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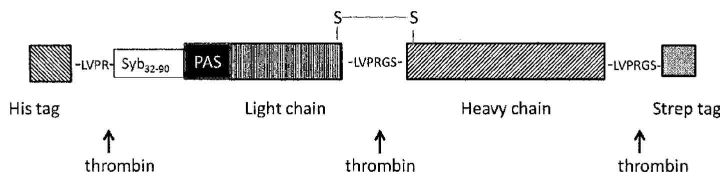
(54) **Title**: NOVEL RECOMBINANT CLOSTRIDIAL NEUROTOXINS WITH INCREASED DURATION OF EFFECT

Figure 1:



(57) **Abstract**: This invention relates to novel recombinant clostridial neurotoxins exhibiting increased duration of effect and to methods for the manufacture of such recombinant clostridial neurotoxins. These novel recombinant clostridial neurotoxins comprise a SNARE complex-binding sequence, and the methods comprise the steps of inserting a nucleic acid sequence coding for a SNARE complex-binding sequence into a nucleic acid sequence coding for a parental clostridial neurotoxin and expression of the recombinant nucleic acid sequence comprising the SNARE complex-binding sequence in a host cell. The invention further relates to novel recombinant single- chain precursor clostridial neurotoxins used in such methods, nucleic acid sequences encoding such recombinant single-chain precursor clostridial neurotoxins, and pharmaceutical compositions comprising the recombinant clostridial neurotoxin with increased duration of effect.

NOVEL RECOMBINANT CLOSTRIDIAL NEUROTOXINS WITH INCREASED DURATION OF EFFECT

FIELD OF THE INVENTION

[0001] This invention relates to novel recombinant clostridial neurotoxins exhibiting increased duration of effect and to methods for the manufacture of such recombinant clostridial neurotoxins. These novel recombinant clostridial neurotoxins comprise a SNARE complex-binding sequence, and the methods comprise the steps of inserting a nucleic acid sequence coding for a SNARE complex-binding sequence into a nucleic acid sequence coding for a parental clostridial neurotoxin and expression of the recombinant nucleic acid sequence comprising the SNARE complex-binding sequence in a host cell. The invention further relates to novel recombinant single-chain precursor clostridial neurotoxins used in such methods, nucleic acid sequences encoding such recombinant single-chain precursor clostridial neurotoxins, and pharmaceutical compositions comprising the recombinant clostridial neurotoxin with increased duration of effect.

BACKGROUND OF THE INVENTION

[0002] Clostridium is a genus of anaerobe gram-positive bacteria, belonging to the Firmicutes. Clostridium consists of around 100 species that include common free-living bacteria as well as important pathogens, such as *Clostridium botulinum* and *Clostridium tetani*. Both species produce neurotoxins, botulinum toxin and tetanus toxin, respectively. These neurotoxins are potent inhibitors of calcium-dependent neurotransmitter secretion of neuronal cells and are among the strongest toxins known to man. The lethal dose in humans lies between 0.1 ng and 1 ng per kilogram of body weight.

[0003] Oral ingestion of botulinum toxin via contaminated food or generation of botulinum toxin in wounds can cause botulism, which is characterised by paralysis of various muscles. Paralysis of the breathing muscles can cause death of the affected individual.

[0004] Although both botulinum neurotoxin (BoNT) and tetanus neurotoxin (TxNT) function via a similar initial physiological mechanism of action, inhibiting neurotransmitter release from the axon of the affected neuron into the synapse, they differ in their clinical response. While the botulinum toxin acts at the neuromuscular junction and other cholinergic synapses in the peripheral nervous system, inhibiting the release of the neurotransmitter acetylcholine and thereby causing flaccid paralysis, the tetanus toxin acts mainly in the central nervous system, preventing the release of the inhibitory neurotransmitters GABA (gamma-aminobutyric acid) and glycine by degrading the protein synaptobrevin. The consequent overactivity in the muscles results in generalized contractions of the agonist and antagonist musculature, termed a tetanic spasm (rigid paralysis).

[0005] While the tetanus neurotoxin exists in one immunologically distinct type, the botulinum neurotoxins are known to occur in seven different immunogenic types, termed BoNT/A through BoNT/H. Most *Clostridium botulinum* strains produce one type of neurotoxin, but strains producing multiple toxins have also been described.

[0006] Botulinum and tetanus neurotoxins have highly homologous amino acid sequences and show a similar domain structure. Their biologically active form comprises two peptide chains, a light chain of about 50 kDa and a heavy chain of about 100 kDa, linked by a disulfide bond. A linker or loop region, whose length varies among different clostridial toxins, is located between the two cysteine residues forming the disulfide bond. This loop region is proteolytically cleaved by an unknown clostridial endoprotease to obtain the biologically active toxin.

[0007] The molecular mechanism of intoxication by TxNT and BoNT appears to be similar as well: entry into the target neuron is mediated by binding of the C-terminal part of the heavy chain to a specific cell surface receptor; the toxin is then taken up

by receptor-mediated endocytosis. The low pH in the so formed endosome then triggers a conformational change in the clostridial toxin which allows it to embed itself in the endosomal membrane and to translocate through the endosomal membrane into the cytoplasm, where the disulfide bond joining the heavy and the light chain is reduced. The light chain can then selectively cleave so called SNARE-proteins, which are essential for different steps of neurotransmitter release into the synaptic cleft, e.g. recognition, docking and fusion of neurotransmitter-containing vesicles with the plasma membrane. TxNT, BoNT/B, BoNT/D, BoNT/F, and BoNT/G cause proteolytic cleavage of synaptobrevin or VAMP (vesicle-associated membrane protein), BoNT/A and BoNT/E cleave the plasma membrane-associated protein SNAP-25, and BoNT/C cleaves the integral plasma membrane protein syntaxin and SNAP-25.

[0008] Clostridial neurotoxins display variable durations of action that are serotype specific. The clinical therapeutic effect of BoNT/A lasts approximately 3 months for neuromuscular disorders and 6 to 12 months for hyperhidrosis. The effects of BoNT/E, on the other hand, last 4 – 6 weeks. The longer lasting therapeutic effect of BoNT/A makes it preferable for clinical use compared to the other serotypes. One possible explanation for the divergent durations of action might be the distinct subcellular localizations of BoNT serotypes. The protease domain of BoNT/A light chain localizes in a punctate manner to the plasma membrane of neuronal cells, co-localizing with its substrate SNAP-25. In contrast, the short-duration BoNT/E serotype is cytoplasmic. Membrane association might protect BoNT/A from cytosolic degradation mechanisms allowing for prolonged persistence of BoNT/A in the neuronal cell.

[0009] In *Clostridium botulinum*, the botulinum toxin is formed as a protein complex comprising the neurotoxic component and non-toxic proteins. The accessory proteins embed the neurotoxic component thereby protecting it from degradation by digestive enzymes in the gastrointestinal tract. Thus, botulinum neurotoxins of most serotypes are orally toxic. Complexes with, for example, 450 kDa or with 900 kDa are obtainable from cultures of *Clostridium botulinum*.

[0010] In recent years, botulinum neurotoxins have been used as therapeutic agents in the treatment of dystonias and spasms. Preparations comprising botulinum toxin complexes are commercially available, e.g. from Ipsen Ltd (Dysport®) or Allergan Inc. (Botox®). A high purity neurotoxic component, free of any complexing proteins, is for example available from Merz Pharmaceuticals GmbH, Frankfurt (Xeomin®).

[0011] Clostridial neurotoxins are usually injected into the affected muscle tissue, bringing the agent close to the neuromuscular end plate, i.e. close to the cellular receptor mediating its uptake into the nerve cell controlling said affected muscle. Various degrees of neurotoxin spread have been observed. The neurotoxin spread is thought to depend on the injected amount and the particular neurotoxin preparation. It can result in adverse side effects such as paralysis in nearby muscle tissue, which can largely be avoided by reducing the injected doses to the therapeutically relevant level. Overdosing can also trigger the immune system to generate neutralizing antibodies that inactivate the neurotoxin preventing it from relieving the involuntary muscle activity. Immunologic tolerance to botulinum toxin has been shown to correlate with cumulative doses.

[0012] At present, clostridial neurotoxins are still predominantly produced by fermentation processes using appropriate Clostridium strains. However, industrial production of clostridial neurotoxin from anaerobic Clostridium culture is a cumbersome and time-consuming process. Due to the high toxicity of the final product, the procedure must be performed under strict containment. During the fermentation process, the single-chain precursors are proteolytically cleaved by an unknown clostridial protease to obtain the biologically active di-chain clostridial neurotoxin. The degree of neurotoxin activation by proteolytic cleavage varies between different strains and neurotoxin serotypes, which is a major consideration for the manufacture due to the requirement of neurotoxin preparations with a well-defined biological activity. Furthermore, during fermentation processes using Clostridium strains the clostridial neurotoxins are produced as protein complexes, in which the neurotoxic component is embedded by accessory proteins. These accessory proteins have no beneficial effect on biological activity or duration of effect. They can however trigger an immune reaction in the patient, resulting in immunity

against the clostridial neurotoxin. Manufacture of recombinant clostridial neurotoxins, which are not embedded by auxiliary proteins, might therefore be advantageous.

