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(54) Title: CELLULAR VAMP CLEAVAGE ASSAY

(57) Abstract: The present invention relates to VAMP epitopes suitable for generating antibodies against a VAMP C-terminal neurotoxin cleavage product, their use to generate antibodies against cleaved VAMP and the use of such antibodies in cellular VAMP cleavage assays based on a gain of signal.

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CELLULAR VAMP CLEAVAGE ASSAY

FIELD OF THE INVENTION

The present invention relates to cell based assays for VAMP cleaving clostridial neurotoxins.

BACKGROUND

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

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

Non-cytotoxic proteases act by proteolytically cleaving intracellular transport proteins known as SNARE proteins (e.g. SNAP25, VAMP, or syntaxin) – see Gerald K (2002) "Cell and Molecular Biology" (4th edition) John Wiley & Sons, Inc. The acronym SNARE derives from the term Soluble NSF Attachment Receptor, where NSF means N-

ethylmaleimide-Sensitive Factor. The acronym SNAP25 derives from the term Synaptosome Associated Protein of 25 kilo daltons. The acronym VAMP derives from the term Vesicle Associated Membrane Protein. SNARE proteins are integral to intracellular vesicle fusion, and thus to secretion of molecules via vesicle transport from a cell. The protease function is a zinc-dependent endopeptidase activity and exhibits high substrate specificity for SNARE proteins. Accordingly, once delivered to a desired target cell, the non-cytotoxic protease is capable of inhibiting cellular secretion from the target cell. The L-chain proteases of clostridial neurotoxins are non-cytotoxic proteases that cleave SNARE proteins. The L-chain proteases of BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X and TeNT cleave VAMPs (also referred to as synaptobrevins), the L-chain proteases of BoNT/A and BoNT/E cleave SNAP25 and the L-chain protease of BoNT/C cleaves both SNAP25 and syntaxin, which result in the inhibition of neurotransmitter release and consequent neuroparalysis (Rossetto, O. et al., "Botulinum neurotoxins: genetic, structural and mechanistic insights." Nature Reviews Microbiology 12.8 (2014): 535-549) (Zhang et al., “Identification and characterization of a novel botulinum neurotoxin”; Nature Communications, 2017, 8:14130).

Clostridial neurotoxins target and enter neurons by binding to their specific receptors through their receptor binding domains (H_C), which are well-defined in the literature (Schiavo, G., Matteoli, M. & Montecucco, *C. Neurotoxins affecting neuroexocytosis*, Physiol Rev, 2000, 80, 717-766). Receptor binding dictates the efficacy and specificity of BoNTs to recognize neurons. BoNT/B, D-C, and G share two homologous synaptic vesicle proteins synaptotagmin I and II (Syt I/II) as their receptors, while BoNT/A, E, D, F and TeNT use another synaptic vesicle protein SV2. In addition to protein receptors, all BoNTs require lipid co-receptor gangliosides, which are abundant on neuronal surfaces.

Clostridial neurotoxins are used in therapy to treat motor and autonomic disorders. Several BoNT/A products (including Botox®, Dysport® and Xeomin®) and one BoNT/B product (Neurobloc®/Myobloc®) are approved by regulatory agencies for use in humans.

Traditionally, the potency of BoNT pharmaceutical products has been quantified in MLD50 (mouse lethal dose 50) units, one unit corresponding to the median lethal intraperitoneal dose in mice. However, the MLD50 unit for botulinum toxins is not a standardised unit. Indeed, assays used by different manufacturers of marketed toxins differ in particular in the choice of dilution buffer (Straughan, D. W., 2006, ATLA 34(3), 305-

313; Hambleton and Pickett, Hambleton, P., and A. M. Pickett., 1994, Journal of the Royal Society of Medicine 87.11: 719). In addition, because of ethical concerns and recent regulations, it is now preferable to avoid the use of animal based potency assays. Cell-based potency assays avoid the requirement for animal testing and related ethical concerns. Following cellular intoxication, the potency of a clostridial neurotoxin can be measured by assessing the degree of SNARE cleavage within the target cell, for example by Western blotting. Alternatively, SNARE cleavage can be detected and quantified using a sandwich ELISA method. Such methods work well for SNAP25 and syntaxin cleavage (see eg. Pellett, Sabine, et al. "Comparison of the primary rat spinal cord cell (RSC) assay and the mouse bioassay for botulinum neurotoxin type A potency determination." Journal of pharmacological and toxicological methods 61.3 (2010):304-310; Fernández-Salas, Ester, et al. "Botulinum neurotoxin serotype A specific cell-based potency assay to replace the mouse bioassay." PLoS One 7.11 (2012): e49516; Kalandakanond S et al. "Cleavage of intracellular substrates of botulinum toxins A, C and D in mammalian target tissue" The Journal of Pharmacology and Experimental Therapeutics (2001):749-755; Peng L et al. "Cytotoxicity of botulinum neurotoxins reveals a direct role of syntaxin 1 and SNAP25 in neuron survival." Nature Communications (2013): 4:1472). However, to date the cleavage product for VAMPs from cellular lysates has proved extremely difficult to detect. Indeed, although VAMP cleavage-specific antibodies that recognise cleaved VAMP are available and suitable for detection of VAMP cleavage in extracellular or cell fraction assays (Hallis, Bassam, B. A. James, and Clifford C. Shone. "Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities." Journal of clinical microbiology 34.8 (1996): 1934-1938; Kegel, B., et al. "An in vitro assay for detection of tetanus neurotoxin activity: Using antibodies for recognizing the proteolytically generated cleavage product." Toxicology in Vitro 21.8 (2007): 1641-1649; Fujita-Yoshigaki, Junko, et al. "Vesicle-associated Membrane Protein 2 Is Essential for cAMP-regulated Exocytosis in Rat Parotid Acinar Cells The Inhibition of cAMP-dependent Amylase Release by Botulinum Neurotoxin B." Journal of Biological Chemistry 271.22 (1996): 13130-13134), these antibodies do not detect cleaved VAMP in cellular studies.

The general consensus in the field was so far that the cleaved VAMP product must be degraded very quickly in the cell and therefore does not contribute to the longevity of BoNT action (Foran, Patrick G., et al. "Evaluation of the Therapeutic Usefulness of

Botulinum Neurotoxin B, C1, E, and F Compared with the Long Lasting Type A Basis for Distinct Durations of Inhibition of Exocytosis in Central Neurons." Journal of biological chemistry 278.2 (2003): 1363-1371). However, Schiavo et al. have shown both VAMP cleavage products are present in small synaptic vesicle fractions prepared from rat cerebral cortex when treated with BoNT/B and TeNT using Coomassie blue staining (Schiavo G., et al (1992), Tetanus and Botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature 359 p832-835). This suggests VAMP products from a cellular source can be present, although the synaptosome preparation may well not contain all the proteases present in a total cell lysate. Dong et al (2004) describe that in PC12 cells expressing YFP-Syb(FL)-CFP, signals from both VAMP products are detectable after cleavage by BoNT/B, and that the YFP-N-terminal cleaved VAMP product disperses into the cytosol and redistributes itself to the nucleus, whereas the CFP-C-terminal product remains localised to the vesicle (Dong M., et al (2004) Using fluorescent sensors to detect botulinum neurotoxin activity in vitro and in living cells. PNAS 101 (41) p14701-14706). This evidence suggests both VAMP products could be present, but as yet unknown cellular processes are hampering the recognition of an antibody to the N-terminal product. It is therefore standard practice to measure VAMP cleavage by disappearance of the full-length band only (see eg. Pellett, Sabine, et al. "A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection of neutralizing serum antibodies." FEBS letters 581.25 (2007): 4803-4808.; Whitemarsh, Regina CM, et al. "Novel application of human neurons derived from induced pluripotent stem cells for highly sensitive botulinum neurotoxin detection Biological Sciences: Applied Biological Sciences." Toxicological Sciences, 2012, 126(2):426–435). However, assays based on the loss of a signal convey a risk of error as there may be discrepancies in total protein loading which would then cause either an over- or under-estimation of VAMP disappearance. A house keeping protein that is unchanged during BoNT treatment can be used to normalise VAMP disappearance to the density of the control protein. The disadvantage here is that the signal between the antibodies needs to be matched and in the linear scale in order to detect any differences for normalisation purposes. Although qualitatively this may be a reasonable indication of BoNT activity, it is not suitable for more detailed quantification and in particular for determining the potency of pharmaceutical BoNT formulations.

There is thus a need for cellular VAMP cleavage assays based on a gain of signal readout.

SUMMARY OF INVENTION

In a first aspect, the invention provides an antigenic polypeptide comprising a VAMP epitope, wherein said antigenic polypeptide consists of 10 to 65 amino acid residues, wherein said VAMP epitope comprises an amino acid sequence which is at least 90% identical to a VAMP sequence comprising at least 8 amino acid residues which are immediately C-terminal to a clostridial neurotoxin cleavage site in said VAMP.

In another aspect, the invention relates to a polypeptide comprising the antigenic polypeptide of the invention, wherein the polypeptide does not comprise a region of greater than 17, preferably 16, more preferably 15 consecutive amino acids having 100% sequence identity to a naturally-occurring VAMP amino acid sequence.

In another aspect, the invention provides an antigenic protein comprising a polypeptide according to the invention covalently linked to a carrier.

In another aspect, the invention provides the use of an antigenic polypeptide or protein according to the invention, to generate antibodies against a C-terminal VAMP cleavage product. In one embodiment, the epitope of the invention is used to generate a polyclonal antibody against a C-terminal VAMP cleavage product. In another embodiment, the epitope of the invention is used to generate a monoclonal antibody against a C-terminal VAMP cleavage product.

In another aspect, the invention provides an antibody that binds to an antigenic polypeptide or protein of the invention.

In another aspect, the invention provides the use of an antibody according to the invention in a gain of signal cellular assay for VAMP cleavage by a VAMP cleaving clostridial neurotoxin.

In another aspect, the invention provides a method for determining cleavage of VAMP by a VAMP cleaving clostridial neurotoxin in a cell, comprising:

a) contacting the cell with the clostridial neurotoxin under conditions suitable for clostridial neurotoxin activity;
b) contacting the cytoplasmic content of said cell with a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by

the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to the invention; and

c) detecting by a suitable means the binding of said first detection antibody to the C-terminal VAMP cleavage product.

In another aspect, the invention provides a method for determining immunoresistance to a VAMP cleaving clostridial neurotoxin in a subject, comprising:

a) adding a VAMP cleaving clostridial neurotoxin to a test sample obtained from the subject;

b) contacting a cell with the test sample of step a) under conditions suitable for clostridial neurotoxin activity;

c) contacting the cytoplasmic content of said cell with a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to the invention;

d) detecting by a suitable means the binding of the first detection antibody to the C-terminal VAMP cleavage product;

e) quantifying the amount of the C-terminal VAMP cleavage product bound to the first detection antibody;

f) repeating steps a) to e) with a negative control sample instead of a test sample; and

g) comparing the amount of the C-terminal VAMP cleavage product bound to said first detection antibody in steps (e) and (f), wherein detection of a lower amount of the C-terminal VAMP cleavage product bound to said first detection antibody in step (e) relative to the amount of the C-terminal VAMP cleavage product bound to said first detection antibody in step (f) is indicative of the presence of neutralizing antibodies against the VAMP cleaving clostridial neurotoxin.

In another aspect, the invention provides a kit comprising a cell which is susceptible to intoxication by a VAMP cleaving neurotoxin; and a first detection antibody against cleaved VAMP, wherein said first detection antibody is an antibody according to the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on a finding by the inventors that it was possible to generate antibodies suitable for use in a cellular VAMP cleavage assay based on a gain of signal readout.

In particular, the inventors have shown that, in order to detect VAMP cleavage *in vitro*, it is key to detect epitopes located on the C-terminal side of BoNT cleavage site. Indeed, the inventors have demonstrated herein the successful detection of a neuronal VAMP2 cleavage product by Western Blot (WB) using antibodies binding epitopes located on the C-terminal side of the BoNT/F and/or BoNT/D and/or BoNT/B cleavage sites, which are adjacent to the BoNT/D and/or BoNT/F and/or BoNT/B cleavage sites. In particular, such antibodies are capable of detecting both full-length VAMP and the cleaved product in a cell lysate. This tool enables the quantitative assessment of the potency of BoNT in a gain of signal cellular assay by monitoring the appearance of the cleaved VAMP product.

In a first aspect, the invention provides an antigenic polypeptide comprising a VAMP epitope, wherein said antigenic polypeptide consists of 10 to 65 amino acid residues, wherein said VAMP epitope comprises an amino acid sequence which is at least 90% identical to a VAMP sequence comprising at least 8 amino acid residues which are immediately C-terminal to a clostridial neurotoxin cleavage site in said VAMP.

The term "clostridial neurotoxin" as used herein means any polypeptide that enters a neuron and inhibits neurotransmitter release. This process encompasses the binding of the neurotoxin to a low or high affinity receptor, the internalisation of the neurotoxin, the translocation of the endopeptidase portion of the neurotoxin into the cytoplasm and the enzymatic modification of the neurotoxin substrate. More specifically, the term "clostridial neurotoxin" encompasses any polypeptide produced by Clostridium bacteria that enters a neuron and inhibits neurotransmitter release, and such polypeptides produced by recombinant technologies or chemical techniques. It is the di-chain form that is the active form of the neurotoxin. The two chains are termed the heavy chain (H-chain), which has a molecular mass of approximately 100 kDa, and the light chain (L-chain), which has a molecular mass of approximately 50 kDa. Clostridial neurotoxins include botulinum neurotoxins (BoNTs) and Tetanus neurotoxin (TeNT). BoNT serotypes A to G can be distinguished based on inactivation by specific neutralising anti-sera, with such

classification by serotype correlating with percentage sequence identity at the amino acid level. BoNT proteins of a given serotype are further divided into different subtypes on the basis of amino acid percentage sequence identity.

An example of a BoNT/A neurotoxin amino acid sequence is provided as SEQ ID NO: 1 (UniProt accession number A5HZZ9). An example of a BoNT/B neurotoxin amino acid sequence is provided as SEQ ID NO: 2 (UniProt accession number B1INP5). An example of a BoNT/C neurotoxin amino acid sequence is provided as SEQ ID NO: 3 (UniProt accession number P18640). An example of a BoNT/D neurotoxin amino acid sequence is provided as SEQ ID NO: 4 (UniProt accession number P19321). An example of a BoNT/E neurotoxin amino acid sequence is provided as SEQ ID NO: 5 (NCBI RefSeq accession number WP_003372387). An example of a BoNT/F neurotoxin amino acid sequence is provided as SEQ ID NO: 6 (UniProt accession number Q57236). An example of a BoNT/G neurotoxin amino acid sequence is provided as SEQ ID NO: 7 (NCBI RefSeq accession number WP_039635782). An example of a BoNT/X neurotoxin amino acid sequence is provided as SEQ ID NO: 41 (Genbank accession number BAQ12790.1). An example of a TeNT amino acid sequence is provided as SEQ ID NO: 8 (UniProt accession number P04958).

The term "H_C domain" as used herein means a functionally distinct region of the neurotoxin heavy chain with a molecular weight of approximately 50 kDa that enables the binding of the neurotoxin to a receptor located on the surface of the target cell. The H_C domain consists of two structurally distinct subdomains, the "H_{CN} subdomain" (N-terminal part of the H_C domain) and the "H_{CC} subdomain" (C-terminal part of the H_C domain), each of which has a molecular weight of approximately 25 kDa.

The term "LH_N domain" as used herein means a neurotoxin that is devoid of the H_C domain and consists of an endopeptidase domain ("L" or "light chain") and the domain responsible for translocation of the endopeptidase into the cytoplasm (H_N domain of the heavy chain).

Exemplary L, H_N, H_{CN} and H_{CC} domains are shown in table 1.

Table 1 – Exemplary L, H_N, H_{CN} and H_{CC} domains

Clostridial neurotoxin	Accession Number	SEQ ID NO	L	H_N	H_{CN}	H_{CC}
BoNT/A1	A5HZZ9	1	1-448	449-872	873-1094	1095-1296
BoNT/B1	B1INP5	2	1-441	442-859	860-1081	1082-1291
BoNT/C1	P18640	3	1-449	450-867	868-1095	1096-1291
BoNT/D	P19321	4	1-442	443-863	864-1082	1083-1276
BoNT/E1	WP_003372387	5	1-423	424-846	847-1069	1070 -1252
BoNT/F1	Q57236	6	1-439	440-865	866-1087	1088-1278
BoNT/G	WP_039635782	7	1-446	447-864	865-1089	1090-1297
BoNT/X	BAQ12790.1	41	1-439	440-891	892-1105	1106-1306
TeNT	P04958	8	1-456	457-880	881-1111	1112-1315

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

Vesicle-associated membrane proteins (VAMPs) are a family of SNARE proteins which have a similar structure and are involved in vesicle fusion and exocytosis, in particular neurotransmitter release. VAMPs are members of a family of SNARE proteins, which is called the Synaptobrevin family and includes members such as VAMP1, VAMP2 (both also known as synaptobrevins), VAMP3 (also known as cellubrevin), VAMP4, VAMP5, VAMP7 (also known as SYBL1, or tetanus-insensitive VAMP), VAMP8 (also known as endobrevin), YKT6, SEC22A and others. VAMP1, VAMP2 and VAMP3 are cleaved by the light chains of BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X and TeNT. BoNT/X also cleaves VAMP4, VAMP5 and YKT6.

The term "VAMP epitope" as used herein means a portion of a VAMP protein to which an antibody binds.

In a preferred embodiment, the antigenic polypeptide of the invention consists of 10 to 65, 10 to 60, 10 to 55, 10 to 50, 10 to 45, 10 to 40, 10 to 35, 10 to 30, 10 to 25, 10 to 20, 10 to 19, 10 to 18, 10 to 17, 10 to 16 or 10 to 15 amino acid residues, preferably 10 to 15 amino acid residues. For example, the antigenic polypeptide of the invention may consist of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65 amino acid residues.

In a preferred embodiment, the antigenic polypeptide of the invention comprises, or consists of, a VAMP epitope which comprises an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a VAMP sequence comprising at least 8, 9, 10, 11, 12, 13, 14, 15, 16 or 17 amino acid residues which are immediately C-terminal to a clostridial neurotoxin cleavage site in said VAMP.

Amino acid sequences of naturally-occurring VAMPs, in particular rat and human VAMP1, VAMP2, VAMP3, VAMP4, VAMP5 and YKT6, and their corresponding clostridial neurotoxin VAMP cleavage sites are shown in table 2 and figure 1.

Table 2 – Clostridial neurotoxin VAMP cleavage sites

VAMP	SEQ ID NO	BoNT/F5 & BoNT/FA	Other BoNT/F	BoNT/D & BoNT/DC	BoNT/B & TeNT	BoNT/G	BoNT/X
VAMP1_Rat (Q63666)	9	Leu56-Glu57	Gln60-Lys61	Lys61-Leu62	*Not cleaved*	Ala83-Ala84	Arg68-Ala69
VAMP1_human (P23763)	10	Leu56-Glu57	Gln60-Lys61	Lys61-Leu62	Gln78-Phe79	Ala83-Ala84	Arg68-Ala69
VAMP2_Rat (P63045)	11	Leu54-Glu55	Gln58-Lys59	Lys59-Leu60	Gln76-Phe77	Ala81-Ala82	Arg66-Ala67
VAMP2_human (P63027)	12	Leu54-Glu55	Gln58-Lys59	Lys59-Leu60	Gln76-Phe77	Ala81-Ala82	Arg66-Ala67
VAMP3_Rat (P63025)	13	Leu41-Glu42	Gln45-Lys46	Lys46-Leu47	Gln63-Phe64	Ala68-Ala69	Arg53-Ala54

VAMP	SEQ ID NO	BoNT/F5 & BoNT/FA	Other BoNT/F	BoNT/D & BoNT/DC	BoNT/B & TeNT	BoNT/G	BoNT/X
VAMP3_human (Q15836)	14	Leu37-Glu38	Gln41-Lys42	Lys42-Leu43	Gln59-Phe60	Ala64-Ala65	Arg49-Ala50
VAMP4_Rat (D4A560)	42	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	Lys87-Ser88
VAMP4_human (O75379)	43	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	Lys87-Ser88
VAMP5_Rat (Q9Z2J5)	44	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	Arg40-Ser41
VAMP5_human (O95183)	45	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	Arg40-Ser41
YKT6_Rat (Q5EGY4)	46	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	Lys173-Ser174
YKT6_human (O15498)	47	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	*Not cleaved*	Lys173-Ser174

In one embodiment, the VAMP is selected from VAMP1, VAMP2, VAMP3, VAMP4, VAMP5, and/or YKT6.

In one embodiment, the VAMP is selected from VAMP1, VAMP2 and/or VAMP3.

In one embodiment, the VAMP is selected from VAMP4, VAMP5 and/or YKT6.

In a preferred embodiment, the VAMP is a human VAMP, more preferably, a human VAMP1, VAMP2, VAMP3, VAMP4, VAMP5, and/or YKT6.

In one embodiment, the VAMP is selected from a human VAMP1, VAMP2 and/or VAMP3.

In one embodiment, the VAMP is selected from a human VAMP4, VAMP5 and/or YKT6.

