 1 μ l of transcription/translation mixture of
[35S] methionine-labeled hSNAP-23 and each modified or wild-type BoNT/A
L-chain at final concentrations of either 1 micromolar or 10 nanomolar, which
was incubated for 60 min at 37 °C in a total volume of 10 μ l of toxin assay
buffer. Reactions were stopped by the addition of an equal volume of double-
10 concentrated sample buffer [120 mM Tris-HCl (pH 6.75), 10% (v/v) β -
mercaptoethanol, 4% (w/v) SDS, 20% (w/v) glycerol, and 0.014% (w/v)
bromophenol blue]. After incubation for 30 min at 37 °C, each sample was
analyzed by SDS-PAGE using 15% Tris-glycine gels (acrylamide/bis-
acrylamide ratio: 73.5:1).

15

Gels were dried and radiolabeled protein visualized employing a FLA-9000
phosphorimager (Fuji Photo Film, Co., Ltd., Tokyo, Japan). Quantification of
radiolabeled protein and its cleavage products was performed with the
Multigaue 3.2 software (Fuji Photo Film). For the determination of the
20 enzyme kinetic parameters of wild-type BoNT/A L-chain and its mutants, the
substrate concentration was varied between 5 and 100 μ M employing
hSNAP-23 produced in *E. coli*. Each of the various substrate concentrations
was endowed by the addition of 1 μ l of radiolabeled hSNAP-23 generated by
in vitro transcription/translation. Incubation was performed in a final volume of
25 μ l of toxin assay buffer. After 2 and 4 min of incubation at 37 °C, aliquots of
25 10 μ l were taken and the enzymatic reaction stopped by mixing with 10 μ l of
prechilled double-concentrated SDS-PAGE sample buffer. The percentage of
cleavage was determined from the turnover of the radiolabeled substrate as
detailed above and used to calculate the initial velocity of substrate hydrolysis.
30 Km, Kcat and Vmax values were calculated by non-linear regression using the
GraphPad Prism 4.03 program (GraphPad Software Inc., San Diego, USA).

The resulting data are shown in Figure 1.

Example 3 – hSNAP-25 cell-free cleavage assay

hSNAP-25 (SEQ ID NO: 3) plasmid for *E. coli* expression (pBN10) has been described in Binz et al. (J Biol Chem., 1994; 269:1617-20). The codon for the carboxyl-terminal glycine-206 is followed by DNA encoding the amino acid sequence VPPTPGHHHHHH (SEQ ID NO: 7). A plasmid for *in vitro* transcription/translation, pSNAP-25His6, was subsequently generated by subcloning the *EcoRI-SalI* fragment of pBN10 in pSP73 (Promega, Mannheim, Germany) cleaved correspondingly.

10

For protein production and purification of SNAP-25, pBN10 was transfected into the *E. coli* strain M15pREP4 (Qiagen, Hilden, Germany) and the same protocol was applied as detailed for BoNT/A L-chain protease in Example 1.

15 Radiolabeled SNAP-25 was generated by *in vitro* transcription/translation using pSNAP-25His6, the SP6 coupled TNT reticulocyte lysate system (Promega), and [³⁵S] methionine (370 KBq/μl, >37 TBq/mmol; Hartmann Analytic, Braunschweig, Germany) according to the manufacturer's instructions.

20 hSNAP25 cleavage assay was performed exactly as described for hSNAP-23 in Example 2.

The resulting data are shown in Figure 1.

25 **Example 4 – Manufacture of LH_N domains containing a modified light chain A (BoNT/A LC) according to the invention**

This Example describes the construction of translocation LH_N domains containing a modified light chain A (BoNT/A LC) displaying hSNAP23 cleaving activity according to the invention. Such LH_N domains can be used to create families of TSI delivery vehicles, by adding appropriate Targeting Moieties.

30

Briefly, BoNT/A LC cloning vectors were firstly constructed, for each mutant of the BoNT/A LC according to the invention, by chemically synthesizing DNA

(GeneArt, ThermoFisher), that encodes said mutant BoNT/A LC and that is optimized for expression into *E. coli*, subcloned into the pCR 4 vector (Invitrogen). In parallel, a BoNT/A H_N domain cloning vector was similarly constructed by chemically synthesizing codon-optimized DNA encoding H_N/A domain (corresponding to amino acid residues 449 to 872 of SEQ ID NO: 8, UniprotKB accession number A5HZZ9), subcloned into a standard vector, such as the pCR 4 vector (Invitrogen). A LH_N linker cloning vector was further constructed by chemically synthesizing codon-optimized DNA encoding said linker subcloned into a standard vector, the pCR 4 vector (Invitrogen). In particular, the LH_N linker VRGIITSKTKSLDKGYNKALNDL (SEQ ID NO: 9) which is suitable for the BoNT/A serotype (it is the inter-domain polypeptidic region that exists between the cysteines of the disulphide bridge between the LC and the H_N domain of BoNT/A) was used for construction of the LH_N linker vector. Alternative LH_N linker constructs can be generated: indeed, as well-known to the skilled practitioner, for generation of a specific protease cleavage site, either the native susceptibility to proteolysis by the LysC protease can be used, or an enterokinase activation site (e.g. DDDDK, SEQ ID NO: 10) can be inserted into the activation loop to generate a sequence such as VDGIIITSKTKSDDDDKKNKALNLQ (SEQ ID NO: 11), or a protease site for any other protease well-known in the art, such as PreScission, Factor Xa, Thrombin, TEV protease, etc., can be inserted into the activation loop.