[0013] Methods for the recombinant expression of clostridial neurotoxins in *E. coli* are well known in the art (see, for example, WO 00/12728, WO 01/14570, or WO 2006/076902). Furthermore, clostridial neurotoxins have been expressed in eukaryotic expression systems, such as in *Pichia pastoris*, *Pichia methanolica*, *Saccharomyces cerevisiae*, insect cells and mammalian cells (see WO 2006/017749).

[0014] Recombinant clostridial neurotoxins may be expressed as single-chain precursors, which subsequently have to be proteolytically cleaved to obtain the final biologically active clostridial neurotoxin. Thus, clostridial neurotoxins may be expressed in high yield in rapidly-growing bacteria as relatively non-toxic single-chain polypeptides.

[0015] Furthermore, it might be advantageous to modify clostridial neurotoxin characteristics regarding biological activity, cell specificity, antigenic potential and duration of effect by genetic engineering to obtain recombinant neurotoxins with new therapeutic properties in specific clinical areas. Genetic modification of clostridial neurotoxins might allow altering the mode of action or expanding the range of therapeutic targets.

[0016] WO 96/39166 discloses analogues of botulinum toxin comprising amino acid residues which are more resistant to degradation in neuromuscular tissue.

[0017] Patent family based on WO 02/08268 (including family member US 6,903,187) discloses a clostridial neurotoxin comprising a structural modification selected from addition or deletion of a leucine-based motif, which alters the biological persistence of the neurotoxin (see also: Fernández-Salas et al., Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 3208-3213; Wang et al., J. Biol. Chem. 286 (2011) 6375-6385). Fernández-Salas et al. initially hypothesized that the increased persistence was due to the membrane-binding properties of the dileucine motif (see Fernández-Salas et al., loc.

cit., p. 3211 and 3213). Wang et al. mention this membrane theory (see Wang et al., loc. cit., p. 6376, left column, last full paragraph, and p. 6383, first full paragraph of "Discussion"), but favor an alternative theory: the protection from degradation by proteolysis (see Wang et al., loc. cit., p. 6384, left column, lines 27ff).

[0018] US 2002/0127247 describes clostridial neurotoxins comprising modifications in secondary modification sites and exhibiting altered biological persistence.

[0019] Botulinum toxin variants exhibiting longer biological half lives in neuromuscular tissue than naturally occurring botulinum toxins would be advantageous in order to reduce administration frequency and the incidence of neutralising antibody generation since immunologic tolerance to botulinum toxin is correlated with cumulative doses.

[0020] Furthermore, BoNT serotypes naturally exhibiting a short duration of action could potentially be effectively used in clinical applications, if their biological persistence could be enhanced. Modified BoNT/E with an increased duration of action could potentially be used in patients exhibiting an immune reaction against BoNT/A. Moreover, BoNT/E was shown to induce a more severe block of pain mediator release from sensory neurons than BoNT/A. In clinical applications where BoNT/A provides only partial pain relief or in just a subset of patients, such as in the treatment of headaches, or where BoNT/E has been found to be more effective than BoNT/A but gives only short-term therapy, such as in the treatment of epilepsy, BoNT/E with an increased duration of effect might prove useful.

[0021] There is a strong demand to produce clostridial neurotoxins with an increased duration of effect, in order to allow for reduction of administration frequency and exploitation of the therapeutic potential of BoNT serotypes, which have so far been considered impractical for clinical application due to the short half-life of the respective clinically relevant effect. Ideally, the duration of effect of a particular clostridial neurotoxin could be adjusted in a tailor-made fashion in order to address any particular features and demands of a given indication, such as the amount of

neurotoxin being administered, frequency of administration etc. To date, such aspects have not been solved satisfactorily.

OBJECTS OF THE INVENTION

[0022] It was an object of the invention to provide recombinant clostridial neurotoxins exhibiting an increased duration of effect and to establish a reliable and accurate method for manufacturing and obtaining such recombinant clostridial neurotoxins. Such a method and novel precursor clostridial neurotoxins used in such methods would serve to satisfy the great need for recombinant clostridial neurotoxins exhibiting an increased duration of effect.

SUMMARY OF THE INVENTION

[0023] The naturally occurring botulinum toxin serotypes display highly divergent durations of effect, probably due to their distinct subcellular localization. BoNT/A exhibiting the longest persistence was shown to localize in the vicinity of the plasma membrane of neuronal cells, whereas the short-duration BoNT/E serotype is cytosolic. However, additional factors such as degradation, diffusion, and/or translocation rates might have an decisive impact on the differences in the duration of effect for the individual botulinum toxin serotypes.

[0024] So far, no generally applicable method for modifying clostridial neurotoxins in order to increase their duration of effect is available. Surprisingly, it has been found that recombinant clostridial neurotoxins having such effects can be obtained by cloning a sequence encoding a SNARE complex-binding sequence into a gene encoding a parental clostridial neurotoxin, and by subsequent heterologous expression of the generated construct in recombinant host cells.

[0025] Thus, in a first aspect, the present invention relates to a recombinant clostridial neurotoxin comprising a SNARE complex-binding sequence derived from a SNARE complex-related protein.

[0026] In another aspect, the present invention relates to a pharmaceutical composition comprising the recombinant clostridial neurotoxin of the present invention.

[0027] In yet another aspect, the present invention relates to the use of the composition of the present invention for cosmetic treatment.

[0028] In another aspect, the present invention relates to a method for the generation of the recombinant clostridial neurotoxin of the present invention, comprising the step of obtaining a recombinant nucleic acid sequence encoding a recombinant single-chain precursor clostridial neurotoxin by the insertion of a nucleic acid sequence encoding said SNARE complex-binding sequence into a nucleic acid sequence encoding a parental clostridial neurotoxin.

[0029] In another aspect, the present invention relates to a recombinant single-chain precursor clostridial neurotoxin comprising a SNARE complex-binding sequence.

[0030] In another aspect, the present invention relates to a nucleic acid sequence encoding the recombinant single-chain precursor clostridial neurotoxin of the present invention.

[0031] In another aspect, the present invention relates to a method for obtaining the nucleic acid sequence of the present invention, comprising the step of inserting a nucleic acid sequence encoding a SNARE complex-binding sequence into a nucleic acid sequence encoding a parental clostridial neurotoxin.

[0032] In another aspect, the present invention relates to a vector comprising the nucleic acid sequence of the present invention, or the nucleic acid sequence obtainable by the method of the present invention.

[0033] In another aspect, the present invention relates to a recombinant host cell comprising the nucleic acid sequence of the present invention, the nucleic acid sequence obtainable by the method of the present invention, or the vector of the present invention.

[0034] In another aspect, the present invention relates to a method for producing the recombinant single-chain precursor clostridial neurotoxin of the present invention, comprising the step of expressing the nucleic acid sequence of the present invention, or the nucleic acid sequence obtainable by the method of the present invention, or the vector of the present invention in a recombinant host cell, or cultivating the recombinant host cell of the present invention under conditions that result in the expression of said nucleic acid sequence.

FIGURES

[0035] **Figure 1** shows a schematic view of construct Syb-PAS-BoNT/A comprising a SNARE-based sequence comprising residues 32-90 of Synaptobrevin-2 ("VAMP") with a His-tag linked to a thrombin cleavage site at the N-terminus of the motif and a 52 amino acid long PAS linker at the C-terminus, which is inserted at the N -terminus of recombinant BoNT/A (rBoNT/A).

[0036] **Figure 2** shows the results of the purification and activation of construct Syb-PAS-BoNT/A: **column 1** (left column): single-chain Syb-PAS-BoNT/A before cleavage with thrombin; **column 2**: Syb-PAS-BoNT/A after cleavage with thrombin; column 3: recombinant wild-type BoNT/A after cleavage with thrombin; **column 4**: molecular weight markers.

[0037] **Figure 3** shows the results of a Digital Abduction Score ("DAS") assay as described in Example 2 using activated Syb-PAS-BoNT/A (■: 2.5 pg; ▲: 5 pg; •: 10 pg) in comparison to Xeomin (▼: 0.6 U).

[0038] **Figure 4** shows a schematic view of construct C-term Syn BoNT/A comprising a SNARE-based sequence comprising residues 186-212 of Syntaxin inserted at the C-terminus of the light chain (Lc) of BoNT/A followed by an AGAG linker and a thrombin cleavage site, which was fused to the N-terminus of the heavy chain of BoNT/A via an AGAG linker.

[0039] **Figure 5** shows the results of the purification and activation of construct C-term Syn BoNT/A: **column 1** (left column): molecular weight markers; **column 2**: single-chain C-term Syn BoNT/A before cleavage with thrombin; **column 3**: C-term Syn BoNT/A after cleavage with thrombin; **column 4**: recombinant wild-type BoNT/A after cleavage with thrombin.