In one embodiment of the antigenic polypeptide of the invention, the VAMP epitope is a BoNT/F cleaved VAMP epitope wherein the at least 8 amino acid residues are immediately C-terminal to a BoNT/F cleavage site in the VAMP.

Examples of BoNT/F VAMP epitopes, more particularly BoNT/F VAMP1, VAMP2 and/or VAMP3 epitopes, include:

- KLSELDDRADALQ (SEQ ID NO: 15)
- QKLSELDDRADALQ (SEQ ID NO: 16)
- KLSELDDRAD (SEQ ID NO: 17)
- KLSELDDRADALQAGAS (SEQ ID NO: 18)
- DQKLSELDDRADALQ (SEQ ID NO: 31).

In one embodiment, a BoNT/F VAMP epitope, in particular a BoNT/F VAMP1, VAMP2 and/or VAMP3 epitope, comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a sequence selected from: SEQ ID NO: 15 to SEQ ID NO: 18, and SEQ ID NO: 31. In a preferred embodiment, a BoNT/F VAMP epitope comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to KLSELDDRADALQ (SEQ ID NO: 15). In a more preferred embodiment, a BoNT/F VAMP epitope comprises or consists of KLSELDDRADALQ (SEQ ID NO: 15).

In one embodiment of the antigenic polypeptide of the invention, the VAMP epitope is a BoNT/D VAMP epitope wherein the at least 8 amino acid residues are immediately C-terminal to a BoNT/D cleavage site in the VAMP.

Examples of BoNT/D VAMP epitopes, more particularly BoNT/D VAMP1, VAMP2 and/or VAMP3 epitopes, include:

- KLSELDDRADALQ (SEQ ID NO: 15)
- LSELDDRADALQ (SEQ ID NO: 19)
- LSELDDRADA (SEQ ID NO: 20)
- LSELDDRADALQAGAS (SEQ ID NO: 21).

In one embodiment, a BoNT/D VAMP epitope, in particular a BoNT/D VAMP1, VAMP2 and/or VAMP3 epitope, comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a sequence selected from: SEQ ID NO: 15, and SEQ ID NO: 19 to SEQ ID NO: 21. In a preferred embodiment, a BoNT/D VAMP epitope comprises or consists of an amino acid sequence

which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to KLSELDDRADALQ (SEQ ID NO:15). In a more preferred embodiment, a BoNT/D VAMP epitope comprises or consists of KLSELDDRADALQ (SEQ ID NO: 15).

In one embodiment of the antigenic polypeptide of the invention, the VAMP epitope is a BoNT/F5 or BoNT/FA cleaved VAMP epitope wherein the at least 8 amino acid residues are immediately C-terminal to a BoNT/F5 or BoNT/FA cleavage site in the VAMP.

Examples of BoNT/F5 or BoNT/FA VAMP epitopes, more particularly BoNT/F5 or BoNT/FA VAMP1, VAMP2 and/or VAMP3 epitopes, include:

- ERDQKLSELDDRA (SEQ ID NO: 32)
- LERDQKLSELDDRA (SEQ ID NO: 33)
- VLERDQKLSELDDRA (SEQ ID NO: 34).

In one embodiment, a BoNT/F5 or BoNT/FA VAMP epitope, in particular a BoNT/F5 or BoNT/FA VAMP1, VAMP2 and/or VAMP3 epitope, comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a sequence selected from: SEQ ID NO: 32 to SEQ ID NO: 34. In a preferred embodiment, a BoNT/F5 or BoNT/FA VAMP epitope comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to ERDQKLSELDDRA (SEQ ID NO: 32). In a more preferred embodiment, a BoNT/F5 or BoNT/FA VAMP epitope comprises or consists of ERDQKLSELDDRA (SEQ ID NO: 32).

In one embodiment of the antigenic polypeptide of the invention, the VAMP epitope is a BoNT/B or TeNT VAMP epitope wherein the at least 8 amino acid residues are immediately C-terminal to a BoNT/B or TeNT cleavage site in the VAMP.

Examples of BoNT/B or TeNT VAMP epitopes, more particularly BoNT/B or TeNT VAMP1, VAMP2 and/or VAMP3 epitopes, include:

- FETSAAKLKRKYW (SEQ ID NO: 22)
- FESSAAKLKRKYW (SEQ ID NO: 23)
- QFETSAAKLKRKYW (SEQ ID NO: 24)
- FETSAAKLKR (SEQ ID NO: 25)

- FETSAAKLKRKYWWKN (SEQ ID NO: 26)
- ETSAAKLKRKYWWK (SEQ ID NO: 48)
- FETSAAKLKRKYWWK (SEQ ID NO: 49)
- QFESSAAKLKRKYW (SEQ ID NO: 50)
- FESSAAKLKR (SEQ ID NO: 51)
- FESSAAKLKRKYWWK (SEQ ID NO: 52).

In one embodiment, a BoNT/B or TeNT VAMP epitope, in particular a BoNT/B or TeNT VAMP1, VAMP2 and/or VAMP3 epitope, comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a sequence selected from: SEQ ID NO: 22 to SEQ ID NO: 26, and SEQ ID NO: 48 to SEQ ID NO: 52. In a preferred embodiment, a BoNT/B or TeNT VAMP epitope comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to FETSAAKLKRKYW (SEQ ID NO: 22) or FETSAAKLKRKYWWK (SEQ ID NO: 49). In a more preferred embodiment, a BoNT/B or TeNT VAMP epitope comprises or consists of FETSAAKLKRKYW (SEQ ID NO: 22) or FETSAAKLKRKYWWK (SEQ ID NO: 49). Surprisingly, antibodies binding the latter epitope allow not only the detection of BoNT/B VAMP cleavage, but also of BoNT/F VAMP cleavage.

In one embodiment of the antigenic polypeptide of the invention, the VAMP epitope is a BoNT/G VAMP epitope wherein the at least 8 amino acid residues are immediately C-terminal to a BoNT/G cleavage site in the VAMP.

Examples of BoNT/G VAMP epitopes, more particularly BoNT/G, VAMP1, VAMP2 and/or VAMP3 epitopes, include:

- AKLKRKYWWKN (SEQ ID NO: 27)
- AAKLKRKYWWKN (SEQ ID NO: 28)
- AKLKRKYWWKNCKM (SEQ ID NO: 29)
- AKLKRKYWWKNLKM (SEQ ID NO: 30).

In one embodiment, a BoNT/G VAMP epitope, in particular a BoNT/G VAMP1, VAMP2 and/or VAMP3 epitope, comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a sequence

selected from: SEQ ID NO: 27 to SEQ ID NO: 30. In a preferred embodiment, a BoNT/G VAMP epitope comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to AKLKRKYWWKN (SEQ ID NO: 27). In a more preferred embodiment, a BoNT/G VAMP epitope comprises or consists of AKLKRKYWWKN (SEQ ID NO: 27).

In one embodiment of the antigenic polypeptide of the invention, the VAMP epitope is a BoNT/X VAMP epitope wherein the at least 8 amino acid residues are immediately C-terminal to a BoNT/X cleavage site in the VAMP.

Examples of BoNT/X VAMP epitopes, more particularly BoNT/X VAMP1, VAMP2 and/or VAMP3 epitopes, include:

- ADALQAGASQF (SEQ ID NO: 53)
- ADALQAGASQ (SEQ ID NO: 54)
- RADALQAGASQF (SEQ ID NO: 55)
- ADALQAGASQFE (SEQ ID NO: 56)
- ADALQAGASVF (SEQ ID NO: 57)
- ADALQAGASV (SEQ ID NO: 58)
- ADALQAGASVFE (SEQ ID NO: 59)
- RADALQAGASVF (SEQ ID NO: 60)
- RADALQAGAS (SEQ ID NO: 61).

In one embodiment, a BoNT/X VAMP epitope, in particular a BoNT/X VAMP1, VAMP2 and/or VAMP3 epitope, comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a sequence selected from: SEQ ID NO: 53 to SEQ ID NO: 61. In a preferred embodiment, a BoNT/X VAMP epitope comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to ADALQAGASQF (SEQ ID NO: 53). In a more preferred embodiment, a BoNT/X VAMP epitope comprises or consists of ADALQAGASQF (SEQ ID NO: 53).

Other examples of BoNT/X VAMP epitopes, and more particularly BoNT/X VAMP4 epitopes, include:

- SESLSDNATAF (SEQ ID NO: 62)
- SESLSDNATA (SEQ ID NO: 63)
- KSESLSDNATAF (SEQ ID NO: 64)
- SESLSDNATAFS (SEQ ID NO: 65).

In one embodiment, a BoNT/X VAMP epitope, in particular a BoNT/X VAMP4 epitope, comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a sequence selected from: SEQ ID NO: 62 to SEQ ID NO: 65. In a preferred embodiment, a BoNT/X VAMP epitope comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to SESLSDNATAF (SEQ ID NO: 62). In a more preferred embodiment, a BoNT/X VAMP epitope comprises or consists of SESLSDNATAF (SEQ ID NO: 62).

Other examples of BoNT/X VAMP epitopes, and more particularly BoNT/X VAMP5 epitopes, include:

- SDQLLDMSSTF (SEQ ID NO: 66)
- SDQLLDMSST (SEQ ID NO: 67)
- RSDQLLDMSSTF (SEQ ID NO: 68)
- SDQLLDMSSTFN (SEQ ID NO: 69)
- SDQLLDMSSAF (SEQ ID NO: 70)
- SDQLLDMSSA (SEQ ID NO: 71)
- RSDQLLDMSSAF (SEQ ID NO: 72)
- SDQLLDMSSAFS (SEQ ID NO: 73)
- RSDQLLDMSS (SEQ ID NO: 74).

In one embodiment, a BoNT/X VAMP epitope, in particular a BoNT/X VAMP5 epitope, comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a sequence selected from: SEQ ID NO: 66 to SEQ ID NO: 74. In a preferred embodiment, a BoNT/X VAMP epitope comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to SDQLLDMSSTF (SEQ ID NO:

66). In a more preferred embodiment, a BoNT/X VAMP epitope comprises or consists of SDQLLDMSSTF (SEQ ID NO: 66).

Other examples of BoNT/X VAMP epitopes, and more particularly BoNT/X YKT6 epitopes, include:

- SEVLGTQSKAF (SEQ ID NO: 75)
- SEVLGTQSKA (SEQ ID NO: 76)
- KSEVLGTQSKAF (SEQ ID NO: 77)
- SEVLGTQSKAFY (SEQ ID NO: 78).

In one embodiment, a BoNT/X VAMP epitope, in particular a BoNT/X YKT6 epitope, comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to a sequence selected from: SEQ ID NO: 75 to SEQ ID NO: 78. In a preferred embodiment, a BoNT/X VAMP epitope comprises or consists of an amino acid sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or is 100% identical to SEVLGTQSKAF (SEQ ID NO: 75). In a more preferred embodiment, a BoNT/X VAMP epitope comprises or consists of SEVLGTQSKAF (SEQ ID NO: 75).

Herein, the "percent sequence identity" between two or more nucleic acid or amino acid sequences is a function of the number of identical nucleotides or amino acids at identical positions shared by the aligned sequences. Thus, % identity may be calculated as the number of identical nucleotides or amino acids at each position in an alignment divided by the total number of nucleotides or amino acids in the aligned sequence, multiplied by 100. Calculations of % sequence identity may also take into account the number of gaps, and the length of each gap that needs to be introduced to optimize alignment of two or more sequences. Sequence comparisons and the determination of percent identity between two or more sequences can be carried out using specific mathematical algorithms, which will be familiar to a skilled person, for example a global alignment mathematical algorithm (such as described by Needleman and Wunsch, J. Mol. Biol. 48(3), 443-453, 1972).

In another aspect, the invention relates to a polypeptide comprising an antigenic polypeptide according to the invention, wherein the polypeptide does not comprise a region

of greater than 17, 16, 15, 14, 13, 12, 11, 10, preferably 16, more preferably 15, consecutive amino acids having 100% sequence identity to a naturally-occurring VAMP amino acid sequence. The skilled person in the art would readily understand that such polypeptide is also antigenic.

In a preferred embodiment, the polypeptide comprises a covalent linker, preferably in its N-terminus and/or in C-terminus. Examples of covalent linkers that are suitable according to the invention are provided below.

In another aspect, the invention provides an antigenic protein comprising a polypeptide according to the invention covalently linked to a carrier.

Preferably, the carrier is a non-immunogenic or weakly immunogenic protein. Examples of suitable carriers include keyhole limpet hemacyanin (KLH), ovalbumin (OVA), thyroglobulin (THY), bovine serum albumin (BSA), soybean trypsin inhibitor (STI) or a multiple attachment peptide (MAP).

In one embodiment, the antigenic protein comprises a covalent linker between the polypeptide of the invention (which may already comprise the linker, as indicated above) and the carrier. Said linker can be one or more amino acids, natural or unnatural, which, as well-known in the art, can form covalent bonds with other amino acids (of the polypeptide and/or carrier) due to the presence of reactive groups present in their N-terminus, C-terminus and/or side chains. Notably, an amino acid having a primary amine group (–NH2) in N-terminus and/or side chain (such as lysine) can react with an amino acid having a carboxyl (–COOH) group in C-terminus and/or side chain (such as aspartic acid or glutamic acid) to form a covalent bond; an amino acid having a sulfhydryl (–SH) group in side chain (such as cysteine or selenocysteine) can react with an amino acid having a sulfhydryl (–SH) group in side chain (such as cysteine or selenocysteine) to form a covalent bond. For example, the covalent linker can be a cysteine added in C-terminus or N-terminus of the polypeptide of the invention, said cysteine forming a disulphide bridge with another cysteine added or present in the carrier. The covalent linker may alternatively, or in addition, be in the form of several amino acids forming a spacer, for example the linker can be a peptide comprising non-charged amino acids with small side-chain R groups, such

as, e.g., glycine, alanine, valine, leucine or serine. Examples of suitable spacers of the invention include G-spacers such as GGG, GGGG and GGGGS or A-spacers such as AAA, AAAA and AAAAV. In one embodiment, the linker consists of about 1 to about 30 amino acid residues, preferably about 2 to about 25 amino acid residues, more preferably about 3 to about 20 amino acid residues, for example, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues.

In another aspect, the invention provides the use of an antigenic polypeptide or protein according to the invention to generate antibodies against a C-terminal VAMP cleavage product. In one embodiment, the epitope of the invention is used to generate a polyclonal antibody against a C-terminal VAMP cleavage product. In another embodiment, the epitope of the invention is used to generate a monoclonal antibody against a C-terminal VAMP cleavage product.

Methods for generating antibodies are well known in the art, see eg. Greenfield, Edward A., ed. *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory Press, 2014; Leenaars, Marlies, and Coenraad FM Hendriksen. "Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations." Ilar Journal 46.3 (2005): 269-279.

Polyclonal antibodies that bind to a VAMP epitope as described herein can be produced by injecting an animal, e.g. a mammal such as a rabbit, a goat, a mouse, a hamster or a monkey, or an egg, such as a chicken egg, with an antigenic polypeptide or protein of the invention. Polyclonal antibodies for a VAMP epitope as disclosed herein can be isolated from the animal (e.g. from the blood) or egg and further purified by well-known techniques, such as protein affinity chromatography to obtain the IgG fraction, or by affinity purification against the VAMP epitope used for producing the antibodies. Several contract research organisations provide custom antibody generation services, for example the company Eurogentec provides a “Speedy 28-day programme” in which they immunise day 0, and then have 3 booster injections day 7, 10 and 18. Medium bleed day 21 and final bleed day 28. This is one example of the general technique of polyclonal antibody production which is well known in the art.

Monoclonal antibodies that bind to a VAMP epitope as described herein can be produced using a hybridoma method. See e.g., Chapter 7, Greenfield, Edward A., ed. *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory Press, 2014. Briefly, a host animal, e.g. a mammal such as a rabbit, a goat, a mouse, a hamster or a monkey, is exposed to one or more injections of an antigenic polypeptide or protein of the invention to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to a cleaved VAMP. The antibody titer in the immunized animal can be monitored over time by standard techniques, such as with an ELISA (enzyme linked immunosorbent assay). Alternatively, the lymphocytes can be immunized in vitro using a suitable cell culture line. At an appropriate time after immunization, e.g., when the antibody titers are highest, antibody-producing cells are isolated from the animal. Generally, either peripheral blood lymphocytes are used, if cells of human origin are desired, or spleen cells or lymph node cells are used, if non-human mammalian sources are desired. The isolated antibody-producing cells are fused with an immortal cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Typically, a murine myeloma cell line is fused with splenocytes harvested from an appropriately immunized mouse to produce the hybridoma. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine (HAT). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, e.g., the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days in culture because they are not transformed). The culture medium in which the hybridoma cells are grown can then be assayed for the presence of monoclonal antibodies that bind a VAMP epitope as described herein. For example, hybridoma supernatants can be screened using a cleaved VAMP positive media in an immunoprecipitation assay, in vitro binding assay, such as, e.g., a radioimmunoassay (RIA) or an enzyme-linked immunoabsorbent assay (ELISA), or in a cell-based activity assay. The binding affinity of a monoclonal antibody can also be determined, e.g., by Scatchard analysis. See, e.g., Peter J. Munson and David Rodbard, Ligand: A Versatile Computerized Approach For Characterization of Ligand-Binding Systems, 107(1) Anal. Biochem. 220-239 (1980). After the desired hybridoma cells are

identified, limiting dilution procedures are used to isolate clones originating from a single cell until a clonal cell line expressing the desired monoclonal antibody is obtained. Alternatively, monoclonal antibodies that bind a VAMP epitope as described herein can be produced by screening a recombinant combinatorial immunoglobulin library, such as, e.g., an antibody phage display library, with an antigenic polypeptide, protein or peptide of the invention. Kits for generating and screening phage display libraries are commercially available, such as, e.g., the Recombinant Phage Antibody System (Amersham GE Healthcare, Piscataway, NJ); and the SurfZAP™ Phage Display Kit (Stratagene, La JoIIa, CA). Additionally, examples of methods and reagents useful in generating and screening antibody display library can be found in, for example, Ladner et al. U.S. Patent 5,223,409; Borrebaeck et al. U.S. Patent 5,712,089; Griffiths et al. U.S. Patent 5,885,793; Griffiths et al. U.S. Patent 5,962,255; McCafferty et al. U.S. Patent 5,969,108; Griffiths et al. U.S. Patent 6,010,884; Jespers et al. U.S. Patent 6,017,732; Borrebaeck et al. U.S. Patent 6,027,930; Johnson et al. U.S. Patent 6,140,471 ; McCafferty et al. U.S. Patent 6,172,197, each of which is hereby incorporated by reference in its entirety.

In another aspect, the invention provides an antibody that binds to an antigenic polypeptide or protein of the invention.

In one embodiment, the antibody is a polyclonal antibody.

In one embodiment, the antibody is a monoclonal antibody.

Binding affinity between the antibody and the antigenic polypeptide or protein can be assessed by determining the equilibrium dissociation constant (K_D) which measures the rate at which new antibody-antigen complexes formed equals the rate at which antibody-antigen complexes dissociate at equilibrium. The equilibrium dissociation constant is expressed in M, and is defined by the Kd/Ka ratio at equilibrium, where Ka is the antibody's association rate constant and Kd is the antibody's dissociation rate constant. K_D = [Ab] x [Ag]/[Ab + Ag], where [Ab] is the molar concentration of the antibody, [Ag] is the molar concentration of the antigen, and [Ab + Ag] is the of molar concentration of the antibody-antigen complex, where all concentrations are of such components when the system is at

equilibrium. The smaller the equilibrium dissociation constant, the more tightly bound the antibody is to its antigen, or the higher the binding affinity between antibody and antigen.

In a one embodiment, the K_D between the antibody of the invention and the antigenic polypeptide or protein epitope is lower than 10^{-6} M. In a preferred embodiment, the K_D between the antibody of the invention and the antigenic polypeptide or protein is lower than 10^{-7} M. In a more preferred embodiment, the K_D between the antibody of the invention and the antigenic polypeptide or protein is lower than 10^{-8} M. In a more preferred embodiment, the K_D between the antibody of the invention and the antigenic polypeptide or protein is lower than 10^{-9} M. In a more preferred embodiment, the K_D between the antibody of the invention and the antigenic polypeptide or protein is lower than 10^{-10} M. In a more preferred embodiment, the K_D between the antibody of the invention and the antigenic polypeptide or protein is lower than 10^{-11} M. In a more preferred embodiment, the K_D between the antibody of the invention and the antigenic polypeptide or protein is lower than 10^{-12} M.

In another aspect, the invention provides the use of an antibody according to the invention in a gain of signal cellular assay for VAMP cleavage by a VAMP cleaving clostridial neurotoxin.

In one embodiment, the use is an *in vitro* or an *ex vivo* use.

In another aspect, the invention provides a method for determining cleavage of VAMP by a VAMP cleaving clostridial neurotoxin in a cell, comprising:

a) contacting the cell with the clostridial neurotoxin under conditions suitable for clostridial neurotoxin activity;
b) contacting the cytoplasmic content of said cell with a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to the invention; and

c) detecting by a suitable means the binding of said first detection antibody to the C-terminal VAMP cleavage product.

In one embodiment, the method according to the invention, further comprises d) quantifying by a suitable means the amount of the C-terminal VAMP cleavage product bound to said first detection antibody.