The LH_N domains were subsequently assembled by cloning, in 2 main steps, the DNA encoding each of the modified BoNT/A LC of the invention upstream of the DNA encoding the LH_N linker, said linker being further upstream of the DNA encoding the H_N/A domain, into a modified pET expression vector (Novagen).

Example 5 – Manufacture of TSI delivery vehicles binding to a non-neuronal cell according to the invention

This Example describes the construction of TSI delivery vehicles by adding a suitable Targeting Moiety (herein, human GHRP) to each C-terminal end of the LH_N domains containing a modified light chain A of the invention, as

described in Example 2 above. To do so, a flexible linker was introduced between the Targeting Moiety and the LH_N domain.

5 Briefly, linker-hGHRP cloning vectors were constructed by chemically synthesizing codon-optimized DNA encoding a flexible linker fused in frame to hGHRP Targeting Moeity, subcloned into the pCR 4 vector (Invitrogen).

10 The TSI constructs were subsequently assembled by cloning the DNA encoding the linker-hGHRP into each of the pET expression vectors containing the LH_N domains described in Example 2, in such manner that the linker-hGHRP is fused in frame to the C-terminal end of each LH_N domain.

For protein expression of each TSI vehicle, 100 ml of modified Terrific Broth (TB) medium containing 0.2% glucosamine and 30 µg/ml kanamycin in a 15 250ml flask were incubated with a single bacterial colony (E. coli BL21 (DE3) transfected with the TSI. Each culture was grown at 37°C, 225 rpm for 16 hours; followed by inoculation of 1L of modified TB containing 0.2% glucosamine and 30 µg/ml kanamycin in a 2L flask with 10ml of the overnight culture. The resulting culture was then grown at 37°C until an approximate 20 OD_{600nm} of 0.5 was reached at which point the temperature was reduced to 16°C. After 1 hour, each culture was induced with 1 mM IPTG and further grown at 16°C for a further 16 hours. Bacteria were collected by centrifugation and frozen at -20°C over night.

25 Subsequent purification of each expressed TSI was performed as follows. Bacteria were defrosted and the cell pellet was sonicated to lyse the cells. After centrifugation, the supernatant was loaded onto a 0.1 M NiSO₄ charged Chelating column equilibrated with 50 mM HEPES pH 7.2 200 mM NaCl. Washing of the column was performed with a buffer containing 40 to 100 mM 30 imidazole (step gradient) to elute the non-bound protein, and with a buffer containing 200 mM imidazole to elute the TSI protein. Fractions containing the desired protein (TSI) were subsequently dialyzed against a buffer containing 50 mM HEPES pH 7.2 200 mM NaCl. The protease was then added (herein LysC) in an appropriate amount to 1 mg of the purified TSI to activate it (i.e.

so that the TSI forms a di-chain, capable of binding to GHRP, translocating the light chain into the cytoplasm and of catalytically cleaving hSNAP23). The resulting mixture was then further purified by loading it to a 0.1 M NiSO₄ charged Chelating column equilibrated with 50 mM HEPES pH 7.2 200 mM NaCl. The column was washed a first time with 50 mM HEPES pH 7.2 200 mM NaCl, then with a buffer containing 40 to 100 mM imidazole to elute the non-specific bound protein and with a buffer containing 200 mM imidazole to elute the activated TSI. Fractions containing the desired activated protein (TSI) were subsequently dialized against a buffer containing 50 mM HEPES pH 7.2 150 mM NaCl. The dialized protein was then concentrated to about 2 mg/ml, aliquoted and finally frozen at -80°C.