[0040] **Figure 6** shows the results of a Digital Abduction Score ("DAS") assay as described in Example 4 using activated C-term Syn BoNT/A (■: 2.5 pg; ▲ (lower curve): 5 pg; ●: 10 pg) in comparison to Xeomin (▲ (upper curve): 0.6 U).

DETAILED DESCRIPTION OF THE INVENTION

[0041] The present invention may be understood more readily by reference to the following detailed description of the invention and the examples included therein.

[0042] In a first aspect, the present invention relates to a recombinant clostridial neurotoxin comprising a SNARE complex-binding sequence derived from a SNARE complex-related protein.

[0043] In the context of the present invention, the term "clostridial neurotoxin" refers to a natural neurotoxin obtainable from bacteria of the class Clostridia, including *Clostridium tetani* and *Clostridium botulinum*, or to a neurotoxin obtainable from alternative sources, including from recombinant technologies or from genetic or chemical modification. Particularly, the clostridial neurotoxins have endopeptidase activity.

[0044] Clostridial neurotoxins are produced as single-chain precursors that are proteolytically cleaved by an unknown clostridial endoprotease within the loop region to obtain the biologically active disulfide-linked di-chain form of the neurotoxin, which comprises two chain elements, a functionally active light chain and a functionally active heavy chain, where one end of the light chain is linked to one end of the heavy chain not via a peptide bond, but via a disulfide bond.

[0045] In the context of the present invention, the term "clostridial neurotoxin light chain" refers to that part of a clostridial neurotoxin that comprises an endopeptidase activity responsible for cleaving one or more proteins that is/are part of the so-called SNARE complex involved in the process resulting in the release of neurotransmitter into the synaptic cleft: In naturally occurring clostridial neurotoxins, the light chain has a molecular weight of approx. 50 kDa.

[0046] In the context of the present invention, the term "clostridial neurotoxin heavy chain" refers to that part of a clostridial neurotoxin that is responsible for entry of the neurotoxin into the neuronal cell: In naturally occurring clostridial neurotoxins, the heavy chain has a molecular weight of approx. 100 kDa.

[0047] In the context of the present invention, the term "functionally active clostridial neurotoxin chain" refers to a recombinant clostridial neurotoxin chain able to perform the biological functions of a naturally occurring *Clostridium botulinum* neurotoxin chain to at least about 50%, particularly to at least about 60%, to at least about 70%, to at least about 80%, and most particularly to at least about 90%, where the biological functions of clostridial neurotoxin chains include, but are not limited to, binding of the heavy chain to the neuronal cell, entry of the neurotoxin into a neuronal cell, release of the light chain from the di-chain neurotoxin, and endopeptidase activity of the light chain. Methods for determining a neurotoxic activity can be found, for example, in WO 95/32738, which describes the reconstitution of separately obtained light and heavy chains of tetanus toxin and botulinum toxin.

[0048] In the context of the present invention, the term “about” or “approximately” means within 20%, alternatively within 10%, including within 5% of a given value or range. Alternatively, especially in biological systems, the term “about” means within about a log (i.e. an order of magnitude), including within a factor of two of a given value.

[0049] In the context of the present invention, the term “recombinant clostridial neurotoxin” refers to a composition comprising a clostridial neurotoxin that is obtained by expression of the neurotoxin in a heterologous cell such as *E. coli*, and including, but not limited to, the raw material obtained from a fermentation process (supernatant, composition after cell lysis), a fraction comprising a clostridial neurotoxin obtained from separating the ingredients of such a raw material in a purification process, an isolated and essentially pure protein, and a formulation for pharmaceutical and/or aesthetic use comprising a clostridial neurotoxin and additionally pharmaceutically acceptable solvents and/or excipients.

[0050] In the context of the present invention, the term “recombinant clostridial neurotoxin” further refers to a clostridial neurotoxin based on a parental clostridial neurotoxin additionally comprising a heterologous SNARE complex-binding sequence, i.e. a SNARE complex-binding sequence that is not naturally occurring in said parental clostridial neurotoxin, in particular a synthetic SNARE complex-binding sequence, or a SNARE complex-binding sequence from a species other than *Clostridium botulinum*, in particular a SNARE complex-binding sequence from a human protein.

[0051] In the context of the present invention, the term “comprises” or “comprising” means “including, but not limited to”. The term is intended to be open-ended, to specify the presence of any stated features, elements, integers, steps or components, but not to preclude the presence or addition of one or more other features, elements, integers, steps, components, or groups thereof. The term “comprising” thus includes the more restrictive terms “consisting of” and “consisting essentially of”.

[0052] In the context of the present invention, the term "SNARE" relates to proteins from a large large protein superfamily consisting of more than 60 members in eukaryotic cells, wherein the term SNARE is an acronym derived from "**S**oluble **N**SF **A**ttachment **P**rotein **Re**ceptor". SNARE proteins are involved in the exocytosis of cellular transport vesicles by mediating vesicle fusion with the cell membrane.

[0053] In the context of the present invention, the term "SNARE motif" relates to a segment in the cytosolic domain of SNARE proteins that consists of 60 to 70 amino acids that are capable of reversibly assembling into tight, four-α-helix bundles called "trans"-SNARE complexes. In human neuronal cells, the "trans" complex consists of three SNARE proteins: Syntaxin-1A and SNAP-25, which are located in the cell membrane, and Synaptobrevin-2, also referred to as vesicle-associated membrane protein 2 (VAMP-2), which is anchored in the vesicular membrane. Syntaxin-1A and Synaptobrevin-2 each contribute one α-helix to the four-α-helix bundle, and SNAP-25 contributes two helices.

[0054] In the context of the present invention, the term "SNARE complex-binding sequence" relates to a sequence that is able to bind to a SNARE complex and that is based on the sequence of a SNARE complex-related protein.

[0055] In the context of the present invention, the term "SNARE complex-related protein" refers to a protein that is either (i) a SNARE protein, or (ii) a protein that is able to bind to a SNARE complex, in particular complexin.

[0056] In particular embodiments, the SNARE complex-related protein is a non-clostridial SNARE complex-related protein. In particular embodiments, the SNARE complex-related protein is selected from: synaptobrevin, particularly synaptobrevin-1 or synaptobrevin-2; syntaxin, particularly syntaxin-1A or syntaxin-4; complexin; SNAP-23; SNAP-25; snapin; endobrevin; and NSF. In particular embodiments, said SNARE complex-related protein is selected from: synaptobrevin-2; syntaxin, particularly syntaxin-1A; and complexin. In particular embodiments, said SNARE complex-related protein is selected from: synaptobrevin-2; syntaxin-1A; and complexin.

[0057] In particular embodiments, said SNARE motif-binding sequence consists of a sequence selected from the group of: (i) a sequence of at least 59 contiguous amino acids comprised in the stretch of amino acid residues from position Leu32 to Trp90 of synaptobrevin-2 (SEQ ID NO: 1); in particular a sequence of at least 26 contiguous amino acids comprised in the stretch of amino acid residues from position Leu32 to Asp57 of synaptobrevin-2; (ii) a sequence of at least 70 contiguous amino acids comprised in the stretch of amino acid residues from position Ser186 to Val255 of syntaxin-1A (SEQ ID NO: 2), in particular a sequence of at least 41 contiguous amino acids comprised in the stretch of amino acid residues from position Ser186 to Gln226 of syntaxin-1A; (iii) a sequence of at least 41 contiguous amino acids comprised in the stretch of amino acid residues from position Lys32 to Ile72 of complexin (SEQ ID NO: 3) in particular a sequence of at least 20 contiguous amino acids comprised in the stretch of amino acid residues from position Ala53 to Ile72 of complexin; and (iv) a mutated sequence obtained from a sequence according to (i) to (iii) by the deletion of a maximum of two amino acid residues of said sequence; the insertion of a maximum of two amino acid residues into said sequence; and/or the conservative exchange of a maximum of three amino acid residues from said sequence, particularly a mutated sequence having a maximum of three, two or one of said deletions, insertions, and/or exchanges, provided that said mutated sequence is still a SNARE complex-binding sequence.

[0058] In particular embodiments, said SNARE complex-binding sequence consists of a fragment sequence of a SNARE protein, particularly synaptobrevin-2; syntaxin-1A; and complexin. The SNARE complex-binding sequence comprises the syntaxin-1A or synaptobrevin-2 fragments of the crystal structure of the cytosolic part of the SNARE complex (PDB entry: 1N7S), or the fragment of complexin of the crystal structure of complexin bound to the cytosolic part of the SNARE complex (PDB entry: 1KIL).