In one embodiment of the method of the invention, step b) comprises contacting the cytoplasmic content of said cell with a second detection antibody against full-length VAMP under conditions suitable for the binding of said second detection antibody to full-length VAMP; step c) comprises detecting by a suitable means the binding of the second detection antibody to full-length VAMP, and step d) comprises quantifying by a suitable means the amount of full-length VAMP bound to said second detection antibody.

In one embodiment, the method is an *in vitro* or an *ex vivo* method.

It will be clear to the person skilled in the art that an increase in the amount of C-terminal VAMP cleavage product bound to the first antibody and/or a decrease in the amount of full-length VAMP bound to the second detection antibody are indicative of an increase in VAMP cleavage by the VAMP cleaving clostridial neurotoxin.

In one embodiment, the second detection antibody is the same as the first detection antibody and binds to the C-terminal VAMP cleavage product and to full-length VAMP.

In an alternative embodiment, the second detection antibody is different from said first detection antibody, and binds to full-length VAMP but not to the C-terminal VAMP cleavage product. Suitably, the second detection antibody binds to a VAMP epitope which is N-terminal to a clostridial neurotoxin cleavage site. Examples of suitable antibodies include commercially available antibodies such as ab3347 (Abcam) or ab181869 (Abcam).

In a particular embodiment, the method for determining cleavage of VAMP by a VAMP cleaving clostridial neurotoxin in a cell, comprises:

a) contacting the cell with the clostridial neurotoxin under conditions suitable for clostridial neurotoxin activity;
b) contacting the cytoplasmic content of said cell with

- a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to the invention which binds to the C-terminal VAMP cleavage product and to full-length VAMP; and with
- a second detection antibody which binds to full-length VAMP but not to the C-terminal VAMP cleavage product;

c) detecting by a suitable means
- the binding of the first antibody to the C-terminal VAMP cleavage product and to full-length VAMP; and
- the binding of the second detection antibody to the full-length VAMP; and

d) quantifying by a suitable means:
- the combined amount of C-terminal VAMP cleavage product and full-length VAMP bound to the first detection antibody; and
- the amount of full-length VAMP bound to the second detection antibody.

It will be clear to the person skilled in the art that a decrease in the amount of full-length VAMP bound to the second detection antibody and no change in the combined amount of full-length and C-terminal VAMP cleavage product bound to the first detection antibody is indicative of VAMP cleavage by the VAMP cleaving clostridial neurotoxin.

In another particular embodiment, the method for determining cleavage of VAMP by a VAMP cleaving clostridial neurotoxin in a cell, comprises:

a) contacting the cell with the clostridial neurotoxin under conditions suitable for clostridial neurotoxin activity;
b) contacting the cytoplasmic content of said cell with a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to the invention which binds to the C-terminal VAMP cleavage product and to full-length VAMP;
c) detecting by a suitable means

- the binding of the first antibody to the C-terminal VAMP cleavage product; and
- the binding of the first detection antibody to the full-length VAMP;

wherein the signal generated from binding of the first detection antibody to the C-terminal VAMP cleavage product can be distinguished from the signal generated from binding of the first detection antibody to full-length VAMP; and

d) quantifying by a suitable means:
   - the amount of C-terminal VAMP cleavage product bound to the first detection antibody; and
   - the amount of full-length VAMP bound to the first detection antibody.

It will be clear to the person skilled in the art that an increase in the amount of C-terminal VAMP cleavage product bound to the first antibody and a decrease in the amount of full-length VAMP bound to the first detection antibody are indicative of an increase in VAMP cleavage by the VAMP cleaving clostridial neurotoxin.

In another aspect, the invention provides a method for determining immunoresistance to a VAMP cleaving clostridial neurotoxin in a subject, comprising:

a) adding a VAMP cleaving clostridial neurotoxin to a test sample obtained from the subject;
b) contacting a cell with the test sample of step a) under conditions suitable for clostridial neurotoxin activity;
c) contacting the cytoplasmic content of said cell with a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to the invention;
d) detecting by a suitable means the binding of the first detection antibody to the C-terminal VAMP cleavage product;
e) quantifying the amount of the C-terminal VAMP cleavage product bound to the first detection antibody;

f) repeating steps a) to e) with a negative control sample instead of a test sample; and

g) comparing the amount of the C-terminal VAMP cleavage product bound to said first detection antibody in steps (e) and (f), wherein detection of a lower amount of the C-terminal VAMP cleavage product bound to said first detection antibody in step (e) relative to the amount of the C-terminal VAMP cleavage product bound to said first detection antibody in step (f) is indicative of the presence of neutralizing antibodies against the VAMP cleaving clostridial neurotoxin.

In one embodiment, step f) further comprises repeating steps a) to e) with a positive control sample.

As used herein, the term "neutralizing antibodies against a VAMP cleaving clostridial neurotoxin" means any antibody that will, under physiological conditions, bind to a region of a VAMP cleaving clostridial neurotoxin in such a manner as to reduce or prevent the VAMP cleaving clostridial neurotoxin from exerting its therapeutic effect in a subject.

In one embodiment, the subject is a mammal. In a preferred embodiment, the subject is a human being.

In one embodiment, the sample is selected from blood, plasma, serum and lymph fluid obtained from the subject.

A test sample can be obtained from a subject prior to exposure to a VAMP cleaving clostridial neurotoxin, after a single treatment with a VAMP cleaving clostridial neurotoxin or after multiple treatments with a VAMP cleaving clostridial neurotoxin. In a particular embodiment, the test sample is from a subject which is resistant to treatment with a VAMP cleaving clostridial neurotoxin.

As used herein, the term "control sample" means any sample in which the presence or absence of the test sample is known and includes both negative and positive control samples. With respect to neutralizing antibodies against VAMP cleaving clostridial neurotoxin, a negative control sample can be obtained from an individual who had never been exposed to the VAMP cleaving clostridial neurotoxin and may include, without limitation, a sample from the same individual supplying the test sample, but taken before undergoing a treatment with a VAMP cleaving clostridial neurotoxin; a sample taken from a different individual never been exposed to a VAMP cleaving clostridial neurotoxin; a

pooled sample taken from a plurality of different individuals never been exposed to a VAMP cleaving clostridial neurotoxin.

With respect to neutralizing antibodies against a VAMP cleaving clostridial neurotoxin, a positive control sample can be obtained from an individual manifesting immunoresistance to the VAMP cleaving clostridial neurotoxin and includes, without limitation, individual testing positive in a patient-based testing assays; individual testing positive in an in vivo bioassay; and individual showing hyperimmunity, e.g., a subject vaccinated against a VAMP cleaving clostridial neurotoxin.

In one embodiment, the method is an *in vitro* or *an ex vivo* method.

In one embodiment of the method for determining immunoresistance, step c) comprises contacting the cytoplasmic content of said cell with a second detection antibody against full-length VAMP under conditions suitable for the binding of said second detection antibody to full-length VAMP; step d) comprises detecting by a suitable means the binding of the second detection antibody to full-length VAMP, and step e) comprises quantifying the amount of full-length VAMP bound to said second detection antibody.

In one embodiment, the second detection antibody is the same as the first detection antibody and binds to the C-terminal VAMP cleavage product and to full-length VAMP.

In an alternative embodiment, the second detection antibody is different from said first detection antibody, and binds to full-length VAMP but not to the C-terminal VAMP cleavage product. Suitably, the second detection antibody binds to a VAMP epitope which is N-terminal to a clostridial neurotoxin cleavage site. Examples of suitable antibodies include commercially available antibodies such as ab3347 (Abcam) or ab181869 (Abcam).

In a particular embodiment, the method for determining immunoresistance to a VAMP cleaving clostridial neurotoxin in a subject, comprises:

a) adding a VAMP cleaving clostridial neurotoxin to a test sample obtained from the subject;
b) contacting a cell with the test sample of step a) under conditions suitable for clostridial neurotoxin activity;
c) contacting the cytoplasmic content of said cell with

- a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to the invention which binds to the C-terminal VAMP cleavage product and to full-length VAMP; and with
- a second detection antibody which binds to full-length VAMP but not to the C-terminal VAMP cleavage product;

d) detecting by a suitable means
- the binding of the first antibody to the C-terminal VAMP cleavage product and to full-length VAMP; and
- the binding of the second detection antibody to the full-length VAMP;

e) quantifying
- the combined amount of C-terminal VAMP cleavage product and full-length VAMP bound to the first detection antibody; and
- the amount of full-length VAMP bound to the second detection antibody;

f) repeating steps a) to e) with a negative control sample instead of a test sample; and

g) comparing the amount of the C-terminal VAMP cleavage product bound to said first detection antibody in steps (e) and (f), wherein detection of a lower combined amount of the C-terminal VAMP cleavage product and full-length VAMP bound to the first detection antibody and/or a higher amount of the full-length VAMP bound to the second detection antibody in step (e) relative to the corresponding amounts in step (f) is indicative of the presence of neutralizing antibodies against the VAMP cleaving clostridial neurotoxin.

In another particular embodiment, the method for determining immunoresistance to a VAMP cleaving clostridial neurotoxin in a subject, comprises:

a) adding a VAMP cleaving clostridial neurotoxin to a test sample obtained from the subject;

b) contacting a cell with the test sample of step a) under conditions suitable for clostridial neurotoxin activity;

c) contacting the cytoplasmic content of said cell with a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to the invention which binds to the C-terminal VAMP cleavage product and to full-length VAMP;

d) detecting by a suitable means

- the binding of the first antibody to the C-terminal VAMP cleavage product and to full-length VAMP; and
- the binding of the first detection antibody to the full-length VAMP;

wherein the signal generated from binding of the first detection antibody to the C-terminal VAMP cleavage product can be distinguished from the signal generated from binding of the first detection antibody to full-length VAMP;

e) quantifying

- the amount of C-terminal VAMP cleavage product bound to the first detection antibody; and
- the amount of full-length VAMP bound to the first detection antibody;

f) repeating steps a) to e) with a negative control sample instead of a test sample; and

g) comparing the amount of the C-terminal VAMP cleavage product bound to said first detection antibody in steps (e) and (f), wherein detection of a lower amount of the C-terminal VAMP cleavage product bound to said first detection antibody and/or a higher amount of the full-length VAMP bound to the first detection antibody in step (e) relative to the corresponding amounts in step (f) is indicative of the presence of neutralizing antibodies against the VAMP cleaving clostridial neurotoxin.

Herein, a "VAMP cleaving clostridial neurotoxin" means a clostridial neurotoxin which binds to a receptor on a target cell, translocates a clostridial light chain (L) into the cytosol, which in turn proteolytically cleaves a VAMP thereby disrupting the secretion of molecules via vesicle transport by the cell.

Preferably, in the methods or use of the invention, the VAMP cleaving clostridial neurotoxin comprises a BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X or TeNT light chain. Suitably, the BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X or TeNT light chain comprises a sequence selected from:

- amino acid residues 1 to 441 of SEQ ID NO: 2, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto,
- amino acid residues 1 to 442 of SEQ ID NO: 4, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto,
- amino acid residues 1 to 439 of SEQ ID NO: 6, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto,
- amino acid residues 1 to 446 of SEQ ID NO: 7, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto,
- amino acid residues 1 to 439 of SEQ ID NO: 41, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto, and
- amino acid residues 1 to 456 of SEQ ID NO: 8, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto.

It is understood that a BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X or TeNT light chain as described herein has the ability to cleave a VAMP.

In one embodiment of methods or uses of the invention, the VAMP cleaving clostridial neurotoxin is selected from a BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X and a TeNT. Suitably, the BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X or TeNT comprises a sequence selected from:

- SEQ ID NO: 2 or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto,

- SEQ ID NO: 4, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto,
- SEQ ID NO: 6, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto,
- SEQ ID NO: 7, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto,
- SEQ ID NO: 41, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto and
- SEQ ID NO: 8, or a polypeptide sequence having at least 70 %, preferably at least 75%, 80%, 85%, 90%, 95% or 99% sequence identity thereto.

It is understood that a BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X or TeNT clostridial neurotoxin as described herein has the ability to bind to a receptor on a target cell, translocate the clostridial light chain into the cytosol and cleave a VAMP.

In one embodiment, the VAMP cleaving clostridial neurotoxin is a mosaic neurotoxin. The term "mosaic neurotoxin" as used in this context refers to a naturally occurring clostridial neurotoxin that comprises at least one functional domain from another type of clostridial neurotoxins (e.g. a clostridial neurotoxin of a different serotype), the clostridial neurotoxin not usually comprising the at least one functional domain. Examples of naturally occurring VAMP cleaving mosaic neurotoxins are BoNT/DC and BoNT/FA. BoNT/DC comprises the L chain and H_N domain of serotype D and the H_C domain of serotype C Nakamura K, et al. "Characterization of the D/C mosaic neurotoxin produced by Clostridium botulinum associated with bovine botulism in Japan." Vet. Microbiol. (2010): 140:147–154., whereas BoNT/FA consists of a BoNT/F5 light chain, a H_N domain closely related to subtype F1 and a BoNT/A1 H_C domain (Pellett, Sabine, et al. "Purification and Characterization of Botulinum Neurotoxin FA from a Genetically Modified Clostridium botulinum Strain." mSphere 1.1 (2016): e00100-15).

In one embodiment, the VAMP cleaving clostridial neurotoxin is a mosaic neurotoxin selected from BoNT/DC and BoNT/FA.

In one embodiment, the VAMP cleaving clostridial neurotoxin is a chimeric neurotoxin. The term "chimeric neurotoxin" as used herein means a neurotoxin comprising one or more domains originating from a first neurotoxin and one or more domains originating from a

second neurotoxin. For example, a chimeric neurotoxin may comprise an LH_N domain originating from a first neurotoxin and a H_C domain originating from a second neurotoxin. Another example of a chimeric neurotoxin is a neurotoxin comprising an LH_NH_{CN} domain originating from a first neurotoxin and a H_{CC} domain originating from a second neurotoxin. Examples of chimeric neurotoxins are provided in GB1607901.4 (not yet published), herein incorporated by reference.

In one embodiment, the VAMP cleaving clostridial neurotoxin is a chimeric neurotoxin which comprises:

- a light chain (L) from a BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X or TeNT,
- a H_N domain from a BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/X or TeNT,
- a H_{CN} domain from a BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/X or TeNT,
- a H_{CC} domain from a BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/X or TeNT, and

  wherein at least two of the domains are from different clostridial neurotoxins.

In one embodiment, the VAMP cleaving clostridial neurotoxin is a chimeric neurotoxin which comprises:

- a LH_N domain from a first clostridial neurotoxin selected from BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X or TeNT,
- a $H_{CN}H_{CC}$ domain from a second clostridial neurotoxin selected from BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/X or TeNT

  wherein the first and second clostridial neurotoxins are different.

In one embodiment, the VAMP cleaving clostridial neurotoxin is a chimeric neurotoxin which comprises:

- a LH_NH_{CN} domain from a first clostridial neurotoxin selected from BoNT/B, BoNT/D, BoNT/F, BoNT/G, BoNT/X or TeNT,
- a H_{CC} domain from a second clostridial neurotoxin selected from BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/X or TeNT

  wherein the first and second clostridial neurotoxins are different.

The VAMP cleaving clostridial neurotoxin can be a modified neurotoxin or a derivative thereof, including but not limited to those described below. A modified neurotoxin or derivative may contain one or more amino acids that has been modified as compared to the native (unmodified) form of the neurotoxin, or may contain one or more inserted amino acids that are not present in the native (unmodified) form of the toxin. By way of example, a modified clostridial neurotoxin may have modified amino acid sequences in one or more domains relative to the native (unmodified) clostridial neurotoxin sequence. Such modifications may modify functional aspects of the neurotoxin, for example biological activity or persistence.

A modified VAMP cleaving clostridial neurotoxin as described herein retains the ability to bind to a receptor on a target cell, to translocate the light chain into the cell cytoplasm and cleave a VAMP.

A modified VAMP cleaving clostridial neurotoxin may have one or more modifications in the amino acid sequence of the heavy chain (such as a modified H_C domain), wherein said modified heavy chain binds to target nerve cells with a higher or lower affinity than the native (unmodified) neurotoxin. Such modifications in the H_C domain can include modifying residues in the ganglioside binding site or in the protein receptor binding site of the H_{CC} domain that alter binding to the ganglioside receptor and/or the protein receptor of the target nerve cell. Examples of such modified neurotoxins are described in WO 2006/027207 and WO 2006/114308, both of which are hereby incorporated by reference in their entirety. For example, the H_{CC} domain from a BoNT/B neurotoxin comprises at least one amino acid residue substitution, addition or deletion which has the effect of increasing the binding affinity of the BoNT/B H_{CC} domain for human Syt II as compared to the natural BoNT/B H_{CC} sequence. Suitable amino acid residue substitution, addition or deletion in the BoNT/B H_{CC} subdomain have been disclosed in WO2013/180799 and in PCT/US2016/024211 which is not yet published (both herein incorporated by reference). Suitable amino acid residue substitution, addition or deletion in the H_{CC} subdomain include substitution mutations selected from the group consisting of: V1118M; Y1183M; E1191M; E1191I; E1191Q; E1191T; S1199Y; S1199F; S1199L; S1201V; E1191C, E1191V, E1191L, E1191Y, S1199W, S1199E, S1199H, W1178Y, W1178Q, W1178A, W1178S, Y1183C, Y1183P and combinations thereof.

In one embodiment, the VAMP cleaving clostridial neurotoxin is a retargeted neurotoxin. The term "retargeted neurotoxin" (also referred to as "targeted secretion inhibitors", "TSIs", "TVEMPs" or "TEMs") as used herein means a clostridial neurotoxin comprising a Targeting Moiety (TM) which binds to a non clostridial receptor. The TM can replace part or all of the H_C or H_{CC} domain of the clostridial neurotoxin heavy chain. Examples of retargeted neurotoxins are disclosed in WO96/33273, WO98/07864, WO00/10598, WO01/21213, WO01/53336; WO02/07759 WO2005/023309, WO2006/026780, WO2006/099590, WO2006/056093, WO2006/059105, WO2006/059113, WO2007/138339, WO2007/106115, WO2007/106799, WO2009/150469, WO2009/150470, WO2010/055358, WO2010/020811, WO2010/138379, WO2010/138395, WO2010/138382, WO2011/020052, WO2011/020056, WO2011/020114 , WO2011/020117, WO2011/20119, WO2012/156743, WO2012/134900, WO2012/134897, WO2012/134904, WO2012/134902, WO2012/135343, WO2012/135448, WO2012/135304, WO2012/134902, WO2014/033441, WO2014/128497, WO2014/053651, WO2015/004464, all of which are herein incorporated by reference.

Examples of cells suitable for use in the methods or use according to the invention include a prokaryotic cell, eg.an E. coli cell, a yeast cell, an insect cell, an animal cell, a mammalian cell, a human cell, a mouse cell, a primate cell, and/or a neuronal cell. Preferably, the cell is a neuronal cell, in particular cells with a high sensitivity to BoNT,

A cell with a high sensitivity to BoNT is a cell which is susceptible to BoNT intoxication. In some embodiments, a cell with a high sensitivity to BoNT is a cell which is susceptible to BoNT intoxication by, e.g., about 500 pM or less, about 400 pM or less, about 300 pM or less, about 200 pM or less, about 100 pM or less, about 90 pM or less, about 80 pM or less, about 70 pM or less, about 60 pM or less, about 50 pM or less, about 40 pM or less, about 30 pM or less, about 20 pM or less, about 10 pM or less, about 9 pM or less, about 8 pM or less, about 7 pM or less, about 6 pM or less, about 5 pM or less, about 4 pM or less, about 3 pM or less, about 2 pM or less, about 1 pM or less, about 0.9 pM or less, about 0.8 pM or less, about 0.7 pM or less, about 0.6 pM or less, about 0.5 pM or less, about 0.4 pM or less, about 0.3 pM or less, about 0.2 pM, about 0.1 pM or less, about 90fM or less, about

80fM or less, about 70fM or less, about 60fM or less, about 50fM or less, about 40fM or less, about 30fM or less, about 20fM or less, or about 10fM or less.

Preferably, the cell has a high sensitivity (as defined above) to a VAMP cleaving BoNT.

In one embodiment, the cell is a primary neuronal cell with a high sensitivity to BoNT, e.g., cortical neurons, hippocampal neurons, and/or spinal cord neurons. For example, the cell is a rat cortical neuron.

In one embodiment, the cell is from a neuronal cell line with a high sensitivity to BoNT, e.g. BE(2)-M17, Kelly, LA1-55n, N1 E-115, N4TG3, N18, Neuro-2a, NG108-15, PC12, SH-SY5Y, SiMa, and/or SK-N-BE(2)-C.

In one embodiment, the cell is a neuronal cell derived from a stem cell, in particular from an induced pluripotent stem cell (iPS cell), eg. i-Cell® Neurons, i-Cell® DopaNeurons iCell Glutamatergic Neurons, iCell MotoNeurons (Cellular dynamics Inc) Cerebral Cortical Neurons, Neural Stem Cells (Axol Biosciences), Peri.4U neurons, CNS.4U neurons, Dopa.4UNeurons (Axiogenesis) , MNP cells (Lonza),Cortical Neurons, Motor Neurons (iStem), and/or iPSC-Derived Neural Cells (MTI-GlobalStem).

In one embodiment, the cell can be modified by recombinant technology to express high levels of VAMP, such as VAMP1, VAMP2 VAMP3, VAMP4, VAMP5 and/or YKT6, more preferably VAMP1, VAMP2 and/or VAMP3.