Clauses

1. A modified botulinum neurotoxin A (BoNT/A) L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:
- 5
- a) at least one amino acid residue change located within a first BoNT/A L-chain protease binding pocket for binding to the P182 /D178 binding site of hSNAP-23;
 - b) wherein said first BoNT/A L-chain protease binding pocket is defined by amino acid residues E148, T307, A308 and Y312 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
 - 10 c) and wherein said at least one amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of asparagine and tyrosine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue E148 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - 15 ii. an amino acid residue selected from the group consisting of phenylalanine, isoleucine and leucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue T307 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - 20 iii. an amino acid residue selected from the group consisting of proline, asparagine, threonine and isoleucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue A308 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - 25 iv. an amino acid residue selected from the group consisting of lysine, valine, methionine and leucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Y312 of wild-type BoNT/A L-chain (SEQ ID NO: 1).
 - 30

2. The modified BoNT/A L-chain protease according to clause 1, further comprising:

a) an amino acid residue change located within a second BoNT/A L-chain protease binding pocket for binding to the R186 binding site of hSNAP-23;

b) wherein said second BoNT/A L-chain protease binding pocket is defined by amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1);

c) and wherein said amino acid residue change comprises:

i. an amino acid residue selected from the group consisting of glutamine, glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue S143 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

3. The modified BoNT/A L-chain protease according to clause 1 or 2, further comprising:

a) at least one amino acid residue change located within a third BoNT/A L-chain protease binding pocket for binding to the K185 binding site of hSNAP-23;

b) wherein said third BoNT/A L-chain protease binding pocket is defined by amino acid residues V304 and G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1);

c) and wherein said at least one amino acid residue change comprises:

i. an amino acid residue selected from the group consisting of glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue V304 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or

ii. an amino acid residue selected from the group consisting of glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds

to amino acid residue G305 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

4. The modified BoNT/ A L-chain protease according to any preceding clause, further comprising:
- a) an amino acid residue change located within a fourth BoNT/A L-chain protease binding pocket for binding to the D189/D192 binding site of hSNAP-23;
 - b) wherein said fourth BoNT/A L-chain protease binding pocket is defined by amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
 - c) and wherein said amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1).
5. The modified BoNT/ A L-chain protease according to any preceding clause, further comprising:
- a) at least one amino acid residue change located within a fifth BoNT/A L-chain protease binding pocket for binding to the K206 binding site of hSNAP-23;
 - b) wherein said fifth BoNT/A L-chain protease binding pocket is defined by amino acid residue Y251, L256, V258, L367 and F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
 - c) and wherein said at least one amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or

- 5 ii. an amino acid residue selected from the group consisting of glutamate, aspartate, glutamine, glycine, alanine and arginine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue L256 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
- 10 iii. an amino acid residue selected from the group consisting of serine, alanine, proline, leucine and glutamate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue V258 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
- 15 iv. an amino acid residue selected from the group consisting of alanine and glycine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue L367 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
- 20 v. an amino acid residue selected from the group consisting of glycine, serine and leucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1).
6. The modified BoNT/A L-chain protease according to any preceding clause, further comprising:
- 25 a) an amino acid residue change located within a sixth BoNT/A L-chain protease binding pocket for binding to the I198 binding site of hSNAP-23;
- b) wherein said sixth BoNT/A L-chain protease binding pocket is defined by amino acid residue K166 of wild-type BoNT/A L-chain
- 30 (SEQ ID NO: 1);
- c) and wherein said amino acid residue change comprises:
- i. an amino acid residue selected from the group consisting of valine, phenylalanine, leucine and isoleucine at the position on the modified L-chain protease amino acid

sequence that corresponds to amino acid residue K166 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

- 5 7. The modified BoNT/A L-chain protease according to any preceding clause, further comprising:
- a) an amino acid residue change located within a seventh BoNT/A L-chain protease binding pocket for binding to the D210 binding site of hSNAP-23;
 - 10 b) wherein said seventh BoNT/A L-chain protease binding pocket is defined by amino acid residue S254 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
 - c) and wherein said amino acid residue change comprises:
 - 15 i. an amino acid residue selected from the group consisting of alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue S254 of wild-type BoNT/A L-chain (SEQ ID NO: 1).
- 20 8. The modified BoNT/A L-chain protease according to any preceding clause, further comprising:
- a) an amino acid residue change located within an eighth BoNT/A L-chain protease binding pocket for binding to the D168 binding site of hSNAP-23;
 - 25 b) wherein said eighth BoNT/A L-chain protease binding pocket is defined by amino acid residue K340 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
 - c) and wherein said amino acid residue change comprises:
 - 30 i. an amino acid residue selected from the group consisting of histidine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue K340 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

9. A modified botulinum neurotoxin A (BoNT/A) L-chain protease that cleaves human SNAP-23 (hSNAP-23) and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain) (SEQ ID NO: 1), that comprises:
- 5 a) an amino acid residue change located within a fourth BoNT/A L-chain protease binding pocket for binding to the D189/D192 binding site of hSNAP-23;
 - b) wherein said fourth BoNT/A L-chain protease binding pocket is defined by amino acid residue Q29 of wild-type BoNT/A L-chain
 - 10 (SEQ ID NO: 1);
 - c) and wherein said amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting of alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino
 - 15 acid residue Q29 of wild-type BoNT/A L-chain (SEQ ID NO: 1).
10. A modified botulinum neurotoxin A (BoNT/A) L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino
- 20 acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:
 - a) an amino acid residue change located within a sixth BoNT/A L-chain protease binding pocket for binding to the I198 binding site of hSNAP-23;
 - 25 b) wherein said sixth BoNT/A L-chain protease binding pocket is defined by amino acid residue K166 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
 - c) and wherein said amino acid residue change comprises:
 - i. an amino acid residue selected from the group consisting
 - 30 of valine, phenylalanine, leucine and isoleucine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue K166 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