[0059] In particular embodiments, said SNARE complex-binding sequence consists of a fragment of either syntaxin-1A or complexin with an alpha-helical structure of from 90 to 100% helical propensity, particularly a fragment from the N-terminal part of the SNARE domain with a 100% helical propensity, wherein in each case the helical

propensity is determined according to a 8ns Molecular Dynamics (MD) Simulation in explicit water using the algorithm Desmond as implemented in the molecular modelling software Maestro (Schrödinger Release 2013-3: Desmond Molecular Dynamics System, version 3.6, D. E. Shaw Research, New York, NY, 2013. Maestro-Desmond Interoperability Tools, version 3.6, Schrödinger, New York, NY, 2013. See also Kevin J. Bowers et al, "Scalable Algorithms for Molecular Dynamics Simulations on Commodity Clusters," Proceedings of the ACM/IEEE Conference on Supercomputing (SC06), Tampa, Florida, 2006, November 11-17). The MD Simulation starting structures are the pre-fusion solution NMR structure of syntaxin-1A (PDB entry: 2M8R) and the crystal structure of complexin bound to the cytosolic part of the SNARE complex (PDB entry: 1KIL), respectively. The SNARE complex-binding sequence may also comprise a fragment of synaptobrevin-2A preferentially from the N-terminal part of the SNARE domain with an alpha-helical propensity of from 10 to 15% according to a 8ns MD Simulation in explicit water of the lipid-bound Synaptobrevin-2 solution NMR structure (PDB entry: 2KOG) using the algorithm mentioned above.

[0060] In the context of the present invention, the term “helical propensity or helicity” refers to the fraction of helix observed in the molecular dynamics simulation: helicity = number of α-helical configurations / total number of configurations; which refers to the total helical content for a given sequence fragment during the trajectory.

[0061] In particular embodiments, said SNARE complex-related protein is synaptobrevin-2, and wherein said SNARE complex-binding sequence comprises the amino acid sequence QAQVDEVVDIMRVNVDKVL (SEQ ID NO: 4), particularly wherein said SNARE complex-binding sequence comprises a sequence selected from: LQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGA-SQFETSAAKLKRKYWW (SEQ ID NO: 5) and LQQTQAQVDEVVDIM-RVNVDKVLERD (SEQ ID NO: 6).

[0062] In particular embodiments, said SNARE complex-related protein is syntaxin-1A, and wherein said SNARE complex-binding sequence comprises the amino acid sequence SISKQALSEIETRHSEIIKLENSIRE (SEQ ID NO: 7), particularly wherein

said SNARE complex-binding sequence comprises a sequence selected from: SISKQALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQ (SEQ ID NO: 8), and SISKQALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVD-YVERAVSDTKKAV (SEQ ID NO: 9).

[0063] In particular embodiments, said SNARE complex-related protein is complexin, and wherein said SNARE complex-binding sequence comprises the amino acid sequence AKMEAEREVMRQGIRDKYGI (SEQ ID NO: 10), particularly wherein said SNARE complex-binding sequence comprises the sequence KKEEERQEALRQAEE-ERKAKYAKMEAEREVMRQGIRDKYGI (SEQ ID NO: 11).

[0064] In particular embodiments, said SNARE complex-binding sequence is inserted at a position within 200 amino acid residues from the N-terminus of the light chain of a clostridial neurotoxin, particularly wherein said SNARE complex-binding sequence is linked via a PAS linker to the mature N-terminus of said light chain.

[0065] In the context of the present invention, the term "PAS linker" refers to a random coil domain sequence consisting of alanine (A), serine (S) and proline (P) residues. These "PAS" sequences (see, for example, Schlapschy et al., Protein Engineering, Design and Selection 26 (2013) 489-501; EP 2 369 005; WO 2011/144756) have been developed in order to extend the plasma half-life of pharmaceutically active proteins.

[0066] In particular embodiments, said PAS linker is a PAS-52 linker, particularly the PAS linker ASPAAPAPASPAAPAPSAPAASPAAPAPASPAAPAPSAPAASPAAPAP-ASPAA (SEQ ID NO: 12).

[0067] In particular embodiments, said recombinant clostridial neurotoxin comprises a SNARE complex-binding sequence selected from the list of: SEQ ID NO 4 to SEQ ID NO 11, wherein said SNARE complex-binding sequence is fused to the N-terminus of a mature light chain of said clostridial neurotoxin.

[0068] In particular embodiments, said SNARE complex-binding sequence is inserted at a position within 10 amino acid residues from the C-terminus of the light chain of a clostridial neurotoxin, particularly wherein said SNARE complex-binding sequence is inserted in the linker region linking said light chain and the heavy chain of said clostridial neurotoxin.

[0069] In particular embodiments, said recombinant clostridial neurotoxin comprises a sequence, which comprises a SNARE complex-binding sequence, selected from the list of: SEQ ID NO 13 to SEQ ID NO 20, wherein said sequence is linking the mature light chain of a clostridial neurotoxin and the heavy chain of said clostridial neurotoxin.

[0070] In particular embodiments, the sequence of said clostridial neurotoxin is selected from the sequence of (i) a *Clostridium botulinum* neurotoxin serotype A, B, C, D, E, F, G, and H, particularly *Clostridium botulinum* neurotoxin serotype A, C and E, particularly *Clostridium botulinum* neurotoxin serotype A, or (ii) from the sequence of a functional variant of a *Clostridium botulinum* neurotoxin of (i), or (iii) from the sequence of a chimeric *Clostridium botulinum* neurotoxin, wherein the clostridial neurotoxin light chain and heavy chain are from different parental clostridial neurotoxin serotypes.

[0071] In the context of the present invention, the term "*Clostridium botulinum* neurotoxin serotype A, B, C, D, E, F, G, and H" refers to neurotoxins found in and obtainable from *Clostridium botulinum*. Currently, eight serologically distinct types, designated serotypes A, B, C, D, E, F, G, and H are known, including certain subtypes (e.g. A1, A2, A3, A4 and A5).

[0072] In particular embodiments the clostridial neurotoxin is selected from a *Clostridium botulinum* neurotoxin serotype A, C and E, in particular from *Clostridium botulinum* neurotoxin serotype A, or from a functional variant of any such *Clostridium botulinum* neurotoxin.

[0073] As already mentioned above, TxNT, BoNT/B, BoNT/D, BoNT/F, and BoNT/G cause proteolytic cleavage of synaptobrevin or VAMP (vesicle-associated membrane protein), BoNT/A and BoNT/E cleave the plasma membrane-associated protein SNAP-25, and BoNT/C cleaves the integral plasma membrane protein syntaxin and SNAP-25. Accordingly, the choice of the SNARE complex-binding sequence to be used for a given clostridial neurotoxin depends on the protein target that is proteolytically cleaved by such clostridial neurotoxin. For example, SNAP-25 should not be chosen as SNARE complex-related protein in order to derive a SNARE complex-binding sequence in accordance with the present invention for *Clostridium botulinum* neurotoxin serotypes A, C or E.

[0074] In the context of the present invention, the term “functional variant of a clostridial neurotoxin” refers to a neurotoxin that differs in the amino acid sequence and/or the nucleic acid sequence encoding the amino acid sequence from a clostridial neurotoxin, but is still functionally active. In the context of the present invention, the term "functionally active" refers to the property of a recombinant clostridial neurotoxin to exhibit a biological activity of at least about 50%, particularly to at least about 60%, at least about 70%, at least about 80%, and most particularly at least about 90% of the biological activity of a naturally occurring parental clostridial neurotoxin, i.e. a parental clostridial neurotoxin without SNARE complex-binding sequence, where the biological functions include, but are not limited to, binding to the neurotoxin receptor, entry of the neurotoxin into a neuronal cell, release of the light chain from the two-chain neurotoxin, and endopeptidase activity of the light chain, and thus inhibition of neurotransmitter release from the affected nerve cell.

[0075] On the protein level, a functional variant will maintain key features of the corresponding clostridial neurotoxin, such as key residues for the endopeptidase activity in the light chain, or key residues for the attachment to the neurotoxin receptors or for translocation through the endosomal membrane in the heavy chain, but may contain one or more mutations comprising a deletion of one or more amino acids of the corresponding clostridial neurotoxin, an addition of one or more amino acids of the corresponding clostridial neurotoxin, and/or a substitution of one or more amino acids of the corresponding clostridial neurotoxin. Particularly, said deleted,

added and/or substituted amino acids are consecutive amino acids. According to the teaching of the present invention, any number of amino acids may be added, deleted, and/or substituted, as long as the functional variant remains biologically active. For example, 1, 2, 3, 4, 5, up to 10, up to 15, up to 25, up to 50, up to 100, up to 200, up to 400, up to 500 amino acids or even more amino acids may be added, deleted, and/or substituted. Accordingly, a functional variant of the neurotoxin may be a biologically active fragment of a naturally occurring neurotoxin. This neurotoxin fragment may contain an N-terminal, C-terminal, and/or one or more internal deletion(s).

[0076] In another embodiment, the functional variant of a clostridial neurotoxin additionally comprises a signal peptide. Usually, said signal peptide will be located at the N-terminus of the neurotoxin. Many such signal peptides are known in the art and are comprised by the present invention. In particular, the signal peptide results in transport of the neurotoxin across a biological membrane, such as the membrane of the endoplasmic reticulum, the Golgi membrane or the plasma membrane of a eukaryotic or prokaryotic cell. It has been found that signal peptides, when attached to the neurotoxin, will mediate secretion of the neurotoxin into the supernatant of the cells. In certain embodiments, the signal peptide will be cleaved off in the course of, or subsequent to, secretion, so that the secreted protein lacks the N-terminal signal peptide, is composed of separate light and heavy chains, which are covalently linked by disulfide bridges, and is proteolytically active.