In one embodiment in which the VAMP cleaving neurotoxin is a BoNT/B, a BoNT/DC or a BoNT/G, the cell expresses high levels of synaptotagmin I and/or synaptotagmin II (Syt I/ Syt II). In one embodiment in which the VAMP cleaving neurotoxin is a BoNT/B, a BoNT/D-C or a BoNT/G, the cell is modified by recombinant technology to express high levels of synaptotagmin I and/or synaptotagmin II (Syt I/ Syt II).

In one embodiment in which the VAMP cleaving neurotoxin is a BoNT/FA, a BoNT/F, a BoNT/D or a TeNT, the cell expresses high levels of synaptic vesicle protein (SV2). In one embodiment in which the VAMP cleaving neurotoxin is a BoNT/FA, a BoNT/F, a BoNT/D or a TeNT, the cell is modified by recombinant technology to express high levels of synaptic vesicle protein (SV2).

As used herein, "conditions suitable for clostridial neurotoxin activity" refers to conditions (e.g. temperature, pH, cofactors, etc) under which the clostridial neurotoxin can bind to a clostridial neurotoxin receptor present on the cell membrane, translocate the clostridial neurotoxin light chain into the cell cytoplasm and cleave a VAMP.

In one embodiment of the methods of the invention, the conditions suitable for clostridial neurotoxin activity can comprise incubation at about 37°C for a period of from about 1 hour to about 48 hours. In one embodiment of the method of the invention, the conditions suitable for clostridial neurotoxin activity can comprise incubation at about 37°C for a period of from about 2 hours to about 36 hours. In one embodiment of the method of the invention, the conditions suitable for clostridial neurotoxin activity can comprise incubation at about 37°C for a period of from about 4 hours to about 24 hours.

For example, conditions suitable for clostridial neurotoxin activity can comprise incubation at 37°C for 24 hours.

As used herein, "conditions suitable for the binding of a first detection antibody to cleaved VAMP" and "conditions suitable for the binding of a second detection antibody to full-length VAMP" refer to conditions (e.g. temperature, pH, cofactors, etc) under which the first and/or second detection antibody can bind to cleaved VAMP and/or full-length VAMP.

In one embodiment of the method of the invention, the conditions suitable for antibody binding can comprise incubation at about 4°C for a period of from about 8 hours to about 48 hours. In one embodiment of the method of the invention, the conditions suitable for antibody binding can comprise incubation at about 4°C for a period of from about 10 hours to about 24 hours. In one embodiment of the method of the invention, the conditions suitable for antibody binding can comprise incubation at about 4°C for a period of from about 12 hours to about 16 hours.

In one embodiment of the method of the invention, the conditions suitable for antibody binding can comprise incubation at about 25°C for a period of from about 30 minutes hour to about 8 hours. In one embodiment of the method of the invention, the conditions suitable for antibody binding can comprise incubation at about 25°C for a period of from about 1 hour to about 4 hours. In one embodiment of the method of the invention, the conditions

suitable for antibody binding can comprise incubation at about 25°C for a period of from about 1,5 hours to about 3 hours.

Means suitable for detecting and quantifying the binding of a detection antibody to cleaved or full-length VAMP are well known in the art. For example, binding of a detection antibody to cleaved or full-length VAMP can be detected and quantified by Western blotting. As each protein runs at a specific molecular weight via SDS-PAGE, the cleaved VAMP will be detected at lower molecular weights than the full-length VAMP. Analysis of the bands by densitometry allows a percentage cleavage readout using both the full-length band and the cleavage band within the same lane on the gel. Alternatively, VAMP cleavage can be detected and quantified using an enzyme-linked immunosorbent assay (ELISA), for example a sandwich ELISA.

In one embodiment of the methods of the invention, the first detection antibody is a polyclonal antibody and the binding of the first detection antibody to the C-terminal VAMP cleavage product is detected and quantified in an enzyme-linked immunosorbent assay.

In one embodiment of the methods of the invention, the first detection antibody is a polyclonal antibody and the binding of the first detection antibody to the C-terminal VAMP cleavage product is detected and quantified in a western blot assay.

In one embodiment of the methods of the invention, the first detection antibody is a monoclonal antibody and the binding of the first detection antibody to the C-terminal VAMP cleavage product is detected and quantified in an enzyme-linked immunosorbent assay.

In one embodiment of the methods of the invention, the first detection antibody is a monoclonal antibody and the binding of the first detection antibody to the C-terminal VAMP cleavage product is detected and quantified in a western blot assay.

In one embodiment of the methods of the invention, the cell is lysed prior to contacting of its cytoplasmic content with the detection antibody(ies).

In an alternative embodiment of the methods of the invention, the cell is permeabilized prior to contacting of its cytoplasmic content with the detection antibody(ies).

In another aspect, the invention provides a kit comprising a cell which is susceptible to intoxication by a VAMP cleaving neurotoxin; and a first detection antibody against cleaved VAMP, wherein said first detection antibody is an antibody according to the invention.

In one embodiment, the kit further comprises a second detection antibody which binds to full-length VAMP but not to the C-terminal VAMP cleavage product. Suitably, the second detection antibody binds to a VAMP epitope which is N-terminal to a clostridial neurotoxin cleavage site. Examples of suitable antibodies include commercially available antibodies such as ab3347 (Abcam) or ab181869 (Abcam).

This disclosure is not limited by the exemplary methods and materials disclosed herein, and any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of this disclosure. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, any nucleic acid sequences are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within this disclosure. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within this disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in this disclosure.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a clostridial neurotoxin" includes a plurality of such candidate agents and reference to "the clostridial neurotoxin" includes reference to one or more

clostridial neurotoxins and equivalents thereof known to those skilled in the art, and so forth.

The invention will now be described, by way of example only, with reference to the following Figures and Examples.

FIGURES

Figure 1 – VAMP sequences with clostridial neurotoxin cleavages sites. (**A**) human and rat VAMP1, VAMP2 and VAMP3 sequences with BoNT/F5 and BoNT/FA, BoNT/F, BoNT/D and BoNT/DC, BoNT/B, BoNT/G, TeNT and BoNT/X cleavage sites. (**B**) human and rat VAMP4, VAMP5 and YKT6 sequences with BoNT/X cleavage sites.

Figure 2 – VAMP sequences with Ab epitopes (i.e. immunogenic epitope regions) and BoNT/F, BoNT/D and BoN/B cleavages sites. The sequences for human and rat VAMP1, VAMP2 and VAMP3 are shown for comparison. Rat and human VAMP2 sequences are identical in the epitope regions selected. The cleavage sites are indicated by arrows: the VAMP2 cleavage points for BoNT/F and BoNT/D are located on adjacent amino acids, Q58-K59 and K59-L60 respectively, while the cleavage point for BoNT/B is located on amino acids Q76-F77 (based on human VAMP2 sequence amino acid position).

Figure 3 – Cell-free cleavage of recombinant VAMP2-GFP with MBP-LF and LH_ND. Recombinant VAMP2-GFP was incubated with 0.01μg/μl LH_ND or MBP-LF for 1hr at 37°C. Equal volumes of sample buffer were added and 0.5μg (Coomassie) and 0.3μg (blots) protein run via SDS-PAGE and either stained with Coomassie or blotted with various anti-VAMP2 antibodies. The cartoon indicates the location of the antibody epitopes. The representation of the recombinant protein and the line length of the epitopes are not to scale. 1 – BSA, 2 – VAMP2-GFP, 3 – Cleaved VAMP2-GFP (aa59/60-end), 4 – Cleaved VAMP2-GFP (aa1-58/59).

Figure 4 – *In vitro* VAMP cleavage after BoNT/F and BoNT/D treatment. Rat cortical neurons grown in 96 well plates until DIV18-21 were treated for 24 hours with either BoNT/F (**A**) or BoNT/D (**B**). Lysates were run via SDS-PAGE and blotted with the

custom-made anti-VAMP2 antibodies: anti-Pep1, anti-Pep2 or anti-Pep3, or with the commercial antibody ab181869. 1 – full-length VAMP2, 2 – cleaved VAMP2. The anti-pep 2 data show the dose dependent disappearance of full-length VAMP2 and the appearance of the lower molecular weight cleaved fragment. Both band signals were used to quantify the dose dependent percentage of VAMP2 cleavage by BoNT/F (**C**) and BoNT/D (**D**).

Figure 5 – (**A**) Rat cortical neurons were treated with natural BoNT/F1 (□), natural BoNT/A1 (●) or recombinant BoNT/FA (Δ) for 24 hours. Cell were lysed, run on SDS-PAGE and blotted for VAMP-2 or SNAP-25 cleavage. Percent SNARE cleavage was determined from the ratio of full-length to cleaved protein by densitometric analysis. Data were fitted using a four-parameter logistic equation and the concentration of BoNT required for 50% maximal SNARE cleavage (pEC50) determined (**B**). Data are mean ± s.e.m. (n=3 (BoNT/F1 and BoNT/A1) or 4 (BoNT/FA) independent experiments in triplicate).

Figure 6 – *in vitro* VAMP cleavage after BoNT/B and BoNT/F treatment. Rat cortical neurons grown until DIV18-21 were treated for 24 hours with either BoNT/F or BoNT/B. Lysates were run via SDS-PAGE and blotted with a new custom-made anti-VAMP2 antibody (anti-Pep4), a BoNT/B cleavage-specific anti-VAMP2 antibody or anti-Pep1, anti-Pep2 or anti-Pep3 antibodies.

EXAMPLES

Example 1: detection of VAMP proteolytic cleavage by BoNT/D and BoNT/F

A - Methods

1. Antibody generation

Antibodies were generated by Eurogentec using their Speedy 28 day programme (https://secure.eurogentec.com/product/research-anti-protein-28-day-speedy-polyclonal-packages.html?country=gbr).Two rabbits per peptide were immunised with the following peptides:

- VAMP PEP1: H2N- SNR RLQ QTQ AQV DEC -CONH2 (SEQ ID NO:39);
- VAMP PEP2: AcNH - KLS ELD DRA DAL Q - CONH2 (SEQ ID NO:15); or
- VAMP PEP3: H2N - CLQ AGA SQ - CONH2 (SEQ ID NO:40).

Animals underwent a first immunisation and three subsequent boosters. A pre-immune bleed, medium bleed and a final bleed were taken.

2. Recombinant protein cleavage

Active constructs containing the light chain and translocation domain of BoNT/D or the equivalent BoNT/F domains fused to a maltose-binding protein (MPB) were generated as previously described (Masuyer et al., "Structure and activity of a functional derivative of Clostridium botulinum neurotoxin B. J Struct Biol", 174, p52-57, 2011; Sutton et al., "Preparation of specifically activatable endopeptidase derivatives of Clostridium botulinum toxins type A, B, and C and their applications. Protein Expression and Purification 40:31–41, 2005). Briefly, either LH_ND (SEQ ID NO: 35) or a fusion protein called MBP-LF (SEQ ID NO: 36) (the latter being a fusion of MBP with the light chain of BoNT/F1 and a C-terminal 6-histidine motif; MPB and the 6-histidine motif being commonly known affinity tags) were diluted to 0.01µg/µl in assay buffer (50mM HEPES pH7.2, 200µM ZnCl2, 1µg/µl BSA, 10mM DTT). VAMP2-GFP (SEQ ID NO: 37) (a fusion protein of amino acids 2-94 of human VAMP2 and the detectable marker green fluorescent protein (GFP)) was diluted to 8µM in assay buffer (50mM HEPES pH7.2, 200µM ZnCl2, 1µg/µl BSA, 10mM DTT). Equal volumes of LH_ND or MBP-LF and VAMP2-GFP (SEQ ID NO: 37) (8µM) were combined and incubated at 37°C for 1 hour. Reactions were stopped by adding 2x reducing sample buffer (NuPage LDS sample buffer, 100mM DTT).

3. Rat Cortical Neuronal Cell Culture

Rat cortical neurons were prepared from E17-E18 CD rat embryos. Dissected cortical tissue was collected into ice-cold Hank's Balanced Salt Solution (HBSS) w/o Ca2+ or Mg2+, and then dissociated in papain solution for 40 minutes at 37°C following the manufacturer's instructions (Worthington Biochemical, NJ, US). Cortical cells were plated on poly-L-ornithine (PLO) coated 96-well plates at a density of 20,000 cells/well in 125 µl Neurobasal media containing 2% B27 supplement, 0.5 mM GlutaMAX, 1% foetal bovine

serum (FBS) and 100 U/ml penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. A further 125 µl Neurobasal media containing 2% B27, 0.5 mM GlutaMAX was added on DIV (days in vitro) 4. Cells were maintained by replacement of half media twice per week. On DIV 11, 1.5 µM cytosine β-D-arabinofuranoside (AraC) was added to the media to prevent proliferation of non-neuronal cells.

4. BoNT treatment

Rat cortical neurons at DIV 18-21 were treated with a concentration range of native BoNT/F1 (Metabiologics, US) (1 nM – 0.1 pM), or BoNT/D (Metabiologics, US) (10 nM – 1 pM) in triplicate wells for 24 hours at 37°C. Media were removed and cells washed once with PBS. Cells were lysed in 40 µl LDS sample buffer (NuPage LDS buffer, 1mM DTT, 1:500 Benzonase) for 10 minutes at room temperature.

5. SDS-PAGE and Western Blot

Neuronal lysates were boiled at 90°C for 5 minutes. 15 µl lysates were loaded per lane to 12% Bis-Tris gels and run in MES buffer at 200V for 50 min. Proteins were transferred to nitrocellulose membranes via a Transblot Turbo (Biorad) using the low MW programme. Membranes were blocked for 1 hour at room temperature with 5% low fat milk/PBS-Tween and then incubated with the custom made anti-Pep1, anti-Pep2 or anti-Pep3 anti-VAMP2 primary antibodies, or with the commercial anti-VAMP2 antibodies (Abcam ab3347 and ab181869), overnight at 4°C. Membranes were washed 3 times in PBS-Tween and incubated with anti-rabbit-HRP secondary antibody for 1 hour at room temperature. Membranes were washed for 3×5mins in PBS-Tween, then developed with SuperSignal West Femto chemiluminescent substrate and visualised using a Syngene PXi system.

B – Results

Assessment of recombinant protein detection

The regions of the chosen three peptide epitopes from VAMP2 relative to BoNT cleavage sites are shown in figure 2. The sequences for human and rat VAMP1, VAMP2 and VAMP3 are shown for comparison. Rat and human VAMP2 sequences are identical in the

epitopes regions selected. The cleavage sites for BoNT/B and BoNT/D are located on adjacent amino acids.

Initially, the antibodies were tested in a cell free assay using recombinant VAMP2-GFP. BoNT/F and BoNT/D substitutes (MBP-LF and LH_ND) containing the enzymatic light chain domains of the toxin were used to cleave the VAMP protein (Figure 3). In addition, two other commercially available VAMP2 antibodies were used as a comparison; ab3347 (epitope aa1-18) and ab181869 (epitope within aa1-100). Figure 3 shows that anti-Pep1 antibody detected full-length VAMP2 and the N-terminal cleaved portion (aa1-58/59) with much reduced signal. As expected, there was no C-terminal cleavage product detection by this antibody, since its epitope was not located on this portion. Anti-Pep2 and Anti-Pep3 antibodies detected both the full-length protein and the C-terminal cleaved products of VAMP2-GFP. Ab3347 only detected full-length VAMP2 and not the N-terminal cleavage fragment whereas ab181869 detected both.

These first results show that the antibodies were able to detect full-length and the expected cleaved products of recombinant VAMP2. The exception was ab3347 which only detected full-length VAMP2 and not the N-terminal cleavage fragment.

Assessment of endogenous protein detection

The next question was whether these antibodies could detect any cleavage products in a neuronal cell assay in which endogenous proteases would be present. Rat primary cortical neurons were treated with either BoNT/F or BoNT/D and lysed for WB analysis (Figure 4). The anti-Pep1 antibody only recognised the full-length protein and there was no detectable cleavage product. The anti-Pep 2 antibody detected both the full-length and the C-terminal cleaved product. The anti-Pep3 antibody showed a weak signal very poor affinity for monomer VAMP within a cell lysate and detected higher molecular weight species which were most likely to be dimers and other proteins (data not shown). The full-length monomer signal was very low but there was a band for the BoNT/F and BoNT/D cleaved C-terminal product. In other words, anti-Pep3 did not detect full-length VAMP but weakly detected the BoNT/F and BoNT/D cleaved C-terminal fragment. This was in contrast to the earlier cell-free results which showed a strong signal from the full-length and cleaved recombinant VAMP. The commercial antibody Ab3347 was not tested *in vitro* due to the absence of cleaved protein detection in the cell-free assay. Despite the positive

binding to the N-terminal cleaved recombinant fragment in the cell-free assay, the commercial antibody ab181869 detected full-length VAMP2 in the cortical lysates, but not a cleaved fragment in the cortical lysates. The Pep 2 data was used to quantify the dose dependent cleavage of VAMP2 by BoNT/F (figure 4C) and BoNT/D (figure 4D).

The inventors have initially shown that, in a cell-free system, both recombinant VAMP cleavage products can be detected. However, when transferred to a cellular lysate, the inventors have also shown that the N-terminal product is not detectable, but there may be other mechanisms involved, apart from degradation, that are yet unknown. In contrast, the inventors have shown that the C-terminal VAMP fragment which is still bound to the vesicle membrane is not degraded or altered in a manner that would prevent antibody binding and detection by Western Blot. The Pep2 epitope is adjacent to the BoNT/D and BoNT/F cleavage site and the antibody generated against this peptide detects both full-length VAMP and the cleaved product. In contrast, the anti-Pep3 antibody, which was generated against a shorter epitope further away from the BoNT F/D cleavage site, also detects, albeit weakly, the cleaved product.

Example 2: detection of VAMP proteolytic cleavage by BoNT/FA and BoNT/F1 in rat cortical neurons

A – Methods

1. Rat Cortical Neuronal Cell Culture

Rat cortical neurons were prepared as detailed in Example 1.

2. BoNT treatment

Rat cortical neurons at DIV 18-21 were treated with a concentration range (1 pM – 1 fM) of recombinant BoNT/FA (SEQ ID NO: 38), or a concentration range (1 nM – 1 pM) of native BoNT/F1 (Metabiologics, US), or a concentration range (1 nM – 1 fM) of native BoNT/A1 (List Biological Laboratories Inc., US), in triplicate wells, for 24 hours, at 37°C. Media were removed and cells washed once with PBS. Cells were lysed in 40 µl LDS sample buffer (NuPage LDS buffer, 1mM DTT, 1:500 Benzonase) for 10 minutes at room temperature.

3. SDS Page and Western blot of rat cortical neurons

Rat cortical neurons were lysed in 40 µl lysis buffer (NuPage LDS sample buffer, 1mM DTT and 1:500 Benzonase) for 10 minutes at room temperature. Samples were boiled at 90°C for 5 minutes and 15 µl lysates loaded per lane to 12% Bis-Tris gels and run in either MOPS buffer at 200 V for 80 min (SNAP-25) or MES buffer at 200 V for 50 min (VAMP2). Proteins were transferred to nitrocellulose membranes via a Transblot Turbo (Biorad) using the mixed MW (SNAP25) or low MW (VAMP2) programmes. Membranes were blocked for 1 hour at room temperature with 5% low fat milk/PBS-Tween and then incubated with either anti-SNAP25 antibody (Sigma S9684 1:4000) or anti-Pep2 (1:500), a custom-made anti-VAMP2 (Eurogentec) antibody as described in Example 1; each primary antibody was incubated overnight at 4°C. Membranes were washed 3 times in PBS-Tween and incubated with anti-rabbit-HRP secondary antibody for 1 hour at room temperature. Membranes were washed for 3×5mins in PBS-Tween, then developed with SuperSignal West Dura or West Femto chemiluminescent substrate and visualised using a Syngene Pxi system. Band densitometry was analysed using Genetools software and % protein cleavage was determined using the ratio of the full-length protein to the cleaved product for both SNAP-25 and VAMP2.

B – Results

Following treatment with BoNT/F1, BoNT/A1 or BoNT/FA for 24 hours, rat cortical neurons were lysed, run on SDS-PAGE and Western blotted for VAMP-2 (BoNT/F1 and BoNT/FA) or SNAP-25 (BoNT/A1). Percent SNARE cleavage was determined from the ratio of full-length to cleaved protein by densitometric analysis.

The results are presented in figure 5. Recombinant BoNT/FA cleaved VAMP-2 with potency pEC50 = 12.75 ± 0.14, n=4. Natural BoNT/F1 cleaved VAMP-2 with potency pEC50 = 10.77 ± 0.12, n=3. Natural BoNT/A1 cleaved SNAP-25 with potency pEC50 = 12.38 ± 0.14, n=3.

Example 3: detection of VAMP proteolytic cleavage by BoNT/B in rat cortical neurons

A – Methods

1. Antibody generation

A monoclonal antibody was generated by Abcam using rabbits immunised with the peptide Pep4: FETSAAKLKRKYWWK (SEQ ID NO:49).

The BoNT/B cleavage-specific anti-VAMP2 antibody (Kegel et al., Toxicology in Vitro; 2007, 21: p1641-1649) was used for comparative study.