11.A modified botulinum neurotoxin A (BoNT/A) L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:

- 5 a) at least one amino acid residue change located within a fifth BoNT/A L-chain protease binding pocket for binding to the K206 binding site of hSNAP-23;
- b) wherein said fifth BoNT/A L-chain protease binding pocket is defined by amino acid residues Y251, L256, V258, L367 and
10 F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- c) and wherein said at least one amino acid residue change comprises:
 - 15 i. an amino acid residue selected from the group consisting of glutamate and aspartate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue Y251 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - 20 ii. an amino acid residue selected from the group consisting of aspartate, glutamine, glycine, alanine and arginine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue L256 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - 25 iii. an amino acid residue selected from the group consisting of serine, alanine, proline, leucine and glutamate at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue V258 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - 30 iv. an amino acid residue selected from the group consisting of alanine and glycine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue L367 of wild-type BoNT/A L-chain (SEQ ID NO: 1); and/ or
 - v. an amino acid residue selected from the group consisting of glycine, serine and leucine at the position on the

modified L-chain protease amino acid sequence that corresponds to amino acid residue F369 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

- 5 12. A modified botulinum neurotoxin A (BoNT/A) L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:
- 10 a) an amino acid residue change located within a seventh BoNT/A L-chain protease binding pocket for binding to the D210 binding site of hSNAP-23;
- b) wherein said seventh BoNT/A L-chain protease binding pocket is defined by amino acid residue S254 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- 15 c) and wherein said amino acid residue change comprises:
- i. an amino acid residue selected from the group consisting of alanine at the position on the modified L-chain protease amino acid sequence that corresponds to amino acid residue S254 of wild-type BoNT/A L-chain (SEQ ID NO: 1).
- 20
13. A modified botulinum neurotoxin A (BoNT/A) L-chain protease that cleaves human SNAP-23 (hSNAP-23), and having a modified amino acid sequence relative to the wild-type BoNT/A L-chain (SEQ ID NO: 1), that comprises:
- 25 a) an amino acid residue change located within an eighth BoNT/A L-chain protease binding pocket for binding to the D168 binding site of hSNAP-23;
- b) wherein said eighth BoNT/A L-chain protease binding pocket is defined by amino acid residue K340 of wild-type BoNT/A L-chain (SEQ ID NO: 1);
- 30 c) and wherein said amino acid residue change comprises:
- i. an amino acid residue selected from the group consisting of histidine at the position on the modified L-chain

protease amino acid sequence that corresponds to amino acid residue K340 of wild-type BoNT/A L-chain (SEQ ID NO: 1).

5 14. The modified botulinum neurotoxin A (BoNT/A) L-chain protease according to any of Clauses 9 to 13, further comprising at least one amino acid residue change located within a different BoNT/A L-chain protease binding pocket, wherein said amino acid residue change and said another BoNT/A L-chain protease binding pocket are defined by
10 the technical features recited in any one of Clauses 1 to 13.

15 15. A nucleic acid construct comprising or consisting of a nucleic acid sequence encoding the modified BoNT/A L-chain protease as defined in any preceding Clause.

15

16. A delivery vehicle, comprising:
a) the modified BoNT/A L chain protease as defined in any Clauses 1 to 14, or the nucleic acid construct of Clause 15; and
b) means for delivering said modified BoNT/A L chain protease, or
20 said nucleic acid construct, into a target cell, preferably into a non-neuronal target cell.

17. The delivery vehicle according to Clause 16, wherein the means b) for delivering said modified BoNT/A L chain protease to a target cell
25 comprises:

i) a targeting moiety that binds the delivery vehicle to the target cell; and
ii) a translocation peptide that translocates the modified BoNT/A L-chain protease or the nucleic acid construct into the target cell,
30 preferably into a non-neuronal target cell.

18. A method of cleaving hSNAP-23, comprising contacting hSNAP-23 with a (BoNT/A) L-chain protease according to any of Clauses 1 to 14, or

with a nucleic acid construct according to Clause 15, or with a delivery vehicle according to Clause 16 or 17.

- 5 19.A (BoNT/A) L-chain protease according to any of Clauses 1 to 14, or a nucleic acid construct according to Clause 15, or a delivery vehicle according to Clause 16 or 17, for use in a method according to Clause 18.