[0077] In particular embodiments, the functional variant has in its clostridium neurotoxin part a sequence identity of at least about 40%, at least about 50%, at least about 60%, at least about 70% or most particularly at least about 80%, and a sequence homology of at least about 60%, at least about 70%, at least about 80%, at least about 90%, or most particularly at least about 95% to the corresponding part in the parental clostridial neurotoxin. Methods and algorithms for determining sequence identity and/or homology, including the comparison of variants having deletions, additions, and/or substitutions relative to a parental sequence, are well known to the practitioner of ordinary skill in the art. On the DNA level, the nucleic acid sequences encoding the functional homologue and the parental clostridial neurotoxin may differ

to a larger extent due to the degeneracy of the genetic code. It is known that the usage of codons is different between prokaryotic and eukaryotic organisms. Thus, when expressing a prokaryotic protein such as a clostridial neurotoxin, in a eukaryotic expression system, it may be necessary, or at least helpful, to adapt the nucleic acid sequence to the codon usage of the expression host cell, meaning that sequence identity or homology may be rather low on the nucleic acid level.

[0078] In the context of the present invention, the term "variant" refers to a neurotoxin that is a chemically, enzymatically, or genetically modified derivative of a corresponding clostridial neurotoxin, including chemically or genetically modified neurotoxin from *C. botulinum*, particularly of *C. botulinum* neurotoxin serotype A, C or E. A chemically modified derivative may be one that is modified by pyruvation, phosphorylation, sulfatation, lipidation, pegylation, glycosylation and/or the chemical addition of an amino acid or a polypeptide comprising between 2 and about 100 amino acids, including modification occurring in the eukaryotic host cell used for expressing the derivative. An enzymatically modified derivative is one that is modified by the activity of enzymes, such as endo- or exoproteolytic enzymes, including modification by enzymes of the eukaryotic host cell used for expressing the derivative. As pointed out above, a genetically modified derivative is one that has been modified by deletion or substitution of one or more amino acids contained in, or by addition of one or more amino acids (including polypeptides comprising between 2 and about 100 amino acids) to, the amino acid sequence of said clostridial neurotoxin. Methods for designing and constructing such chemically or genetically modified derivatives and for testing of such variants for functionality are well known to anyone of ordinary skill in the art.

[0079] In particular embodiments, said recombinant clostridial neurotoxin shows increased duration of effect relative to an identical clostridial neurotoxin without said SNARE complex-binding sequence.

[0080] In the context of the present invention, the term "increased duration of effect" or "increased duration of action" refers to a longer lasting denervation mediated by a clostridial neurotoxin of the present invention. For example, as disclosed herein,

administration of a disulfide-linked di-chain clostridial neurotoxin comprising a SNARE complex-binding sequence results in localized paralysis for a longer period of time relative to administration of an identical disulfide-linked di-chain clostridial neurotoxin without the SNARE complex-binding sequence.

[0081] In the context of the present invention, the term “increased duration of effect/action” is defined as a more than about 20%, particularly more than about 50%, more particularly more than about 90% increased duration of effect of the recombinant neurotoxin of the present invention relative to the identical neurotoxin without the SNARE complex-binding sequence.

[0082] In the context of the present invention the term “denervation” refers to denervation resulting from administration of a chemodenervating agent, for example a neurotoxin.

[0083] In the context of the present invention, the term ”localized denervation” or “localized paralysis” refers to denervation of a particular anatomical region, usually a muscle or a group of anatomically and/or physiologically related muscles, which results from administration of a chemodenervating agent, for example a neurotoxin, to the particular anatomical region.

[0084] Without wishing to be bound by theory, the recombinant clostridial neurotoxins of the present invention might show increased biological half-life, reduced degradation rates, decreased diffusion rates, increased uptake by neuronal cells, and/or modified intracellular translocation rates, in each case relative to an identical parental clostridial neurotoxin without said SNARE complex-binding sequence.

[0085] In particular embodiments, the increased duration of effect is due to increased plasma membrane localization.

[0086] In the context of the present invention, the term “plasma membrane localization” refers to the localization, i.e. an increased local concentration, in the vicinity of the plasma membrane, where the proteins of the SNARE complex are set

to mediate the fusion of secretory vesicles with the plasma membrane as a key step of exocytosis. Without wishing to be bound by theory, it can be hypothesized that such increased plasma membrane localization increases the duration of the blocking effect of said recombinant neurotoxin on neurotransmitter release and chemodenervation. Additionally, increased plasma membrane localization may increase the biological half-life of the recombinant clostridial neurotoxin.

[0087] In the context of the present invention, the term "biological half-life" specifies the lifespan of a protein, for example of a clostridial neurotoxin, *in vivo*. In the context of the present invention, the term "biological half-life" refers to the period of time, by which half of a protein pool is degraded *in vivo*. For example it refers to the period of time, by which half of the amount of clostridial neurotoxin of one administered dosage is degraded.

[0088] In the context of the present invention, the term "increased biological half-life" is defined as a more than about 20%, particularly more than about 50%, more particularly more than about 90% increased biological half-life of the recombinant neurotoxin of the present invention relative to the identical neurotoxin without the SNARE complex-binding sequence.

[0089] In the context of the present invention, the term "reduced degradation rate" means that increased plasma membrane localization may protect the recombinant neurotoxin of the present invention against degradation processes in the cytosol of the neuron such as, for example, the attack of proteases or modifying enzymes like E3 ligases. Because of this protection the half-life of the light chain in the neuron may be extended resulting in a longer duration of the therapeutic effect.

[0090] In particular embodiments, the recombinant clostridial neurotoxin is for the use in the treatment of a disease requiring improved chemodenervation, wherein the recombinant clostridial neurotoxin causes longer lasting denervation relative to an identical clostridial neurotoxin without said SNARE complex-binding sequence.

[0091] In particular other embodiments, the recombinant clostridial neurotoxin is for use in the treatment of (a) patients showing an immune reaction against BoNT/A, or (b) headache or epilepsy, wherein the recombinant clostridial neurotoxin is of serotype E.

[0092] In another aspect, the present invention relates to a pharmaceutical composition comprising the recombinant clostridial neurotoxin of the present invention.

[0093] In particular embodiments, the recombinant clostridial neurotoxin of the present invention or the pharmaceutical composition of the present invention is for use in the treatment of a disease or condition taken from the list of: cervical dystonia (spasmodic torticollis), blepharospasm, severe primary axillary hyperhidrosis, achalasia, lower back pain, benign prostate hypertrophy, chronic focal painful neuropathies, migraine and other headache disorders.

[0094] Additional indications where treatment with botulinum neurotoxins is currently under investigation and where the pharmaceutical composition of the present invention may be used, include pediatric incontinence, incontinence due to overactive bladder, and incontinence due to neurogenic bladder, anal fissure, spastic disorders associated with injury or disease of the central nervous system including trauma, stroke, multiple sclerosis, Parkinson's disease, or cerebral palsy, focal dystonias affecting the limbs, face, jaw or vocal cords, temporomandibular joint (TMJ) pain disorders, diabetic neuropathy, wound healing, excessive salivation, vocal cord dysfunction, reduction of the Masseter muscle for decreasing the size of the lower jaw, treatment and prevention of chronic headache and chronic musculoskeletal pain, treatment of snoring noise, assistance in weight loss by increasing the gastric emptying time.

[0095] Most recently, clostridial neurotoxins have been evaluated for the treatment of other new indications, for example painful keloid, diabetic neuropathic pain, refractory knee pain, trigeminal neuralgia trigger-zone application to control pain, scarring after cleft-lip surgery, cancer and depression.

[0096] In yet another aspect, the present invention relates to the use of the composition of the present invention for cosmetic treatment.

[0097] In the context of the present invention, the term "cosmetic treatment" relates to uses in cosmetic or aesthetic applications, such as the treatment of wrinkles, crow's feet, frown lines etc..

[0098] In another aspect, the present invention relates to a method for the generation of the recombinant clostridial neurotoxin of the present invention, comprising the step of obtaining a recombinant nucleic acid sequence encoding a recombinant single-chain precursor clostridial neurotoxin by the insertion of a nucleic acid sequence encoding said SNARE complex-binding sequence, into a nucleic acid sequence encoding a parental clostridial neurotoxin.

[0099] In the context of the present invention, the term "recombinant nucleic acid sequence" refers to a nucleic acid, which has been generated by joining genetic material from two different sources.

[00100] In the context of the present invention, the term "single-chain precursor clostridial neurotoxin" refers to a single-chain precursor for a disulfide-linked di-chain clostridial neurotoxin, comprising a functionally active clostridial neurotoxin light chain, a functionally active neurotoxin heavy chain, and a loop region linking the C-terminus of the light chain with the N-terminus of the heavy chain.