2. Rat Cortical Neuronal Cell Culture

Rat cortical neurons were prepared from E17-E18 CD rat embryos. Dissected cortical tissue was collected into ice-cold Hank's Balanced Salt Solution (HBSS) w/o Ca2+ or Mg2+, and then dissociated in papain solution for 40 minutes at 37°C following the manufacturer's instructions (Worthington Biochemical, NJ, US). Cortical cells were plated on poly-L-ornithine (PLO) coated 96-well plates at a density of 20,000 cells/well in 125 µl Neurobasal media containing 2% B27 supplement, 0.5 mM GlutaMAX, 1% foetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. A further 125 µl Neurobasal media containing 2% B27, 0.5 mM GlutaMAX was added on DIV (days in vitro) 4. Cells were maintained by replacement of half media twice per week. On DIV 11, 1.5 µM cytosine β-D-arabinofuranoside (AraC) was added to the media to prevent proliferation of non-neuronal cells.

3. BoNT treatment

Rat cortical neurons were cultured in T25 flasks and treated with 1nM and 10pM of BoNT/B (provided by obtained from List Biological Laboratories, Inc.) (SEQ ID NO:2) for 24 hours at 37°C. Media were removed and cells washed once with PBS. Cells were lysed in 1.5ml NuPage sample buffer (NuPage LDS buffer, 1mM DTT, 1:500 Benzonase) for 10 minutes at room temperature.

4. SDS-PAGE and Western Blot

Neuronal lysates were boiled at 90°C for 5 minutes. 15 µl lysates were loaded per lane to 12% Bis-Tris gels and run in MES buffer at 200V for 50 min. Proteins were transferred to nitrocellulose membranes via a Transblot Turbo (Biorad) using the low MW programme. Membranes were blocked for 1 hour at room temperature with 5% low fat milk/PBS-Tween and then incubated with the custom made anti-Pep1, anti-Pep2, anti-Pep3 or anti-Pep4 antibodies, or with the BoNT-B cleavage-specific antibody, overnight at 4°C. Membranes were washed 3 times in PBS-Tween and incubated with anti-rabbit-HRP secondary antibody for 1 hour at room temperature. Membranes were washed for 3×5mins in PBS-Tween, then developed with SuperSignal West Femto chemiluminescent substrate and visualised using a Syngene PXi system.

B - Results

Based on the results obtained in the above-described Examples 1 and 2, which implied the location of the epitope was key to detection of cleaved VAMP *in vitro*, a new monoclonal antibody was generated against the epitope adjacent to the BoNT/B cleavage site located on the C-Terminal side.

This antibody was tested in the same rat cortical assay following BoNT/B and BoNT/F treatment and compared with anti-Pep1, anti-Pep2, anti-Pep3 and a BoNT/B cleavage-specific antibody (Figure 6). The epitope regions for all comparison antibodies were located on the N-terminal side of the BoNT/B cleavage site. Figure 6 show that the location of the epitope of the new antibody directed against Pep4 enabled detection of the full-length VAMP2, as well as of the cleaved products for both BoNT/B and BoNT/F treatment. In contrast, the anti-Pep2 and anti-Pep3 antibodies only detected the BoNT/F cleaved product but not the BoNT/B cleaved product. The anti-Pep1 antibody did not detect any cleavage product as expected. The BoNT/B cleavage-specific antibody also did not detect any BoNT/B cleavage product in these cell lysates.

Overall, the present data show that an important consideration for cleaved VAMP detection is the location of the antibody epitope. Only antibodies raised against epitopes located on the membrane-bound VAMP fragment, post-cleavage, were able to detect the fragment. By locating the monoclonal antibody epitope towards the C-terminal end of VAMP, it was

hypothesized that this region should be present in the VAMP fragments produced by the VAMP-cleaving neurotoxin serotypes B, D and F. This proved to be the case, enabling to generate a single antibody (anti-Pep4 Mab) which provided positive results for BoNT/B and BoNT/F treated neurons. Besides, since TeNT shares the same cleavage site as BoNT/B and BoNT/D cleavage site is in close vicinity to BoNT/F cleavage site, it is expected that this antibody will also be applicable to TeNT and BoNT/D cleavage.

An additional advantage of the Pep4 epitope region is that antibodies directed against this region can detect both full length and cleaved VAMP with similar sensitivity. The ability to simultaneously detect both protein forms within the same sample provides a robust tool for normalisation, without the need to blot for additional housekeeping proteins. This provides a very useful and straightforward gain of signal Western blot assay for quantification of BoNT potency in cell models.

The present data also show differences in VAMP detection between cell-free recombinant protein assays and a whole cell model. It was precisely this inability to detect cellular cleaved VAMP which formed the basis of the hypothesis that VAMP degradation in the cell occurred very quickly (Foran et al., "Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E and F compared with the long-lasting type A". J. Biol Chem 278 (2) pp1363 – 1371 2003). In contrast to the antibodies of the present invention, the majority of commercially available VAMP antibodies are raised against epitopes within the N-terminal region of the protein and therefore the N-terminal VAMP fragment was the focus of those earlier studies. Although it is shown herein that the smaller C-terminal VAMP fragment is not degraded in a cell, the larger N-terminal fragment was not detected either. It is interesting to note however that, our cell-free results show that not all commercial antibodies are able to detect the expected N-terminal fragment even when it is present in a cell-free system lacking any proteases. From the present data, it can be concluded that the VAMP degradation hypothesis most certainly relates only to the N-terminal fragment, and that the C-terminal VAMP fragment is not degraded and remains bound to the vesicle membrane.

SEQUENCE INFORMATION

- SEQ ID NO: 1 - BoNT/A1 - UniProtKB Accession Number P10845 (*Clostridium botulinum)*

MPFVNKQFNYKDPVNGVDIAYIKIPNAGQMQPVKAFKIHNKIWVIPERDTFT
NPEEGDLNPPPEAKQVPVSYYDSTYLSTDNEKDNYLKGVTKLFERIYSTDLG
RMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVIQPDGSYRSEELNLVIIGPSADI
IQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPLLGAGKFA
TDPAVTLAHELIHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFG
GHDAKFIDSLQENEFRLYYYNKFKDIASTLNKAKSIVGTTASLQYMKNVFKE
KYLLSEDTSGKFSVDKLKFDKLYKMLTEIYTEDNFVKFFKVLNRKTYLNFDK
AVFKINIVPKVNYTIYDGFNLRNTNLAANFNGQNTEINNMNFTKLKNFTGLFE
FYKLLCVRGIITSKTKSLDKGYNKALNDLCIKVNNWDLFFSPSEDNFTNDLNK
GEEITSDTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENLSSDIIGQLELMPNI
ERFPNGKKYELDKYTMFHYLRAQEFEHGKSRIALTNSVNEALLNPSRVYTFF
SSDYVKKVNKATEAAMFLGWVEQLVYDFTDETSEVSTTDKIADITIIIPYIGPA
LNIGNMLYKDDFVGALIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVLTVQTI
DNALSKRNEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAI
INYQYNQYTEEEKNNINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMNS
MIPYGVKRLEDFDASLKDALLKYIYDNRGTLIGQVDRLKDKVNNTLSTDIPFQ
LSKYVDNQRLLSTFTEYIKNIINTSILNLRYESNHLIDLSRYASKINIGSKVNFD
PIDKNQIQLFNLESSKIEVILKNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYT
IINCMENNSGWKVSLNYGEIIWTLQDTQEIKQRVVFKYSQMINISDYINRWIF
VTITNNRLNNSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIK
YFNLFDKELNEKEIKDLYDNQSNSGILKDFWGDYLQYDKPYYMLNLYDPNK
YVDVNNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGTKFIIKKYASGNKDNI
VRNNDRVYINVVVKNKEYRLATNASQAGVEKILSALEIPDVGNLSQVVVMK
SKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNIAKLVASNWYNRQIERSSR
TLGCSWEFIPVDDGWGERPL

- SEQ ID NO: 2 - BoNT/B1 - UniProtKB Accession Number P10844 (*Clostridium botulinum)*

MPVTINNFNYNDPIDNNNIIMMEPPFARGTGRYYKAFKITDRIWIIPERYTFGY
KPEDFNKSSGIFNRDVCEYYDPDYLNTNDKKNIFLQTMIKLFNRIKSKPLGEK
LLEMIINGIPYLGDRRVPLEEFNTNIASVTVNKLISNPGEVERKKGIFANLIIFGP
GPVLNENETIDIGIQNHFASREGFGGIMQMKFCPEYVSVFNNVQENKGASIFN
RRGYFSDPALILMHELIHVLHGLYGIKVDDLPIVPNEKKFFMQSTDAIQAEEL
YTFGGQDPSIITPSTDKSIYDKVLQNFRGIVDRLNKVLVCISDPNININIYKNKF
KDKYKFVEDSEGKYSIDVESFDKLYKSLMFGFTETNIAENYKIKTRASYFSDS
LPPVKIKNLLDNEIYTIEEGFNISDKDMEKEYRGQNKAINKQAYEEISKEHLAV
YKIQMCKSVKAPGICIDVDNEDLFFIADKNSFSDDLSKNERIEYNTQSNYIEND
FPINELILDTDLISKIELPSENTESLTDFNVDVPVYEKQPAIKKIFTDENTIFQYL
YSQTFPLDIRDISLTSSFDDALLFSNKVYSFFSMDYIKTANKVVEAGLFAGWV
KQIVNDFVIEANKSNTMDKIADISLIVPYIGLALNVGNETAKGNFENAFEIAGA
SILLEFIPELLIPVVGAFLLESYIDNKNKIIKTIDNALTKRNEKWSDMYGLIVAQ
WLSTVNTQFYTIKEGMYKALNYQAQALEEIIKYRYNIYSEKEKSNINIDFNDI
NSKLNEGINQAIDNINNFINGCSVSYLMKKMIPLAVEKLLDFDNTLKKNLLNY
IDENKLYLIGSAEYEKSKVNKYLKTIMPFDLSIYTNDTILIEMFNKYNSEILNNI
ILNLRYKDNNLIDLSGYGAKVEVYDGVELNDKNQFKLTSSANSKIRVTQNQN
IIFNSVFLDFSVSFWIRIPKYKNDGIQNYIHNEYTIINCMKNNSGWKISIRGNRII
WTLIDINGKTKSVFFEYNIREDISEYINRWFFVTITNNLNNAKIYINGKLESNTD
IKDIREVIANGEIIFKLDGDIDRTQFIWMKYFSIFNTELSQSNIEERYKIQSYSEY
LKDFWGNPLMYNKEYYMFNAGNKNSYIKLKKDSPVGEILTRSKYNQNSKYI
NYRDLYIGEKFIIRRKSNSQSINDDIVRKEDYIYLDFFNLNQEWRVYTYKYFK
KEEEKLFLAPISDSDEFYNTIQIKEYDEQPTYSCQLLFKKDEESTDEIGLIGIHRF
YESGIVFEEYKDYFCISKWYLKEVKRKPYNLKLGCNWQFIPKDEGWTE

- SEQ ID NO: 3 - BoNT/C1 - UniProtKB Accession Number P18640 (*Clostridium botulinum)*

MPITINNFNYSDPVDNKNILYLDTHLNTLANEPEKAFRITGNIWVIPDRFSRNS
NPNLNKPPRVTSPKSGYYDPNYLSTDSDKDPFLKEIIKLFKRINSREIGEELIYR

LSTDIPFPGNNNTPINTFDFDVDFNSVDVKTRQGNNWVKTGSINPSVIITGPRE
NIIDPETSTFKLTNNTFAAQEGFGALSIISISPRFMLTYSNATNDVGEGRFSKSE
FCMDPILILMHELNHAMHNLYGIAIPNDQTISSVTSNIFYSQYNVKLEYAEIYA
FGGPTIDLIPKSARKYFEEKALDYYRSIAKRLNSITTANPSSFNKYIGEYKQKLI
RKYRFVVESSGEVTVNRNKFVELYNELTQIFTEFNYAKIYNVQNRKIYLSNVY
TPVTANILDDNVYDIQNGFNIPKSNLNVLFMGQNLSRNPALRKVNPENMLYL
FTKFCHKAIDGRSLYNKTLDCRELLVKNTDLPFIGDISDVKTDIFLRKDINEET
EVIYYPDNVSVDQVILSKNTSEHGQLDLLYPSIDSESEILPGENQVFYDNRTQN
VDYLNSYYYLESQKLSDNVEDFTFTRSIEEALDNSAKVYTYFPTLANKVNAG
VQGGLFLMWANDVVEDFTTNILRKDTLDKISDVSAIIPYIGPALNISNSVRRG
NFTEAFAVTGVTILLEAFPEFTIPALGAFVIYSKVQERNEIIKTIDNCLEQRIKR
WKDSYEWMMGTWLSRIITQFNNISYQMYDSLNYQAGAIKAKIDLEYKKYSG
SDKENIKSQVENLKNSLDVKISEAMNNINKFIRECSVTYLFKNMLPKVIDELN
EFDRNTKAKLINLIDSHNIILVGEVDKLKAKVNNSFQNTIPFNIFSYTNNSLLK
DIINEYFNNINDSKILSLQNRKNTLVDTSGYNAEVSEEGDVQLNPIFPFDFKLG
SSGEDRGKVIVTQNENIVYNSMYESFSISFWIRINKWVSNLPGYTIIDSVKNNS
GWSIGIISNFLVFTLKQNEDSEQSINFSYDISNNAPGYNKWFFVTVTNNMMGN
MKIYINGKLIDTIKVKELTGINFSKTITFEINKIPDTGLITSDSDNINMWIRDFYIF
AKELDGKDINILFNSLQYTNVVKDYWGNDLRYNKEYYMVNIDYLNRYMYA
NSRQIVFNTRRNNNDFNEGYKIIIKRIRGNTNDTRVRGGDILYFDMTINNKAY
NLFMKNETMYADNHSTEDIYAIGLREQTKDINDNIIFQIQPMNNTYYYASQIF
KSNFNGENISGICSIGTYRFRLGGDWYRHNYLVPTVKQGNYASLLESTSTHW
GFVPVSE

- SEQ ID NO: 4 - BoNT/D - UniProtKB Accession Number P19321 (*Clostridium botulinum)*

MTWPVKDFNYSDPVNDNDILYLRIPQNKLITTPVKAFMITQNIWVIPERFSSDT
NPSLSKPPRPTSKYQSYYDPSYLSTDEQKDTFLKGIIKLFKRINERDIGKKLINY
LVVGSPFMGDSSTPEDTFDFTRHTTNIAVEKFENGSWKVTNIITPSVLIFGPLP
NILDYTASLTLQGQQSNPSFEGFGTLSILKVAPEFLLTFSDVTSNQSSAVLGKSI
FCMDPVIALMHELTHSLHQLYGINIPSDKRIRPQVSEGFFSQDGPNVQFEELYT
FGGLDVEIIPQIERSQLREKALGHYKDIAKRLNNINKTIPSSWISNIDKYKKIFSE

KYNFDKDNTGNFVVNIDKFNSLYSDLTNVMSEVVYSSQYNVKNRTHYFSRH
YLPVFANILDDNIYTIRDGFNLTNKGFNIENSGQNIERNPALQKLSSESVVDLF
TKVCLRLTKNSRDDSTCIKVKNNRLPYVADKDSISQEIFENKIITDETNVQNYS
DKFSLDESILDGQVPINPEIVDPLLPNVNMEPLNLPGEEIVFYDDITKYVDYLN
SYYYLESQKLSNNVENITLTTSVEEALGYSNKIYTFLPSLAEKVNKGVQAGLF
LNWANEVVEDFTTNIMKKDTLDKISDVSVIIPYIGPALNIGNSALRGNFNQAF
ATAGVAFLLEGFPEFTIPALGVFTFYSSIQEREKIIKTIENCLEQRVKRWKDSY
QWMVSNWLSRITTQFNHINYQMYDSLSYQADAIKAKIDLEYKKYSGSDKENI
KSQVENLKNSLDVKISEAMNNINKFIRECSVTYLFKNMLPKVIDELNKFDLRT
KTELINLIDSHNIILVGEVDRLKAKVNESFENTMPFNIFSYTNNSLLKDIINEYF
NSINDSKILSLQNKKNALVDTSGYNAEVRVGDNVQLNTIYTNDFKLSSSGDKI
IVNLNNNILYSAIYENSSVSFWIKISKDLTNSHNEYTIINSIEQNSGWKLCIRNG
NIEWILQDVNRKYKSLIFDYSESLSHTGYTNKWFFVTITNNIMGYMKLYINGE
LKQSQKIEDLDEVKLDKTIVFGIDENIDENQMLWIRDFNIFSKELSNEDINIVYE
GQILRNVIKDYWGNPLKFDTEYYIINDNYIDRYIAPESNVLVLVQYPDRSKLY
TGNPITIKSVSDKNPYSRILNGDNIILHMLYNSRKYMIIRDTDTIYATQGGECS
QNCVYALKLQSNLGNYGIGIFSIKNIVSKNKYCSQIFSSFRENTMLLADIYKPW
RFSFKNAYTPVAVTNYETKLLSTSSFWKFISRDPGWVE

- SEQ ID NO: 5 - BoNT/E – Accession number WP_003372387 (*Clostridium botulinum)*

MPKINSFNYNDPVNDRTILYIKPGGCQEFYKSFNIMKNIWIIPERNVIGTTPQDF
HPPTSLKNGDSSYYDPNYLQSDEEKDRFLKIVTKIFNRINNNLSGGILLEELSK
ANPYLGNDNTPDNQFHIGDASAVEIKFSNGSQDILLPNVIIMGAEPDLFETNSS
NISLRNNYMPSNHGFGSIAIVTFSPEYSFRFNDNSMNEFIQDPALTLMHELIHS
LHGLYGAKGITTKYTITQKQNPLITNIRGTNIEEFLTFGGTDLNIITSAQSNDIY
TNLLADYKKIASKLSKVQVSNPLLNPYKDVFEAKYGLDKDASGIYSVNINKF
NDIFKKLYSFTEFDLATKFQVKCRQTYIGQYKYFKLSNLLNDSIYNISEGYNIN
NLKVNFRGQNANLNPRIITPITGRGLVKKIIRFCKNIVSVKGIRKSICIEINNGEL
FFVASENSYNDDNINTPKEIDDTVTSNNNYENDLDQVILNFNSESAPGLSDEK
LNLTIQNDAYIPKYDSNGTSDIEQHDVNELNVFFYLDAQKVPEGENNVNLTSS
IDTALLEQPKIYTFFSSEFINNVNKPVQAALFVSWIQQVLVDFTTEANQKSTVD

KIADISIVVPYIGLALNIGNEAQKGNFKDALELLGAGILLEFEPELLIPTILVFTI
KSFLGSSDNKNKVIKAINNALKERDEKWKEVYSFIVSNWMTKINTQFNKRKE
QMYQALQNQVNAIKTIIESKYNSYTLEEKNELTNKYDIKQIENELNQKVSIAM
NNIDRFLTESSISYLMKLINEVKINKLREYDENVKTYLLNYIIQHGSILGESQQE
LNSMVTDTLNNSIPFKLSSYTDDKILISYFNKFFKRIKSSSVLNMRYKNDKYV
DTSGYDSNININGDVYKYPTNKNQFGIYNDKLSEVNISQNDYIIYDNKYKNFSI
SFWVRIPNYDNKIVNVNNEYTIINCMRDNNSGWKVSLNHNEIIWTLQDNAGI
NQKLAFNYGNANGISDYINKWIFVTITNDRLGDSKLYINGNLIDQKSILNLGNI
HVSDNILFKIVNCSYTRYIGIRYFNIFDKELDETEIQTLYSNEPNTNILKDFWGN
YLLYDKEYYLLNVLKPNNFIDRRKDSTLSINNIRSTILLANRLYSGIKVKIQRV
NNSSTNDNLVRKNDQVYINFVASKTHLFPLYADTATTNKEKTIKISSSGNRFN
QVVVMNSVGNNCTMNFKNNNGNNIGLLGFKADTVVASTWYYTHMRDHTN
SNGCFWNFISEEHGWQEK

- SEQ ID NO: 6 - BoNT/F - UniProtKB Accession Number YP_001390123 (*Clostridium botulinum)*

MPVVINSFNYNDPVNDDTILYMQIPYEEKSKKYYKAFEIMRNVWIIPERNTIG
TDPSDFDPPASLENGSSAYYDPNYLTTDAEKDRYLKTTIKLFKRINSNPAGEV
LLQEISYAKPYLGNEHTPINEFHPVTRTTSVNIKSSTNVKSSIILNLLVLGAGPD
IFENSSYPVRKLMDSGGVYDPSNDGFGSINIVTFSPEYEYTFNDISGGYNSSTE
SFIADPAISLAHELIHALHGLYGARGVTYKETIKVKQAPLMIAEKPIRLEEFLTF
GGQDLNIITSAMKEKIYNNLLANYEKIATRLSRVNSAPPEYDINEYKDYFQWK
YGLDKNADGSYTVNENKFNEIYKKLYSFTEIDLANKFKVKCRNTYFIKYGFL
KVPNLLDDDIYTVSEGFNIGNLAVNNRGQNIKLNPKIIDSIPDKGLVEKIVKFC
KSVIPRKGTKAPPRLCIRVNNRELFFVASESSYNENDINTPKEIDDTTNLNNNY
RNNLDEVILDYNSETIPQISNQTLNTLVQDDSYVPRYDSNGTSEIEEHNVVDL
NVFFYLHAQKVPEGETNISLTSSIDTALSEESQVYTFFSSEFINTINKPVHAALFI
SWINQVIRDFTTEATQKSTFDKIADISLVVPYVGLALNIGNEVQKENFKEAFEL
LGAGILLEFVPELLIPTILVFTIKSFIGSSENKNKIIKAINNSLMERETKWKEIYS
WIVSNWLTRINTQFNKRKEQMYQALQNQVDAIKTVIEYKYNNYTSDERNRL
ESEYNINNIREELNKKVSLAMENIERFITESSIFYLMKLINEAKVSKLREYDEG
VKEYLLDYISEHRSILGNSVQELNDLVTSTLNNSIPFELSSYTNDKILILYFNKL

YKKIKDNSILDMRYENNKFIDISGYGSNISINGDVYIYSTNRNQFGIYSSKPSEV
NIAQNNDIIYNGRYQNFSISFWVRIPKYFNKVNLNNEYTIIDCIRNNNSGWKIS
LNYNKIIWTLQDTAGNNQKLVFNYTQMISISDYINKWIFVTITNNRLGNSRIYI
NGNLIDEKSISNLGDIHVSDNILFKIVGCNDTRYVGIRYFKVFDTELGKTEIETL
YSDEPDPSILKDFWGNYLLYNKRYYLLNLLRTDKSITQNSNFLNINQQRGVY
QKPNIFSNTRLYTGVEVIIRKNGSTDISNTDNFVRKNDLAYINVVDRDVEYRL
YADISIAKPEKIIKLIRTSNSNNSLGQIIVMDSIGNNCTMNFQNNNGGNIGLLGF
HSNNLVASSWYYNNIRKNTSSNGCFWSFISKEHGWQEN

- SEQ ID NO: 7 - BoNT/G - UniProtKB Accession Number WP_039635782 (*Clostridium botulinum)*

MPVNIKNFNYNDPINNDDIIMMEPFNDPGPGTYYKAFRIIDRIWIVPERFTYGF
QPDQFNASTGVFSKDVYEYYDPTYLKTDAEKDKFLKTMIKLFNRINSKPSGQ
RLLDMIVDAIPYLGNASTPPDKFAANVANVSINKKIIQPGAEDQIKGLMTNLII
FGPGPVLSDNFTDSMIMNGHSPISEGFGARMMIRFCPSCLNVFNNVQENKDTS
IFSRRAYFADPALTLMHELIHVLHGLYGIKISNLPITPNTKEFFMQHSDPVQAE
ELYTFGGHDPSVISPSTDMNIYNKALQNFQDIANRLNIVSSAQGSGIDISLYKQI
YKNKYDFVEDPNGKYSVDKDKFDKLYKALMFGFTETNLAGEYGIKTRYSYF
SEYLPPIKTEKLLDNTIYTQNEGFNIASKNLKTEFNGQNKAVNKEAYEEISLEH
LVIYRIAMCKPVMYKNTGKSEQCIIVNNEDLFFIANKDSFSKDLAKAETIAYN
TQNNTIENNFSIDQLILDNDLSSGIDLPNENTEPFTNFDDIDIPVYIKQSALKKIF
VDGDSLFEYLHAQTFPSNIENLQLTNSLNDALRNNNKVYTFFSTNLVEKANT
VVGASLFVNWVKGVIDDFTSESTQKSTIDKVSDVSIIIPYIGPALNVGNETAKE
NFKNAFEIGGAAILMEFIPELIVPIVGFFTLESYVGNKGHIIMTISNALKKRDQK
WTDMYGLIVSQWLSTVNTQFYTIKERMYNALNNQSQAIEKIIEDQYNRYSEE
DKMNINIDFNDIDFKLNQSINLAINNIDDFINQCSISYLMNRMIPLAVKKLKDF
DDNLKRDLLEYIDTNELYLLDEVNILKSKVNRHLKDSIPFDLSLYTKDTILIQV
FNNYISNISSNAILSLSYRGGRLIDSSGYGATMNVGSDVIFNDIGNGQFKLNNS
ENSNITAHQSKFVVYDSMFDNFSINFWVRTPKYNNNDIQTYLQNEYTIISCIKN
DSGWKVSIKGNRIIWTLIDVNAKSKSIFFEYSIKDNISDYINKWFSITITNDRLG
NANIYINGSLKKSEKILNLDRINSSNDIDFKLINCTDTTKFVWIKDFNIFGRELN
ATEVSSLYWIQSSTNTLKDFWGNPLRYDTQYYLFNQGMQNIYIKYFSKASMG

ETAPRTNFNNAAINYQNLYLGLRFIIKKASNSRNINNDNIVREGDYIYLNIDNIS
DESYRVYVLVNSKEIQTQLFLAPINDDPTFYDVLQIKKYYEKTTYNCQILCEK
DTKTFGLFGIGKFVKDYGYVWDTYDNYFCISQWYLRRISENINKLRLGCNWQ
FIPVDEGWTE

- SEQ ID NO: 8 - TeNT - UniProtKB Accession Number P04958 (*Clostridium tetani)*

MPITINNFRYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGT
KPEDFNPPSSLIEGASEYYDPNYLRTDSDKDRFLQTMVKLFNRIKNNVAGEAL
LDKIINAIPYLGNSYSLLDKFDTNSNSVSFNLLEQDPSGATTKSAMLTNLIIFGP
GPVLNKNEVRGIVLRVDNKNYFPCRDGFGSIMQMAFCPEYVPTFDNVIENITS
LTIGKSKYFQDPALLLMHELIHVLHGLYGMQVSSHEIIPSKQEIYMQHTYPISA
EELFTFGGQDANLISIDIKNDLYEKTLNDYKAIANKLSQVTSCNDPNIDIDSYK
QIYQQKYQFDKDSNGQYIVNEDKFQILYNSIMYGFTEIELGKKFNIKTRLSYFS
MNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMRVNTNAFRNVDGS
GLVSKLIGLCKKIIPPTNIRENLYNRTASLTDLGGELCIKIKNEDLTFIAEKNSFS
EEPFQDEIVSYNTKNKPLNFNYSLDKIIVDYNLQSKITLPNDRTTPVTKGIPYA
PEYKSNAASTIEIHNIDDNTIYQYLYAQKSPTTLQRITMTNSVDDALINSTKIYS
YFPSVISKVNQGAQGILFLQWVRDIIDDFTNESSQKTTIDKISDVSTIVPYIGPA
LNIVKQGYEGNFIGALETTGVVLLLEYIPEITLPVIAALSIAESSTQKEKIIKTID
NFLEKRYEKWIEVYKLVKAKWLGTVNTQFQKRSYQMYRSLEYQVDAIKKII
DYEYKIYSGPDKEQIADEINNLKNKLEEKANKAMININIFMRESSRSFLVNQMI
NEAKKQLLEFDTQSKNILMQYIKANSKFIGITELKKLESKINKVFSTPIPFSYSK
NLDCWVDNEEDIDVILKKSTILNLDINNDIISDISGFNSSVITYPDAQLVPGING
KAIHLVNNESSEVIVHKAMDIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTN
EYSIISSMKKHSLSIGSGWSVSLKGNNLIWTLKDSAGEVRQITFRDLPDKFNA
YLANKWVFITITNDRLSSANLYINGVLMGSAEITGLGAIREDNNITLKLDRCN
NNNQYVSIDKFRIFCKALNPKEIEKLYTSYLSITFLRDFWGNPLRYDTEYYLIP
VASSSKDVQLKNITDYMYLTNAPSYTNGKLNIYYRRLYNGLKFIIKRYTPNNE
IDSFVKSGDFIKLYVSYNNNEHIVGYPKDGNAFNNLDRILRVGYNAPGIPLYK
KMEAVKLRDLKTYSVQLKLYDDKNASLGLVGTHNGQIGNDPNRDILIASNW
YFNHLKDKILGCDWYFVPTDEGWTND

- SEQ ID NO: 9 - VAMP1_Rat (Q63666)

MSAPAQPPAEGTEGAAPGGGPPGPPPNTTSNRRLQQTQAQVEEVVDIMRVNV
DKVLERDQKLSELDDRADALQAGASVFESSAAKLKRKYWWKNCKMMIML
GAICAIIVVVIVIYIFT

- SEQ ID NO: 10 - VAMP1_human (P23763)

MSAPAQPPAEGTEGTAPGGGPPGPPPNMTSNRRLQQTQAQVEEVVDIIRVNV
DKVLERDQKLSELDDRADALQAGASQFESSAAKLKRKYWWKNCKMMIML
GAICAIIVVVIVIYFFT

- SEQ ID NO: 11 - VAMP2_Rat (P63045)

MSATAATVPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVD
KVLERDQKLSELDDRADALQAGASQFETSAAKLKRKYWWKNLKMMIILGVI
CAIILIIIIVYFST

- SEQ ID NO: 12 - VAMP2_human (P63027)

MSATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVD
KVLERDQKLSELDDRADALQAGASQFETSAAKLKRKYWWKNLKMMIILGVI
CAIILIIIIVYFST

- SEQ ID NO: 13 - VAMP3_Rat (P63025)

MSTGVPSGSSAATGSNRRLQQTQNQVDEVVDIMRVNVDKVLERDQKLSELD
DRADALQAGASQFETSAAKLKRKYWWKNCKMWAIGISVLVIIVIIIIVWCVS

- SEQ ID NO: 14 - VAMP3_human (Q15836)

MSTGPTAATGSNRRLQQTQNQVDEVVDIMRVNVDKVLERDQKLSELDDRA
DALQAGASQFETSAAKLKRKYWWKNCKMWAIGITVLVIFIIIIIVWVVSS

- SEQ ID NO: 15 – VAMP epitope
  KLSELDDRADALQ

- SEQ ID NO: 16 – VAMP epitope
  QKLSELDDRADALQ

- SEQ ID NO: 17 – VAMP epitope KLSELDDRAD

- SEQ ID NO: 18 – VAMP epitope KLSELDDRADALQAGAS

- SEQ ID NO: 19 – VAMP epitope LSELDDRADALQ

- SEQ ID NO: 20 – VAMP epitope LSELDDRADA

- SEQ ID NO: 21 – VAMP epitope LSELDDRADALQAGAS

- SEQ ID NO: 22 – VAMP epitope FETSAAKLKRKYW

- SEQ ID NO: 23 – VAMP epitope FESSAAKLKRKYW

- SEQ ID NO: 24 – VAMP epitope QFETSAAKLKRKYW

- SEQ ID NO: 25 – VAMP epitope FETSAAKLKR

- SEQ ID NO: 26 – VAMP epitope FETSAAKLKRKYWWKN

- SEQ ID NO: 27 – VAMP epitope AKLKRKYWWKN

- SEQ ID NO: 28 – VAMP epitope
  AAKLKRKYWWKN

- SEQ ID NO: 29 – VAMP epitope
  AKLKRKYWWKNCKM

- SEQ ID NO: 30 – VAMP epitope
  AKLKRKYWWKNLKM

- SEQ ID NO: 31 – VAMP epitope
  DQKLSELDDRADALQ

- SEQ ID NO: 32 – VAMP epitope
  ERDQKLSELDDRA

- SEQ ID NO: 33 – VAMP epitope
  LERDQKLSELDDRA

- SEQ ID NO: 34 – VAMP epitope
  VLERDQKLSELDDRA

- SEQ ID NO: 35- LH_ND

MGSMTWPVKDFNYSDPVNDNDILYLRIPQNKLITTPVKAFMITQNIWVIPERF
SSDTNPSLSKPPRPTSKYQSYYDPSYLSTDEQKDTFLKGIIKLFKRINERDIGKK
LINYLVVGSPFMGDSSTPEDTFDFTRHTTNIAVEKFENGSWKVTNIITPSVLIFG
PLPNILDYTASLTLQGQQSNPSFEGFGTLSILKVAPEFLLTFSDVTSNQSSAVLG
KSIFCMDPVIALMHELTHSLHQLYGINIPSDKRIRPQVSEGFFSQDGPNVQFEE
LYTFGGLDVEIIPQIERSQLREKALGHYKDIAKRLNNINKTIPSSWISNIDKYKK
IFSEKYNFDKDNTGNFVVNIDKFNSLYSDLTNVMSEVVYSSQYNVKNRTHYF
SRHYLPVFANILDDNIYTIRDGFNLTNKGFNIENSGQNIERNPALQKLSSESVV
DLFTKVCVDKSEEKLYDDDDKDRWGSSLQCIKVKNNRLPYVADKDSISQEIF
ENKIITDETNVQNYSDKFSLDESILDGQVPINPEIVDPLLPNVNMEPLNLPGEEI

VFYDDITKYVDYLNSYYYLESQKLSNNVENITLTTSVEEALGYSNKIYTFLPS
LAEKVNKGVQAGLFLNWANEVVEDFTTNIMKKDTLDKISDVSVIIPYIGPALN
IGNSALRGNFNQAFATAGVAFLLEGFPEFTIPALGVFTFYSSIQEREKIIKTIENC
LEQRVKRWKDSYQWMVSNWLSRITTQFNHINYQMYDSLSYQADAIKAKIDL
EYKKYSGSDKENIKSQVENLKNSLDVKISEAMNNINKFIRECSVTYLFKNMLP
KVIDELNKFDLRTKTELINLIDSHNIILVGEVDRLKAKVNESFENTMPFNIFSYT
NNSLLKDIINEYFNLEAHHHHHHHHHH

- SEQ ID NO: 36 - MBP-LF

MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVA
ATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKL
IAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFT
WPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNAD
TDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFV
GVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEEL
AKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALK
DAQTNSSSNNNNNNNNNNLGIEGRISEFGSMPVAINSFNYNDPVNDDTILYM
QIPYEEKSKKYYKAFEIMRNVWIIPERNTIGTNPSDFDPPASLKNGSSAYYDPN
YLTTDAEKDRYLKTTIKLFKRINSNPAGKVLLQEISYAKPYLGNDHTPIDEFSP
VTRTTSVNIKLSTNVESSMLLNLLVLGAGPDIFESCCYPVRKLIDPDVVYDPSN
YGFGSINIVTFSPEYEYTFNDISGGHNSSTESFIADPAISLAHELIHALHGLYGA
RGVTYEETIEVKQAPLMIAEKPIRLEEFLTFGGQDLNIITSAMKEKIYNNLLAN
YEKIATRLSEVNSAPPEYDINEYKDYFQWKYGLDKNADGSYTVNENKFNEIY
KKLYSFTESDLANKFKVKCRNTYFIKYEFLKVPNLLDDDIYTVSEGFNIGNLA
VNNRGQSIKLNPKIIDSIPDKGLVEKIVKFAVDKLAAALEHHHHHH

- SEQ ID NO :37 (recombinant VAMP2-GFP)

GPLGSSATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRV
NVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKRKYWWKNLKLEN
VSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGK
LPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDD
GNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMA

DKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSA
LSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

- SEQ ID NO: 38 - recombinant BoNT/FA

MPVVINSFNYDDPVNDNTIIYIRPPYYETSNTYFKAFQIMDNVWIIPERYRLGI
DPSLFNPPVSLKAGSDGYFDPNYLSTNTEKNKYLQIMIKLFKRINSKPAGQILL
EEIKNAIPYLGNSYTQEEQFTTNNRTVSFNVKLANGNIVQQMANLIIWGPGPD
LTTNKTGGIIYSPYQSMEATPYKDGFGSIMTVEFSPEYATAFNDISIASHSPSLF
IKDPALILMHELIHVLHGLYGTYITEYKITPNVVQSYMKVTKPITSAEFLTFGG
RDRNIVPQSIQSQLYNKVLSDYKRIASRLNKVNTATALINIDEFKNLYEWKYQ
FAKDSNGVYSVDLNKFEQLYKKIYSFTEFNLAYEFKIKTRLGYLAENFGPFYL
PNLLDDSIYTEVDGFNIGALSINYQGQNIGSDINSIKKLQGQGVVSRVVRLCKS
VIPRKGTKAPPRLCITVNNRDLFFIASQESYGENTINTYKEIDDTTTLDPSFEDI
LDKVILNFNEQVIPQMPNRNVSTDIQKDNYIPKYDYNRTDIIDSYEVGRNYNT
FFYLNAQKFSPNESNITLTSSFDTGLLEGSKVYTFFSSDFINNINKPVQALLFIE
WVKQVIRDFTTEATKTSTVDKLKDISLVVPYIGLALNIGDEIYKQHFAEAVEL
VGAGLLLEFSPEFLIPTLLIFTIKGYLTGSIRDKDKIIKTLDNALNVRDQKWKEL
YRWVVSKWLTTINTQFNKRKEQMYKALKNQATAIKKIIENKYNNYTTDEKS
KIDSSYNINEIERTLNEKINLAMKNIEQFITESSIAYLINIINNETIQKLKSYDDLV
RRYLLGYIRNHSSILGNSVEELNSKVNNHLDNGIPFELSSYTNDSLLIRYFNKN
YGELKYNCILNIKYEMDRDKLVDSSGYRSRINIGTGVKFSEIDKNQVQLSNLE
SSKIEVILNNGVIYNSMYENFSTSFWIRIPKYFRNINNEYKIISCMQNNSGWEV
SLNFSNMNSKIIWTLQDTEGIKKTVVFQYTQNINISDYINRWIFVTITNNRLSNS
KIYINGRLINEESISDLGNIHASNNIMFKLDGCRDPHRYIWIKYFNLFDKELNK
KEIKDLYDNQSNSGILKDFWGDYLQYDKPYYMLNLYDPNKYLDVNNVGIRG
YMYLKGPRGRIVTTNIYLNSTLYMGTKFIIKKYASGNKDNIVRNNDRVYINV
VVKNKEYRLATNASQAGVEKILSAVEIPDVGNLSQVVVMKSENDQGIRNKC
KMNLQDNNGNDIGFIGFHQFNNIAKLVASNWYNRQIGKASRTFGCSWEFIPV
DDGWGESSLHHHHHHHHHH

- SEQ ID NO: 39 – Pep1
SNRRLQQTQAQVDEC

- SEQ ID NO: 40 – Pep3
  CLQAGASQ

- SEQ ID NO:41 – BoNT/X Genbank Accession Number BAQ12790 (*Clostridium botulinum*)

MKLEINKFNYNDPIDGINVITMRPPRHSDKINKGKGPFKAFQVIKNIWIVPERY
NFTNNTNDLNIPSEPIMEADAIYNPNYLNTPSEKDEFLQGVIKVLERIKSKPEG
EKLLELISSSIPLPLVSNGALTLSDNETIAYQENNNIVSNLQANLVIYGPGPDIA
NNATYGLYSTPISNGEGTLSEVSFSPFYLKPFDESYGNYRSLVNIVNKFVKREF
APDPASTLMHELVHVTHNLYGISNRNFYYNFDTGKIETSRQQNSLIFEELLTF
GGIDSKAISSLIIKKIIETAKNNYTTLISERLNTVTVENDLLKYIKNKIPVQGRLG
NFKLDTAEFEKKLNTILFVLNESNLAQRFSILVRKHYLKERPIDPIYVNILDDN
SYSTLEGFNISSQGSNDFQGQLLESSYFEKIESNALRAFIKICPRNGLLYNAIYR
NSKNYLNNIDLEDKKTTSKTNVSYPCSLLNGCIEVENKDLFLISNKDSLNDINL
SEEKIKPETTVFFKDKLPPQDITLSNYDFTEANSIPSISQQNILERNEELYEPIRN
SLFEIKTIYVDKLTTFHFLEAQNIDESIDSSKIRVELTDSVDEALSNPNKVYSPF
KNMSNTINSIETGITSTYIFYQWLRSIVKDFSDETGKIDVIDKSSDTLAIVPYIGP
LLNIGNDIRHGDFVGAIELAGITALLEYVPEFTIPILVGLEVIGGELAREQVEAI
VNNALDKRDQKWAEVYNITKAQWWGTIHLQINTRLAHTYKALSRQANAIK
MNMEFQLANYKGNIDDKAKIKNAISETEILLNKSVEQAMKNTEKFMIKLSNS
YLTKEMIPKVQDNLKNFDLETKKTLDKFIKEKEDILGTNLSSSLRRKVSIRLNK
NIAFDINDIPFSEFDDLINQYKNEIEDYEVLNLGAEDGKIKDLSGTTSDINIGSDI
ELADGRENKAIKIKGSENSTIKIAMNKYLRFSATDNFSISFWIKHPKPTNLLNN
GIEYTLVENFNQRGWKISIQDSKLIWYLRDHNNSIKIVTPDYIAFNGWNLITIT
NNRSKGSIVYVNGSKIEEKDISSIWNTEVDDPIIFRLKNNRDTQAFTLLDQFSIY
RKELNQNEVVKLYNYYFNSNYIRDIWGNPLQYNKKYYLQTQDKPGKGLIRE
YWSSFGYDYVILSDSKTITFPNNIRYGALYNGSKVLIKNSKKLDGLVRNKDFI
QLEIDGYNMGISADRFNEDTNYIGTTYGTTHDLTTDFEIIQRQEKYRNYCQLK
TPYNIFHKSGLMSTETSKPTFHDYRDWVYSSAWYFQNYENLNLRKHTKTNW
YFIPKDEGWDED

- SEQ ID NO: 42 - VAMP4_Rat (D4A560)