[00101] In the context of the present invention, the term "recombinant single-chain precursor clostridial neurotoxin" refers to a single-chain precursor clostridial neurotoxin comprising a heterologous SNARE complex-binding sequence, i.e. a SNARE complex-binding sequence from a species other than *Clostridium botulinum*.

[00102] In particular embodiments, the recombinant single-chain precursor clostridial neurotoxin comprises a protease cleavage site in said loop region.

[00103] Single-chain precursor clostridial neurotoxins have to be proteolytically cleaved to obtain the final biologically active clostridial neurotoxins. Proteolytic cleavage may either occur during heterologous expression by host cell enzymes, or by adding proteolytic enzymes to the raw protein material isolated after heterologous expression. Naturally occurring clostridial neurotoxins usually contain one or more cleavage signals for proteases which post-translationally cleave the single-chain precursor molecule, so that the final di- or multimeric complex can form. At present, clostridial neurotoxins are still predominantly produced by fermentation processes using appropriate Clostridium strains. During the fermentation process, the single-chain precursors are proteolytically cleaved by an unknown clostridial protease to obtain the biologically active di-chain clostridial neurotoxin. In cases, where the single-chain precursor molecule is the precursor of a protease, autocatalytic cleavage may occur. Alternatively, the protease can be a separate non-clostridial enzyme expressed in the same cell. WO 2006/076902 describes the proteolytic cleavage of a recombinant clostridial neurotoxin single-chain precursor at a heterologous recognition and cleavage site by incubation of the *E. coli* host cell lysate. The proteolytic cleavage is carried out by an unknown *E. coli* protease. In certain applications of recombinant expression, modified protease cleavage sites have been introduced recombinantly into the interchain region between the light and heavy chain of clostridial toxins, e.g. protease cleavage sites for human thrombin or non-human proteases (see WO 01/14570).

[00104] In particular embodiments, the protease cleavage site is a site that is cleaved by a protease selected from the list of: a protease selected from the list of: thrombin, trypsin, enterokinase, factor Xa, plant papain, insect papain, crustacean papain, human rhinovirus 3C protease, human enterovirus 3C protease, tobacco etch virus protease, Tobacco Vein Mottling Virus, subtilisin and caspase 3.

[00105] In a particular embodiment, the recombinant single-chain precursor clostridial neurotoxin further comprises a binding tag, particularly selected from the group comprising: glutathione-S-transferase (GST), maltose binding protein (MBP), a His-tag, a StrepTag, or a FLAG-tag.

[00106] In the context of the present invention, the term "parental clostridial neurotoxin" refers to an initial clostridial neurotoxin without a heterologous SNARE complex-binding sequence, selected from a natural clostridial neurotoxin, a functional variant of a natural clostridial neurotoxin or a chimeric clostridial neurotoxin, wherein the clostridial neurotoxin light chain and heavy chain are from different clostridial neurotoxin serotypes.

[00107] In particular embodiments, the method for the generation of the recombinant clostridial neurotoxin of the present invention further comprises the step of heterologously expressing said recombinant nucleic acid sequence in a host cell, particularly in a bacterial host cell, more particularly in an *E. coli* host cell.

[00108] In certain embodiments, the *E. coli* cells are selected from *E. coli* XL1-Blue, Nova Blue, TOP10, XL10-Gold, BL21, and K12.

[00109] In particular embodiments, the method for the generation of the recombinant clostridial neurotoxin of the present invention additionally comprises at least one of the steps of (i) generating a disulfide-linked di-chain recombinant clostridial neurotoxin comprising a SNARE complex-binding sequence by causing or allowing contacting of said recombinant single-chain precursor clostridial neurotoxin with an endoprotease and (ii) purification of said recombinant single-chain precursor clostridial neurotoxin or said disulfide-linked di-chain recombinant clostridial neurotoxin by chromatography.

[00110] In particular embodiments, the recombinant single-chain precursor clostridial neurotoxin, or the recombinant disulfide-linked di-chain clostridial neurotoxin, is purified after expression, or in the case of the recombinant disulfide-linked di-chain clostridial neurotoxin, after the cleavage reaction. In particular such embodiments, the protein is purified by chromatography, particularly by immunoaffinity chromatography, or by chromatography on an ion exchange matrix, a hydrophobic interaction matrix, or a multimodal chromatography matrix, particularly a strong ion exchange matrix, more particularly a strong cation exchange matrix.

[00111] In the context of the present invention, the term "causing ... contacting of said recombinant single-chain precursor clostridial neurotoxin ...with an endoprotease" refers to an active and/or direct step of bringing said neurotoxin and said endoprotease in contact, whereas the term "allowing contacting of a recombinant single-chain precursor clostridial neurotoxin ...with an endoprotease" refers to an indirect step of establishing conditions in such a way that said neurotoxin and said endoprotease are getting in contact to each other.

[00112] In the context of the present invention, the term "endoprotease" refers to a protease that breaks peptide bonds of non-terminal amino acids (i.e. within the polypeptide chain). As they do not attack terminal amino acids, endoproteases cannot break down peptides into monomers.

[00113] In particular embodiments, cleavage of the recombinant single-chain precursor clostridial neurotoxin is near-complete.

[00114] In the context of the present invention, the term "near-complete" is defined as more than about 95% cleavage, particularly more than about 97.5%, more particularly more than about 99% as determined by SDS-PAGE and subsequent Western Blot or reversed phase chromatography.

[00115] In particular embodiments, cleavage of the recombinant single-chain precursor clostridial neurotoxin occurs at a heterologous cleavage signal located in the loop region of the recombinant precursor clostridial neurotoxin.

[00116] In particular embodiments, the cleavage reaction is performed with crude host cell lysates containing said single-chain precursor protein.

[00117] In other particular embodiments, the single-chain precursor protein is purified or partially purified, particularly by a first chromatographic enrichment step, prior to the cleavage reaction.

[00118] In the context of the present invention, the term "purified" relates to more than about 90% purity. In the context of the present invention, the term "partially purified" relates to purity of less than about 90% and an enrichment of more than about two fold.

[00119] In another aspect, the present invention relates to a recombinant single-chain clostridial neurotoxin, which is a precursor for the recombinant clostridial neurotoxin of the present invention Thus, in such aspect, the present invention relates to a recombinant single-chain precursor clostridial neurotoxin comprising a SNARE complex-binding sequence.

[00120] In particular embodiments, said recombinant single-chain clostridial neurotoxin has the amino acid sequence as found in SEQ ID NO: 8 or SEQ ID NO: 10 (see Table 1).

[00121] In another aspect, the present invention relates to a nucleic acid sequence encoding the recombinant single-chain clostridial neurotoxin of the present invention.

[00122] In another aspect, the present invention relates to a method for obtaining the nucleic acid sequence of the present invention, comprising the step of inserting a nucleic acid sequence encoding a SNARE complex-binding sequence into a nucleic acid sequence encoding a parental clostridial neurotoxin.

[00123] In another aspect, the present invention relates to a vector comprising the nucleic acid sequence of the present invention, or the nucleic acid sequence obtainable by the method of the present invention.

[00124] In another aspect, the present invention relates to a recombinant host cell comprising the nucleic acid sequence of the present invention, the nucleic acid sequence obtainable by the method of the present invention, or the vector of the present invention.

[00125] In certain embodiments, the recombinant host cells are selected from *E. coli* XL1-Blue, Nova Blue, TOP10, XL10-Gold, BL21, and K12.

[00126] In another aspect, the present invention relates to a method for producing the recombinant single-chain precursor clostridial neurotoxin of the present invention, comprising the step of expressing the nucleic acid sequence of the present invention, or the nucleic acid sequence obtainable by the method of the present invention, or the vector of the present invention in a recombinant host cell, or cultivating the recombinant host cell of the present invention under conditions that result in the expression of said nucleic acid sequence.

EXAMPLES

Example 1: Generation and Purification of a Synaptobrevin-PAS-BoNT/A fusion protein (Syb-PAS-BoNT/A)

[00127] A SNARE-based sequence comprising residues 32-90 of Synaptobrevin-2 ("VAMP") with a His-tag linked to a thrombin cleavage site at the N-terminus of the motif and a 52 amino acid long PAS linker at the C-terminus was synthetically produced and after digestion with NdeI/ SwaI inserted at the N -terminus of recombinant BoNT/A (rBoNT/A) (**Figure 1**). The correct cloning was verified by sequencing.

[00128] Expression was performed in expression strain E. coli BL21. Purification was done using a combination of affinity, ion exchange and size exclusion chromatography, followed by activation using thrombin. **Figure 2** summarizes the results of purification and activation.

Example 2: Determination of Biological Activity of Syb-PAS-BoNT/A

[00129] In this in vivo test, the biological activity of a sample is determined. Adequate e dosages of the Synaptobrevin-PAS-BoNT/A fusion protein (Example 1), in a volume of 20 µl in each case, are injected into the M. gastrocnemius of the right hind paw of four mice in each case. The resulting effect is measured over time by determining the digit abduction when lifting the mouse and is quantified by using a score from 0 to 4 (Aoki, K.R.; Toxicon 39 (2001) 1815-1820). A score of 0 corresponds to maximum digit abduction, while a score of 4 corresponds to maximum paralysis, where digit abduction is completely absent. Scores 1, 2 or 3 describe intermediate states between these two extremes. In **Figure 3**, the results of the DAS test with Syb-PAS-BoNT/A are shown.