MPPKFKRHLNDDDVTGSVKSERRNLLEDDSDEEEDFFLRGPSGPRFGPRNDKI
KHVQNQVDEVIDVMQENITKVIERGERLDELQDKSESLSDNATAFSNRSKQLR
RQMWWRGCKIKAIMALAAAILLLMIITQIILHLKK

- SEQ ID NO: 43 - VAMP4_human (O75379)

MPPKFKRHLNDDDVTGSVKSERRNLLEDDSDEEEDFFLRGPSGPRFGPRNDKI
KHVQNQVDEVIDVMQENITKVIERGERLDELQDKSESLSDNATAFSNRSKQLR
RQMWWRGCKIKAIMALVAAILLLVIIILIVMKYRT

- SEQ ID NO: 44 - VAMP5_Rat (Q9Z2J5)

MAGKELERCQRQADQVTEIMLNNFDKVLERDGKLSELQQRSDQLLDMSSAFS
KTTKTLAQQKRWENIRCRVYLGLAVAGGLLLILVVLLVIFLPSGEDSSKP

- SEQ ID NO: 45 - VAMP5_human (O95183)

MAGIELERCQQQANEVTEIMRNNFGKVLERGVKLAELQQRSDQLLDMSSTFN
KTTQNLAQKKCWENIRYRICVGLVVVGVLLIILIVLLVVFLPQSSDSSSAPRTQ
DAGIASGPGN

- SEQ ID NO: 46 - YKT6_Rat (Q5EGY4)

MKLYSLSVFYKGEPKAVLLKAAYDVSSFSFFQRSSVQEFMTFTSQLIVERSAK
GSRASVKEQEYLCHVYVRSDSLAGVVIADSEYPSRVAFTLLEKVLDEFSKQVD
RIDWPVGSPATIHYTALDGHLSRYQNPREADPMSKVQAELDETKIILHNTMES
LLERGEKLDDLVSKSEVLGTQSKAFYKTARKQNSCCAIM

- SEQ ID NO: 47 -YKT6_human (O15498)

MKLYSLSVLYKGEAKVVLLKAAYDVSSFSFFQRSSVQEFMTFTSQLIVERSSK
GTRASVKEQDYLCHVYVRNDSLAGVVIADNEYPSRVAFTLLEKVLDEFSKQV
DRIDWPVGSPATIHYPALDGHLSRYQNPREADPMTKVQAELDETKIILHNTME
SLLERGEKLDDLVSKSEVLGTQSKAFYKTARKQNSCCAIM

- SEQ ID NO: 48 - VAMP epitope ETSAAKLKRKYWWK

- SEQ ID NO: 49 - VAMP epitope FETSAAKLKRKYWWK

- SEQ ID NO: 50 -VAMP epitope QFESSAAKLKRKYW

- SEQ ID NO: 51 -VAMP epitope FESSAAKLKR

- SEQ ID NO: 52 -VAMP epitope FESSAAKLKRKYWWK

- SEQ ID NO: 53 -VAMP epitope ADALQAGASQF

- SEQ ID NO: 54 -VAMP epitope ADALQAGASQ

- SEQ ID NO: 55 -VAMP epitope RADALQAGASQF

- SEQ ID NO: 56-VAMP epitope ADALQAGASQFE

- SEQ ID NO: 57-VAMP epitope ADALQAGASVF

- SEQ ID NO: 58-VAMP epitope ADALQAGASV

- SEQ ID NO: 59-VAMP epitope ADALQAGASVFE

- SEQ ID NO: 60 -VAMP epitope RADALQAGASVF

- SEQ ID NO: 61 -VAMP epitope RADALQAGAS

- SEQ ID NO: 62 -VAMP epitope SESLSDNATAF

- SEQ ID NO: 63 -VAMP epitope SESLSDNATA

- SEQ ID NO: 64 -VAMP epitope KSESLSDNATAF

- SEQ ID NO: 65 -VAMP epitope SESLSDNATAFS

- SEQ ID NO: 66 -VAMP epitope SDQLLDMSSTF

- SEQ ID NO: 67 -VAMP epitope SDQLLDMSST

- SEQ ID NO: 68 -VAMP epitope RSDQLLDMSSTF

- SEQ ID NO: 69 -VAMP epitope SDQLLDMSSTFN

- SEQ ID NO: 70 -VAMP epitope SDQLLDMSSAF

- SEQ ID NO: 71 -VAMP epitope SDQLLDMSSA

- SEQ ID NO: 72 -VAMP epitope RSDQLLDMSSAF

- SEQ ID NO: 73 -VAMP epitope SDQLLDMSSAFS

- SEQ ID NO: 74 -VAMP epitope RSDQLLDMSS

- SEQ ID NO: 75 -VAMP epitope SEVLGTQSKAF

- SEQ ID NO: 76 -VAMP epitope SEVLGTQSKA

- SEQ ID NO: 77 -VAMP epitope KSEVLGTQSKAF

- SEQ ID NO: 78 -VAMP epitope SEVLGTQSKAFY

CLAIMS

1. An antigenic polypeptide comprising a VAMP epitope, wherein said antigenic polypeptide consists of 10 to 65 amino acid residues, wherein said VAMP epitope comprises an amino acid sequence which is at least 90% identical to a VAMP sequence comprising at least 8 amino acid residues which are immediately C-terminal to a clostridial neurotoxin cleavage site in said VAMP.

2. The antigenic polypeptide of claim 1, wherein said polypeptide consists of 10 to 17 amino acid residues, preferably 10 to 16 amino acid residues, more preferably 10 to 15 amino acid residues.

3. The antigenic polypeptide according to claim 1 or 2, wherein said VAMP is selected from VAMP1, VAMP2, VAMP3, VAMP4, VAMP5 and/or YKT6.

4. The antigenic polypeptide according to any one of claims 1 to 3, wherein said VAMP epitope comprises or consists of an amino acid sequence which is at least 90% identical to a VAMP sequence selected from: SEQ ID NO: 15 to SEQ ID NO: 34, and SEQ ID NO: 48 to SEQ ID NO: 78.

5. The antigenic polypeptide according to any one of claims 1 to 4, wherein said VAMP epitope is a VAMP1, VAMP2 and/or VAMP3 epitope and is selected from:
   - a BoNT/F or BoNT/D VAMP epitope comprising or consisting of KLSELDDRADALQ (SEQ ID NO: 15);
   - a BoNT/F5 or BoNT/FA VAMP epitope comprising or consisting of ERDQKLSELDDRA (SEQ ID NO: 32);
   - a BoNT/B or TeNT VAMP epitope comprising or consisting of FETSAAKLKRKYW (SEQ ID NO: 22) or FETSAAKLKRKYWWK (SEQ ID NO: 49);
   - a BoNT/G VAMP epitope comprising or consisting of AKLKRKYWWKN (SEQ ID NO: 27); and

- a BoNT/X VAMP epitope comprising or consisting of ADALQAGASQF (SEQ ID NO: 53).

6. The antigenic polypeptide according to any one of claims 1 to 4, wherein said VAMP epitope is a VAMP4, VAMP5 and/or YKT6 epitope and is selected from:
   - a BoNT/X VAMP epitope comprising or consisting of SESLSDNATAF (SEQ ID NO: 62), SDQLLDMSSTF (SEQ ID NO: 66), or SEVLGTQSKAF (SEQ ID NO: 75).

7. A polypeptide comprising an antigenic polypeptide of any one of the preceding claims, wherein the polypeptide does not comprise a region of greater than 17, preferably 16, more preferably 15 consecutive amino acids having 100% sequence identity to a naturally-occurring VAMP amino acid sequence.

8. An antigenic protein comprising a polypeptide according to any one of claims 1 to 6, or a polypeptide according to claim 7, covalently linked to a carrier.

9. Use of an antigenic polypeptide according to any one of claims 1 to 6, or a polypeptide according to claim 7, or an antigenic protein according to claim 8, to generate antibodies against a C-terminal VAMP cleavage product.

10. An antibody that binds to an antigenic polypeptide according to any one of claim 1 to 6, or to a polypeptide according to claim 7, or to an antigenic protein according to claim 8.

11. The antibody according to claim 10, wherein said antibody is a polyclonal antibody.

12. The antibody according to claim 10, wherein said antibody is a monoclonal antibody.

13. The antibody according to any one of claims 10 to 12, wherein the K_D between said antibody and said epitope is lower than 10^{-7} M.

14. Use of an antibody according to any one of claims 10 to 13 in a gain of signal cellular assay for VAMP cleavage by a VAMP cleaving clostridial neurotoxin.

15. A method for determining cleavage of VAMP by a VAMP cleaving clostridial neurotoxin in a cell, comprising:
    a. contacting the cell with the clostridial neurotoxin under conditions suitable for clostridial neurotoxin activity;
    b. contacting the cytoplasmic content of said cell with a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to any one of claims 10 to 13; and
    c. detecting by a suitable means the binding of said first detection antibody to the C-terminal VAMP cleavage product.

16. The method according to claim 15, further comprising d) quantifying by a suitable means the amount of the C-terminal VAMP cleavage product bound to said first detection antibody.

17. A method for determining immunoresistance to a VAMP cleaving clostridial neurotoxin in a subject, comprising:
    a. adding a VAMP cleaving clostridial neurotoxin to a test sample obtained from the subject;
    b. contacting a cell with the test sample of step a) under conditions suitable for clostridial neurotoxin activity;
    c. contacting the cytoplasmic content of said cell with a first detection antibody against the C-terminal VAMP cleavage product following cleavage of a VAMP by the VAMP cleaving clostridial neurotoxin under conditions suitable for the binding of the first detection antibody to the C-terminal VAMP cleavage product, wherein said first detection antibody is an antibody according to any one of claims 10 to 13;

d. detecting by a suitable means the binding of the first detection antibody to the C-terminal VAMP cleavage product;
e. quantifying the amount of the C-terminal VAMP cleavage product bound to the first detection antibody;
f. repeating steps a) to e) with a negative control sample instead of a test sample; and
g. comparing the amount of the C-terminal VAMP cleavage product bound to said first detection antibody in steps (e) and (f), wherein detection of a lower amount of the C-terminal VAMP cleavage product bound to said first detection antibody in step (e) relative to the amount of the C-terminal VAMP cleavage product bound to said first detection antibody in step (f) is indicative of the presence of neutralizing antibodies against the VAMP cleaving clostridial neurotoxin.

18. A kit comprising a cell which is susceptible to intoxication by a VAMP cleaving neurotoxin; and a first detection antibody against cleaved VAMP, wherein said first detection antibody is an antibody according to any one of claims 10 to 13.

WO 2018/073288

Figure 1A

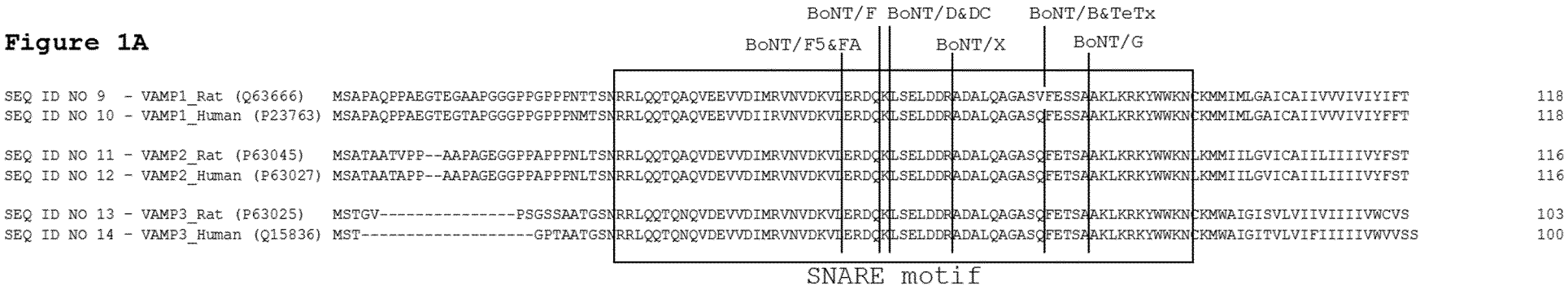
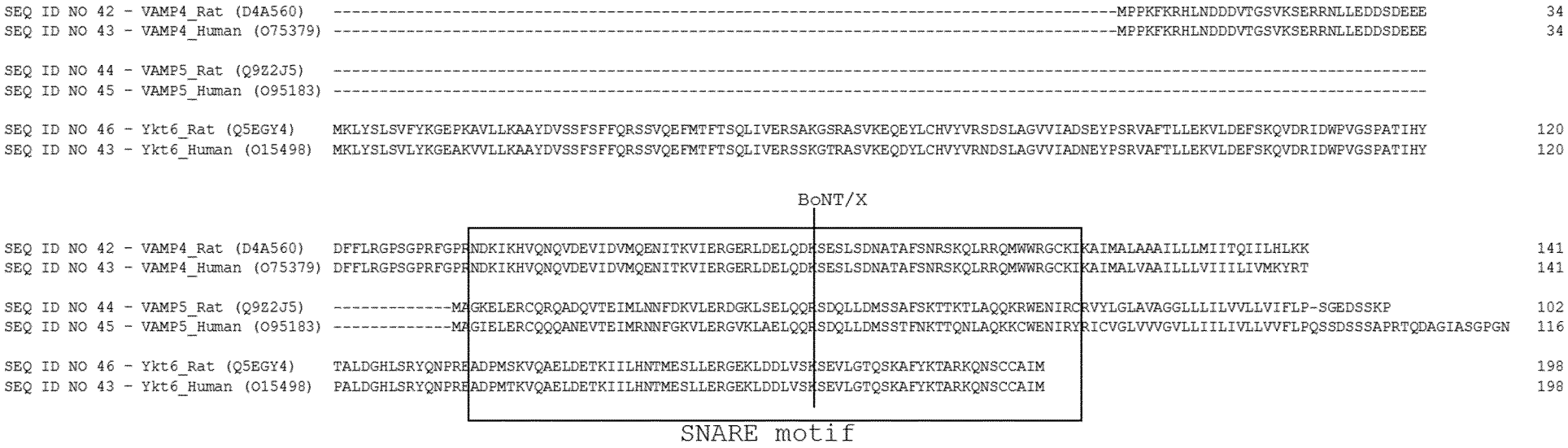


Figure 1B



PCT/EP2017/076569

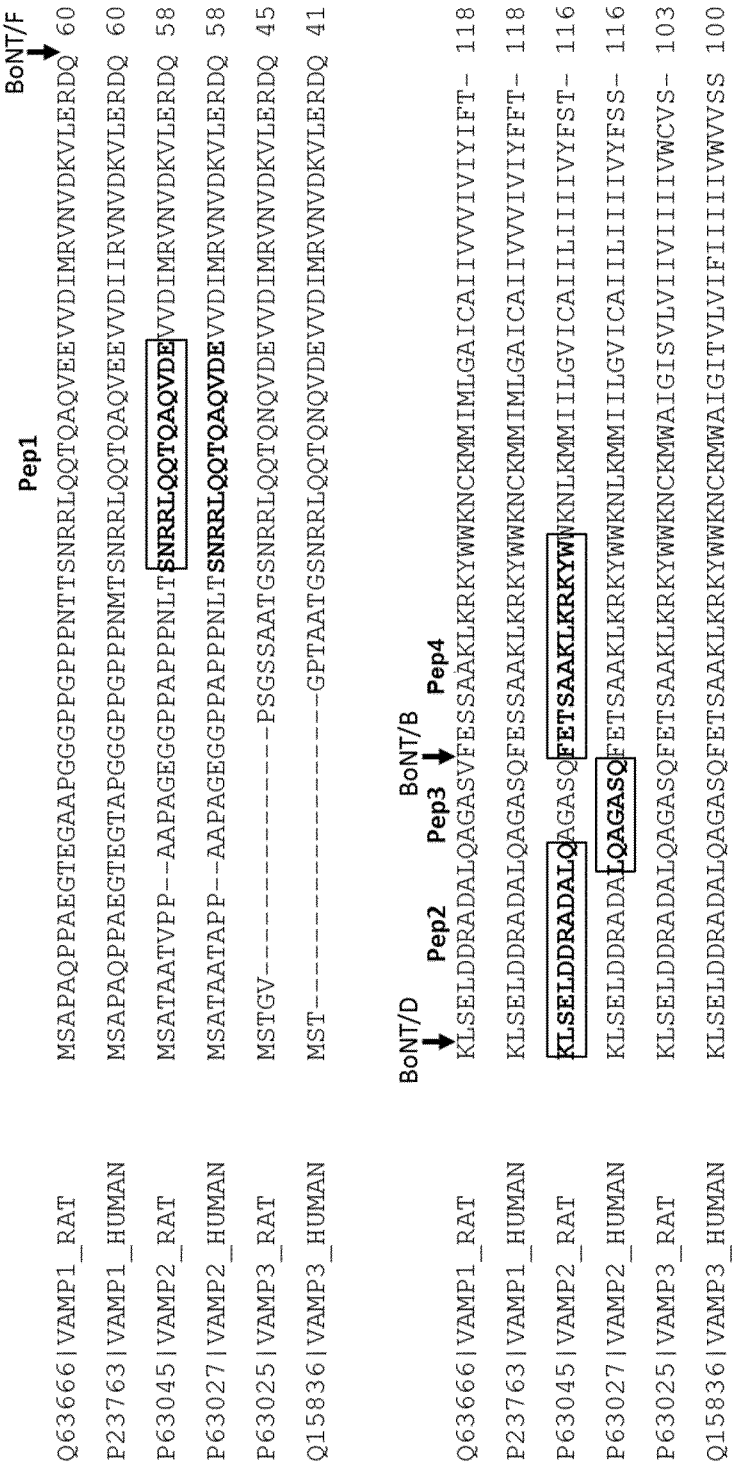

Pep1
BoNT/F
Q63666|VAMP1_RAT MSAPAQPPAEGTEGAAPGGGPPGPPPNTTSNRRLQQTQAQVEEVVDIMRVNVDKVLERDQ 60
P23763|VAMP1_HUMAN MSAPAQPPAEGTEGTAPGGGPPGPPPNMTSNRRLQQTQAQVEEVVDIIRVNVDKVLERDQ 60
P63045|VAMP2_RAT MSATAATVPP--AAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQ 58
P63027|VAMP2_HUMAN MSATAATAPP--AAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQ 58
P63025|VAMP3_RAT MSTGV----------PSGSSAATGSNRRLQQTQNQVDEVVDIMRVNVDKVLERDQ 45
Q15836|VAMP3_HUMAN MST----------GPTAATGSNRRLQQTQNQVDEVVDIMRVNVDKVLERDQ 41
BoNT/D
Pep2
Pep3
BoNT/B
Pep4
Q63666|VAMP1_RAT KLSELDDRADALQAGASVFESSAAKLKRKYWWKNCKMMIMLGAICAIIVVVIVIYIFT- 118
P23763|VAMP1_HUMAN KLSELDDRADALQAGASQFESSAAKLKRKYWWKNCKMMIMLGAICAIIVVVIVIYFFT- 118
P63045|VAMP2_RAT KLSELDDRADALQAGASQFETSAAKLKRKYWWKNLKMMIILGVICAIILIIIIVYFST- 116
P63027|VAMP2_HUMAN KLSELDDRADALQAGASQFETSAAKLKRKYWWKNLKMMIILGVICAIILIIIIVYFSS- 116
P63025|VAMP3_RAT KLSELDDRADALQAGASQFETSAAKLKRKYWWKNCKMWAIGISVLVIVIIIIVWCVS- 103
Q15836|VAMP3_HUMAN KLSELDDRADALQAGASQFETSAAKLKRKYWWKNCKMWAIGITVLVIFIIIIVWVVSS 100

FIGURE 2

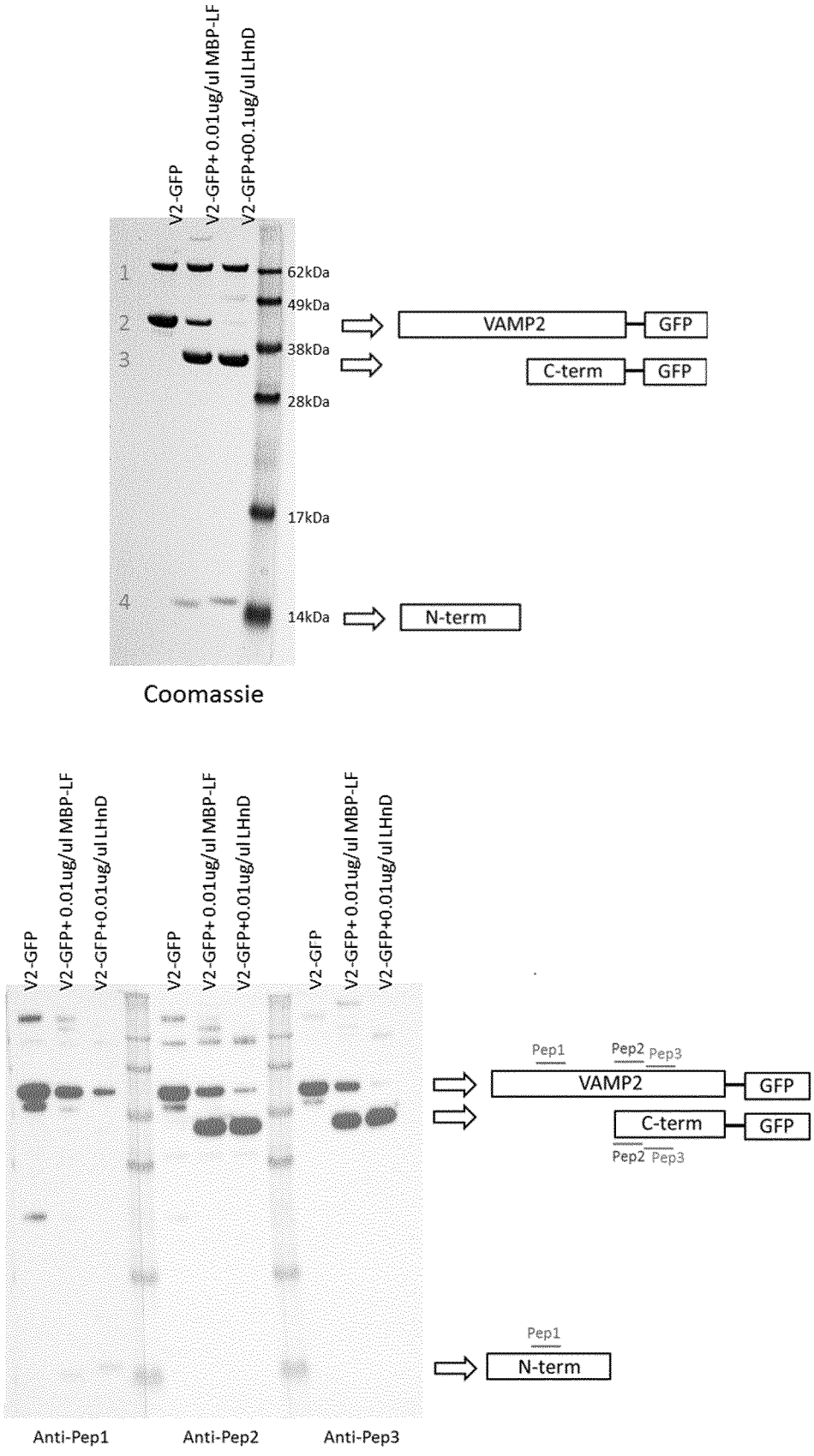

V2-GFP
V2-GFP+ 0.01ug/ul MBP-LF
V2-GFP+00.1ug/ul LHnD
1
2
3
4
62kDa
49kDa
38kDa
28kDa
17kDa
14kDa
VAMP2
GFP
C-term
GFP
N-term
Coomassie
V2-GFP
V2-GFP+ 0.01ug/ul MBP-LF
V2-GFP+0.01ug/ul LHnD
Pep1
Pep2
Pep3
VAMP2
GFP
C-term
GFP
Pep2
Pep3
Pep1
N-term
Anti-Pep1
Anti-Pep2
Anti-Pep3

FIGURE 3

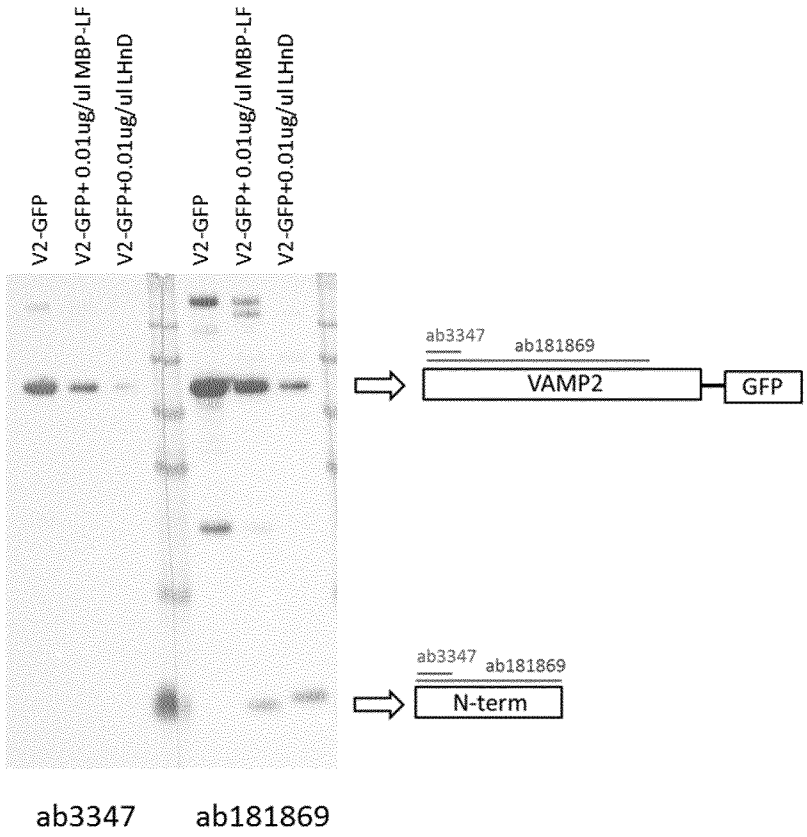
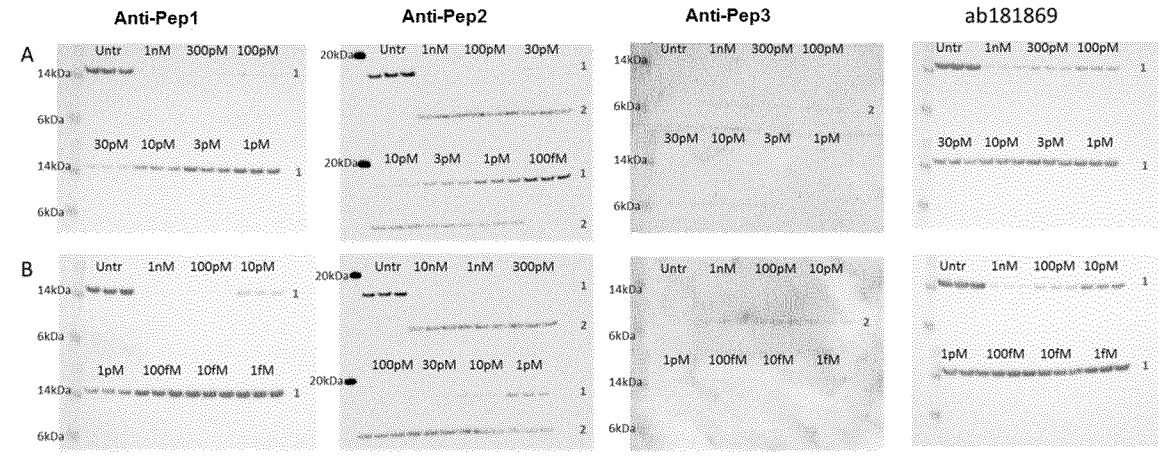
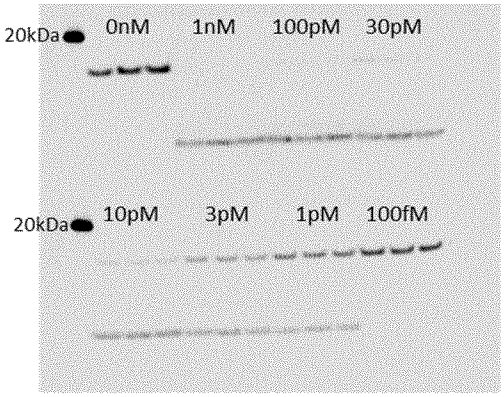
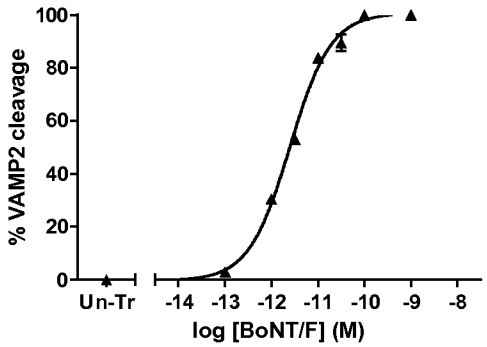


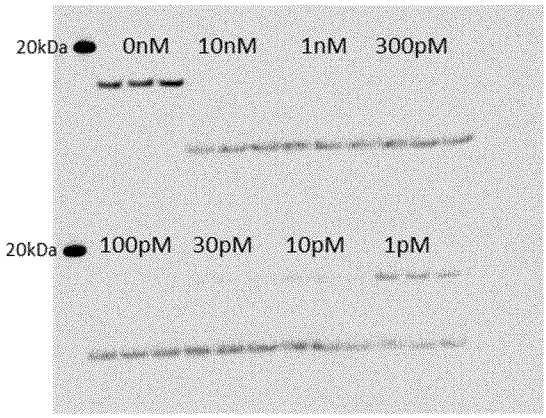
FIGURE 3 (continued)


Anti-Pep1
Anti-Pep2
Anti-Pep3
ab181869
A
B
Untr 1nM 300pM 100pM
30pM 10pM 3pM 1pM
Untr 1nM 100pM 30pM
10pM 3pM 1pM 100fM
Untr 1nM 100pM 10pM
1pM 100fM 10fM 1fM
Untr 10nM 1nM 300pM
100pM 30pM 10pM 1pM
14kDa
6kDa
20kDa


20kDa
0nM 1nM 100pM 30pM
20kDa
10pM 3pM 1pM 100fM

C


% VAMP2 cleavage
100
80
60
40
20
0
Un-Tr
-14 -13 -12 -11 -10 -9 -8
log [BoNT/F] (M)


20kDa
0nM 10nM 1nM 300pM
20kDa
100pM 30pM 10pM 1pM

D

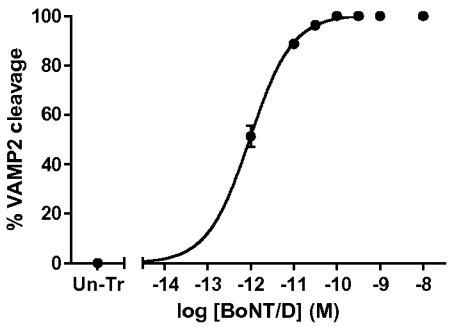
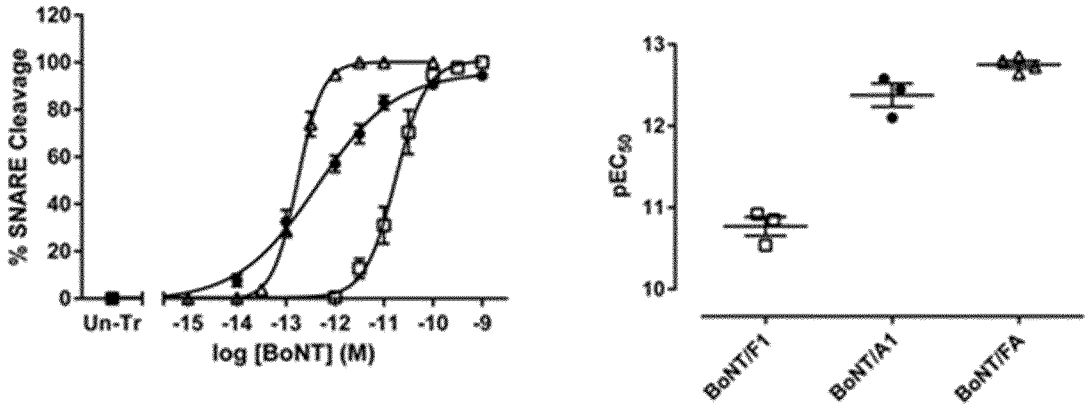

% VAMP2 cleavage
100
80
60
40
20
0
Un-Tr
-14 -13 -12 -11 -10 -9 -8
log [BoNT/D] (M)

FIGURE 4



Toxin	pEC_{50}	*n*
BoNT/A1	12.38 ± 0.14	3
BoNT/F1	10.77 ± 0.12	3
BoNT/FA	12.75 ± 0.09	4

FIGURE 5

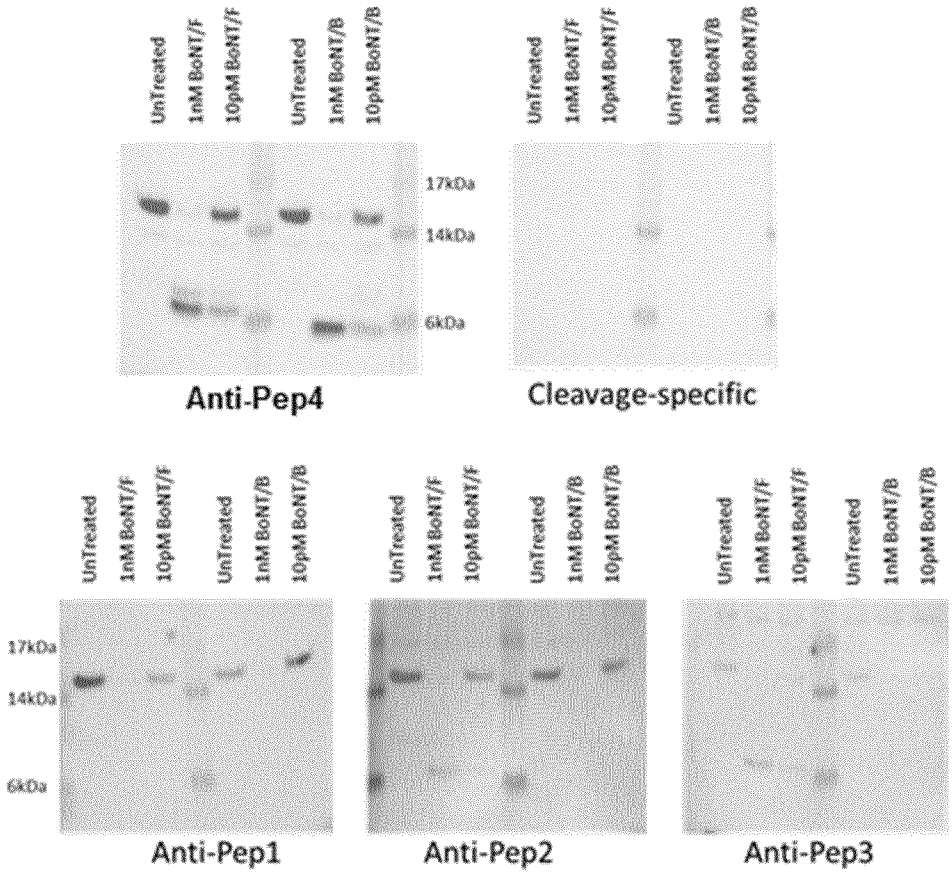

UnTreated
1nM BoNT/F
10pM BoNT/F
UnTreated
1nM BoNT/B
10pM BoNT/B
17kDa
14kDa
6kDa
Anti-Pep4
Cleavage-specific
Anti-Pep1
Anti-Pep2
Anti-Pep3

FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/076569

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/37 G01N33/569
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q G01N C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOGHADDAM M M ET AL: "Cloning and expression of a region of vesicle associated membrane protein2 (VAMP2) gene and its use as a recombinant peptide substrate for assaying clostridial neurotoxins in contaminated biologicals", BIOLOGICALS, ACADEMIC PRESS LTD., LONDON, GB, vol. 38, no. 1, 1 January 2010 (2010-01-01), pages 113-119, XP026978062, ISSN: 1045-1056 [retrieved on 2010-01-01]	1,3-5, 10-12
Y	page 115, left-hand column, paragraph 4; figures 4,6 page 116, right-hand column, paragraph 3	2,7-9, 13-18

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 March 2018	03/04/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Wiesner, Martina

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 763 131 A1 (MICROBIOLOGICAL RES AUTHORITY [GB]) 19 March 1997 (1997-03-19) paragraph [0027] - paragraph [0029]; figure 1; sequence 11 paragraph [0032] -----	1,3-5, 10-12 2,7-9, 13-18
Y	US 2012/164657 A1 (JOHNSON ERIC A [US] ET AL) 28 June 2012 (2012-06-28) paragraph [0119] - paragraph [0121]; figure 13 paragraph [0159] - paragraph [0160] paragraph [0173] - paragraph [0176] -----	2,7-9, 13-18
Y	THOMAS BINZ ET AL: "Clostridial Neurotoxins: Mechanism of SNARE Cleavage and Outlook on Potential Substrate Specificity Reengineering", TOXINS, vol. 2, no. 4, 13 April 2010 (2010-04-13), pages 665-682, XP055357445, DOI: 10.3390/toxins2040665 figure 2 -----	2,7-9, 13-18
Y	STEFAN SIKORRA ET AL: "Substrate Recognition Mechanism of VAMP/Synaptobrevin-cleaving Clostridial Neurotoxins", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 283, no. 30, 25 July 2008 (2008-07-25), pages 21145-21152, XP055441234, ISSN: 0021-9258, DOI: 10.1074/jbc.M800610200 figure 2 -----	2,7-9, 13-18
A	HALLIS B ET AL: "Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities", JOURNAL OF CLINICAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 34, no. 8, 1 August 1996 (1996-08-01), pages 1934-1938, XP002976172, ISSN: 0095-1137 page 1935, left-hand column, paragraph 2 - right-hand column, paragraph 2; figures 1,2 ----- -/--	1-5,7-18

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WICTOME M ET AL: "Development of an in vitro bioassay for Clostridium botulinum type B neurotoxin in foods that is more sensitive than the mouse bioassay", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 65, no. 9, 1 September 1999 (1999-09-01), pages 3787-3792, XP002314355, ISSN: 0099-2240 page 3788, left-hand column, paragraph 3 - right-hand column, paragraph 2 page 3789, right-hand column, paragraph 3 - page 3790, left-hand column, paragraph 2 -----	1-5,7-18
A	KEGEL ET AL: "An in vitro assay for detection of tetanus neurotoxin activity: Using antibodies for recognizing the proteolytically generated cleavage product", TOXICOLOGY IN VITRO, ELSEVIER SCIENCE, GB, vol. 21, no. 8, 12 November 2007 (2007-11-12), pages 1641-1649, XP022340114, ISSN: 0887-2333, DOI: 10.1016/J.TIV.2007.06.015 page 1643, left-hand column, paragraph 3; figures 1-4 -----	1-5,7-18
A	SUZANNE R KALB ET AL: "Discovery of a novel enzymatic cleavage site for botulinum neurotoxin F5", FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 586, no. 2, 28 November 2011 (2011-11-28), pages 109-115, XP028439969, ISSN: 0014-5793, DOI: 10.1016/J.FEBSLET.2011.11.033 [retrieved on 2011-12-09] the whole document -----	1-5,7-18

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/076569

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-5, 7-18(all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-5, 7-18(all partially)

An antigenic polypeptide comprising a VAMP epitope, wherein said antigenic polypeptide consists of 10 to 65 amino acid residues, wherein said VAMP epitope comprises an amino acid sequence which is at least 90% identical to a VAMP sequence comprising at least 8 amino acid residues which are immediately C-terminal to a clostridial neurotoxin cleavage site in said VAMP, wherein said VAMP epitope comprises or consists of an amino acid sequence which is at least 90% identical to VAMP sequence SEQ ID NO: 15.

2-6. claims: 1-5, 7-18(all partially)

An antigenic polypeptide comprising a VAMP epitope, wherein said antigenic polypeptide consists of 10 to 65 amino acid residues, wherein said VAMP epitope comprises an amino acid sequence which is at least 90% identical to a VAMP sequence comprising at least 8 amino acid residues which are immediately C-terminal to a clostridial neurotoxin cleavage site in said VAMP, wherein said VAMP epitope comprises or consists of an amino acid sequence which is at least 90% identical to a VAMP sequence selected from SEQ ID NO: 22, 27, 32, 49, and 53.

7-9. claims: 1-4, 6-18(all partially)

An antigenic polypeptide comprising a VAMP epitope, wherein said antigenic polypeptide consists of 10 to 65 amino acid residues, wherein said VAMP epitope comprises an amino acid sequence which is at least 90% identical to a VAMP sequence comprising at least 8 amino acid residues which are immediately C-terminal to a clostridial neurotoxin cleavage site in said VAMP, wherein said VAMP epitope comprises or consists of an amino acid sequence which is at least 90% identical to a VAMP sequence selected from SEQ ID NO: 62, 66, and 75.

10-51. claims: 1-4, 7-18(all partially)

An antigenic polypeptide comprising a VAMP epitope, wherein said antigenic polypeptide consists of 10 to 65 amino acid residues, wherein said VAMP epitope comprises an amino acid sequence which is at least 90% identical to a VAMP sequence comprising at least 8 amino acid residues which are immediately C-terminal to a clostridial neurotoxin cleavage site in said VAMP, wherein said VAMP epitope comprises or consists of an amino acid sequence which is at least 90% identical to a VAMP sequence selected from SEQ ID NO: 16-21,

23-26, 28-31, 33, 34, 48, 50-52, 54-61, 63-65, 67-74, and 76-78.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/076569

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0763131 A1	19-03-1997	AT 183779 T	15-09-1999
		AU 687564 B2	26-02-1998
		CA 2191895 A1	14-12-1995
		DE 69511693 D1	30-09-1999
		DE 69511693 T2	09-03-2000
		DK 0763131 T3	13-12-1999
		EP 0763131 A1	19-03-1997
		JP 4246259 B2	02-04-2009
		JP H10504801 A	12-05-1998
		WO 9533850 A1	14-12-1995
US 2012164657 A1	28-06-2012	NONE	