Example 3: Generation and Purification of a C-terminal syntaxin BoNT/A fusion protein BoNT/A (C-term Syn BoNT/A)

[00130] A SNARE-based sequence comprising residues 186-212 of Syntaxin was inserted at the C-terminus of the light chain (Lc) of BoNT/A followed by an AGAG linker and the thrombin cleavage site, which was fused to the N-terminus of the heavy chain of BoNT/A via an AGAG linker (**Figure 4**). The correct cloning was verified by sequencing. Expression was performed in expression strain E. coli Bl21.

[00131] Purification was done using a combination of affinity, ion exchange and size exclusion chromatography, followed by activation using thrombin. **Figure 5** summarizes the results of purification and activation.

Example 4: Determination of the biological activity of C-term Syn BoNT/A

[00132] In this in vivo test, the biological activity of a sample is determined. Adequate dosages of the C-term Syn BoNT/A fusion protein (Example 3), in a volume of 20 µl in each case, are injected into the M. gastrocnemius of the right hind

paw of four mice in each case. The resulting effect is measured over time by determining the digit abduction when lifting the mouse and is quantified by using a score from 0 to 4 (Aoki, K.R.; Toxicon 39 (2001) 1815-1820). A score of 0 corresponds to maximum digit abduction, while a score of 4 corresponds to maximum paralysis, where digit abduction is completely absent. Scores 1, 2 or 3 describe intermediate states between these two extremes. In **Figure 6**, the results of the DAS test with C-term Syn-BoNT/A are shown.

Table 1: Sequences

SEQ ID NO 1: Synaptobrevin

>sp|P63027|VAMP2_HUMAN Vesicle-associated membrane protein 2 OS=Homo sapiens GN=VAMP2 PE=1 SV=3

MSATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLE
RDQKLSELDDRADALQAGASQFETSAAKLKRKYWWKNLKMMIILGVICAIILIIIIVYFS
T

SEQ ID NO 2: Syntaxin

>sp|Q16623|STX1A_HUMAN Syntaxin-1A OS=Homo sapiens GN=STX1A PE=1 SV=1

MKDRTQELRTAKDSDDDDDVAVTVDRDRFMDEFFEQVEEIRGFIDKIAENVEEVKR
KHSAILASPNPDEKTKEELEELMSDIKKTANKVRSKLKSIEQSIEQEEGLNRSSADLRI
RKTQHSTLSRKFVEVMSEYNATQSDYRERCKGRIQRQLEITGRTTTSEELEDMLES
GNPAIFASGIIMDSSISKQALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQGEMI
DRIEYNVEHAVDYVERAVSDTKKAVKYQSKARRKKIMIIICCVILGIVIASTVGGIFA

SEQ ID NO 3: Complexin

>sp|Q6PUV4|CPLX2_HUMAN Complexin-2 OS=Homo sapiens GN=CPLX2 PE=1 SV=2

MDFVMKQALGGATKDMGKMLGGEEEKDPDAQKKEEERQEALRQQEEERKAKHA
RMEAEREKVRQQIRDKYGLKKKEEKEAEEKAALEQPCEGSLTRPKKAIPAGCGDEE
EEEEESILDTVLKYLPGPLQDMFKK

SEQ ID NO 4: QAQVDEVVDIMRVNVDKVL

SEQ ID NO 5: LQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQA-GASQFETSAAKLKRKYWW

SEQ ID NO 6: LQQTQAQVDEVVDIMRVNVDKVLERD

SEQ ID NO 7: SISKQALSEIETRHSEIIKLENSIRE

SEQ ID NO 8: SISKQALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQ

SEQ ID NO 9: SISKQALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQGE-MIDRIEYNVEHAVDYVERAVSDTKKAV

SEQ ID NO 10: AKMEAEREVMRQGIRDKYGI

SEQ ID NO 11: KKEEERQEALRQAEEERKAKYAKMEAEREVMRQGIRDKYGI

SEQ ID No 12 ASPAAPAPASPAAPAPSAPAASPAAPAPASPAAPAPSAPA-ASPAAPAPASPAA

SEQ ID NO 13: QAQVDEVVDIMRVNVDKVLAGAGLVPRGSAGAG

SEQ ID NO 14: LQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQA-GASQFETSAAKLKRKYWWAGLVPRGSAGAG

SEQ ID NO 15: LQQTQAQVDEVVDIMRVNVDKVLERDAGLVPRGSAGAG

SEQ ID NO 16: SISKQALSEIETRHSEIIKLENSIREAGLVPRGSAGAG

SEQ ID NO 17: SISKQALSEIETRHSEIIKLENSIRELHDMFMDMAMLVE-SQAGLVPRGSAGAG

SEQ ID NO 18: SISKQALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQGE-MIDRIEYNVEHAVDYVERAVSDTKKAVAGLVPRGSAGAG

SEQ ID NO 19: AKMEAEREVMRQGIRDKYGIAGLVPRGSAGAG

SEQ ID NO 20: KKEEERQEALRQAEEERKAKYAK-MEAEREVMRQGIRDKYGIAGLVPRGSAGAG

CLAIMS

1. A recombinant clostridial neurotoxin comprising a SNARE complex-binding sequence derived from a SNARE complex-related protein, particularly wherein said SNARE complex-related protein is selected from: synaptobrevin, particularly synaptobrevin-2; syntaxin, particularly syntaxin-1A; and complexin.

2. The recombinant clostridial neurotoxin of claim 1, wherein said SNARE complex-binding sequence consists of a sequence selected from the group of: (i) a sequence of at least 59 contiguous amino acids comprised in the stretch of amino acid residues from position Leu32 to Trp90 of synaptobrevin-2 (SEQ ID NO: 1); in particular a sequence of at least 26 contiguous amino acids comprised in the stretch of amino acid residues from position Leu32 to Asp57 of synaptobrevin-2; (ii) a sequence of at least 70 contiguous amino acids comprised in the stretch of amino acid residues from position Ser186 to Val255 of syntaxin-1A (SEQ ID NO: 2), in particular a sequence of at least 41 contiguous amino acids comprised in the stretch of amino acid residues from position Ser186 to Gln226 of syntaxin-1A; (iii) a sequence of at least 41 contiguous amino acids comprised in the stretch of amino acid residues from position Lys32 to Ile72 of complexin (SEQ ID NO: 3), in particular a sequence of at least 20 contiguous amino acids comprised in the stretch of amino acid residues from position Ala53 to Ile72 of complexin; and (iv) a mutated sequence obtained from a sequence according to (i) to (iii) by the deletion of a maximum of two amino acid residues of said sequence; the insertion of a maximum of two amino acid residues into said sequence; and/or the conservative exchange of a maximum of three amino acid residues from said sequence, particularly a mutated sequence having a maximum of three, two or one of said deletions, insertions, and/or exchanges, provided that said mutated sequence is still a SNARE complex-binding sequence.

3. The recombinant clostridial neurotoxin of claim 1 or 2, wherein said SNARE complex-binding sequence consists of a fragment of syntaxin-1A or complexin with an alpha-helical structure of from 90 to 100% helical propensity according to a 8ns Molecular Dynamics (MD) Simulation in explicit water using the algorithm Desmond, or a fragment of synaptobrevin with at least 15% helical propensity according to a 8ns MD Simulation in explicit water of the lipid-bound Synaptobrevin-2 solution NMR structure.

4. The recombinant clostridial neurotoxin of any one of claims 1 to 3, wherein (i) said SNARE complex-related protein is synaptobrevin-2, and wherein said SNARE complex-binding sequence comprises the amino acid sequence QAQVDEVVDIMRVNVDKVL (SEQ ID NO: 4), particularly wherein said SNARE complex-binding sequence comprises a sequence selected from: LQQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETSAAK-LKRKYWW (SEQ ID NO: 5) and LQQTQAQVDEVVDIMRVNVDKVLERD (SEQ ID NO: 6); wherein (ii) said SNARE complex-related protein is syntaxin-1A, and wherein said SNARE complex-binding sequence comprises the amino acid sequence SISKQALSEIETRHSEIIKLENSIRE (SEQ ID NO: 7), particularly wherein said SNARE complex-binding sequence comprises a sequence selected from: SISKQALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQ (SEQ ID NO: 8), and SISKQALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQGEMIDRIEYNVE-HAVDYVERAVSDTKKAV (SEQ ID NO: 9); or wherein (iii) said SNARE complex-related protein is complexin, and wherein said SNARE complex-binding sequence comprises the amino acid sequence AKMEAEREVMRQGIRDKYGI (SEQ ID NO: 10), particularly wherein said SNARE complex-binding sequence comprises the sequence KKEEERQEALRQAEEERKAKYAKMEAEREVMRQGIRDKYGI (SEQ ID NO: 11)

5. The recombinant clostridial neurotoxin of any one of claims 1 to 4, wherein said SNARE complex-binding sequence is inserted at a position within 200 amino acid residues from the N-terminus of the light chain of a clostridial neurotoxin, particularly wherein said SNARE complex-binding sequence is linked via a PAS linker, particularly a PAS-52 linker, particularly the PAS linker ASPAAPAPASPAA-PAPSAPAASPAAPAPASPAAPAPSAPAASPAAPAPASPAA (SEQ ID NO: 12), to the mature N-terminus of said light chain, particularly wherein said recombinant clostridial neurotoxin comprises a SNARE complex-binding sequence selected from the list of: SEQ ID NO: 4 to 11, wherein said SNARE complex-binding sequence is fused to the N-terminus of a mature light chain of said clostridial neurotoxin.

6. The recombinant clostridial neurotoxin of any one of claims 1 to 4, wherein said SNARE complex-binding sequence is inserted at a position within 10 amino acid residues from the C-terminus of the light chain of a clostridial neurotoxin, particularly wherein said SNARE complex-binding sequence is inserted in the linker region linking said light chain and the heavy chain of said clostridial neurotoxin, particularly wherein said recombinant clostridial neurotoxin comprises a sequence, which comprises a SNARE complex-binding sequence, selected from the list of: SEQ ID NO: 13 to 20, wherein said sequence is linking the mature light chain of a clostridial neurotoxin and the heavy chain of said clostridial neurotoxin.

7. The recombinant clostridial neurotoxin of any one of claims 1 to 6, wherein said clostridial neurotoxin is selected from (i) a *Clostridium botulinum* neurotoxin serotype A, B, C, D, E, F, G, and H, particularly *Clostridium botulinum* neurotoxin serotype A, C and E, more particularly *Clostridium botulinum* neurotoxin serotype A or (ii) from a functional variant of a *Clostridium botulinum* neurotoxin of (i), or (iii) from a chimeric *Clostridium botulinum* neurotoxin, wherein the clostridial

neurotoxin light chain and heavy chain are from different clostridial neurotoxin serotypes.

8. The recombinant clostridial neurotoxin of any one of claims 1 to 7, wherein said recombinant clostridial neurotoxin shows increased duration of effect relative to an identical clostridial neurotoxin without said SNARE complex-binding sequence, particularly wherein the increased duration of effect is due to increased plasma membrane localization.

9. The recombinant clostridial neurotoxin of any one of claims 1 to 8 for the use in the treatment of a disease requiring improved chemodenervation, wherein the recombinant clostridial neurotoxin causes longer lasting denervation relative to an identical clostridial neurotoxin without said SNARE complex-binding sequence.

10. A pharmaceutical composition comprising the recombinant clostridial neurotoxin of any one of claims 1 to 9.

11. Use of the recombinant clostridial neurotoxin of any one of claims 1 to 9 for cosmetic treatment.

12. A method for the generation of a recombinant clostridial neurotoxin according to any one of claims 1 to 9, comprising the step of obtaining a recombinant nucleic acid sequence encoding a recombinant single-chain precursor clostridial neurotoxin by the insertion of a nucleic acid sequence encoding said SNARE complex-binding sequence into a nucleic acid sequence encoding a parental clostridial neurotoxin, particularly a method further comprising the step of heterologously expressing said recombinant nucleic acid sequence in a host cell, particularly in a bacterial host cell, more particularly in an *E. coli* host cell.

13. A recombinant single-chain precursor clostridial neurotoxin comprising a SNARE complex-binding sequence.

14. A nucleic acid sequence encoding the recombinant single-chain precursor clostridial neurotoxin of claim 13.

15. A method for obtaining the nucleic acid sequence of claim 14, comprising the step of inserting a nucleic acid sequence encoding a SNARE complex-binding sequence into a nucleic acid sequence encoding a parental clostridial neurotoxin.

16. A vector comprising the nucleic acid sequence of claim 14, or the nucleic acid sequence obtainable by the method of claim 15.

17. A recombinant host cell comprising the nucleic acid sequence of claim 14, the nucleic acid sequence obtainable by the method of claim 15, or the vector of claim 16.

18. A method for producing the recombinant single-chain precursor clostridial neurotoxin of claim 13, comprising the step of expressing the nucleic acid sequence of claim 14, or the nucleic acid sequence obtainable by the method of claims 15, or the vector of claim 16 in a recombinant host cell, or cultivating the recombinant host cell of claim 17 under conditions that result in the expression of said nucleic acid sequence.

Figure 1:

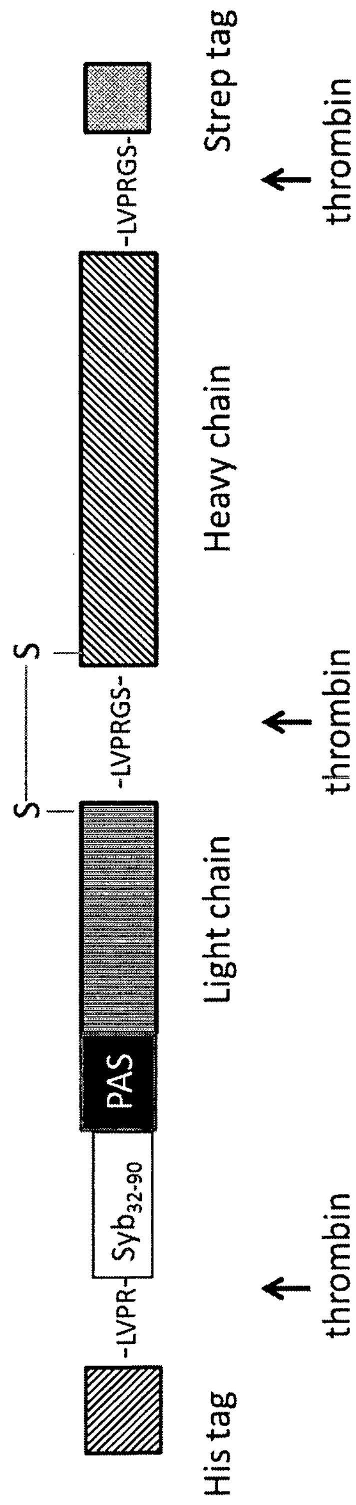


Figure 2:

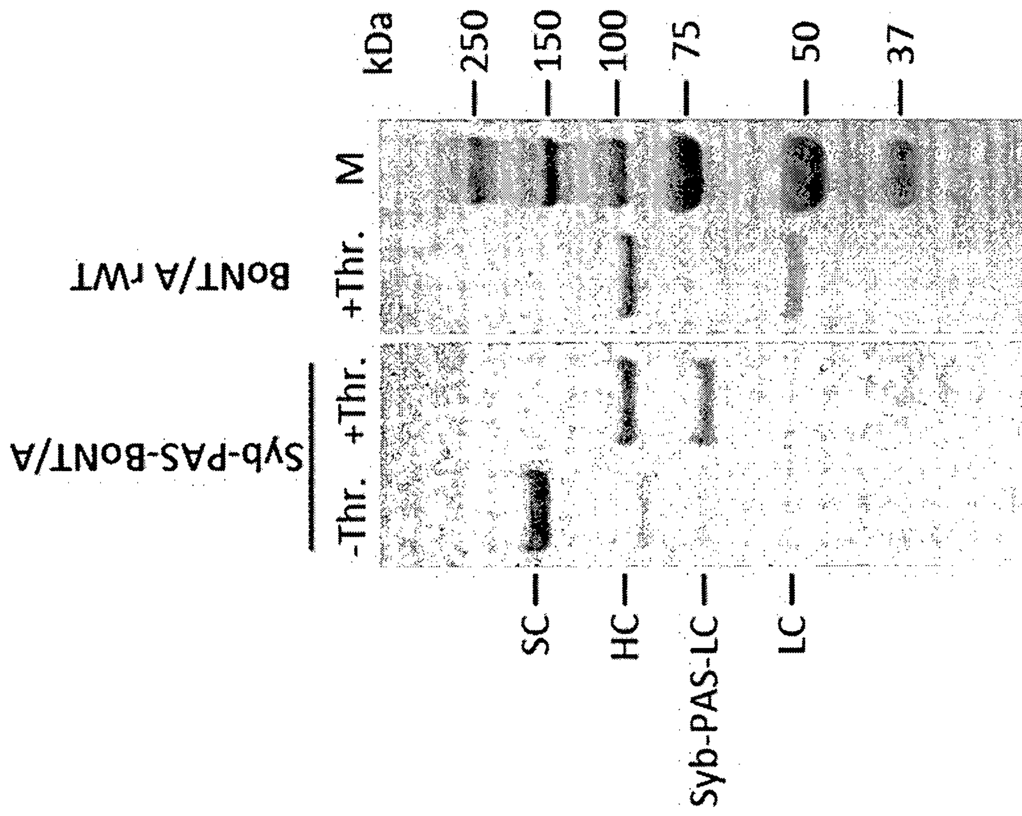


Figure 3:

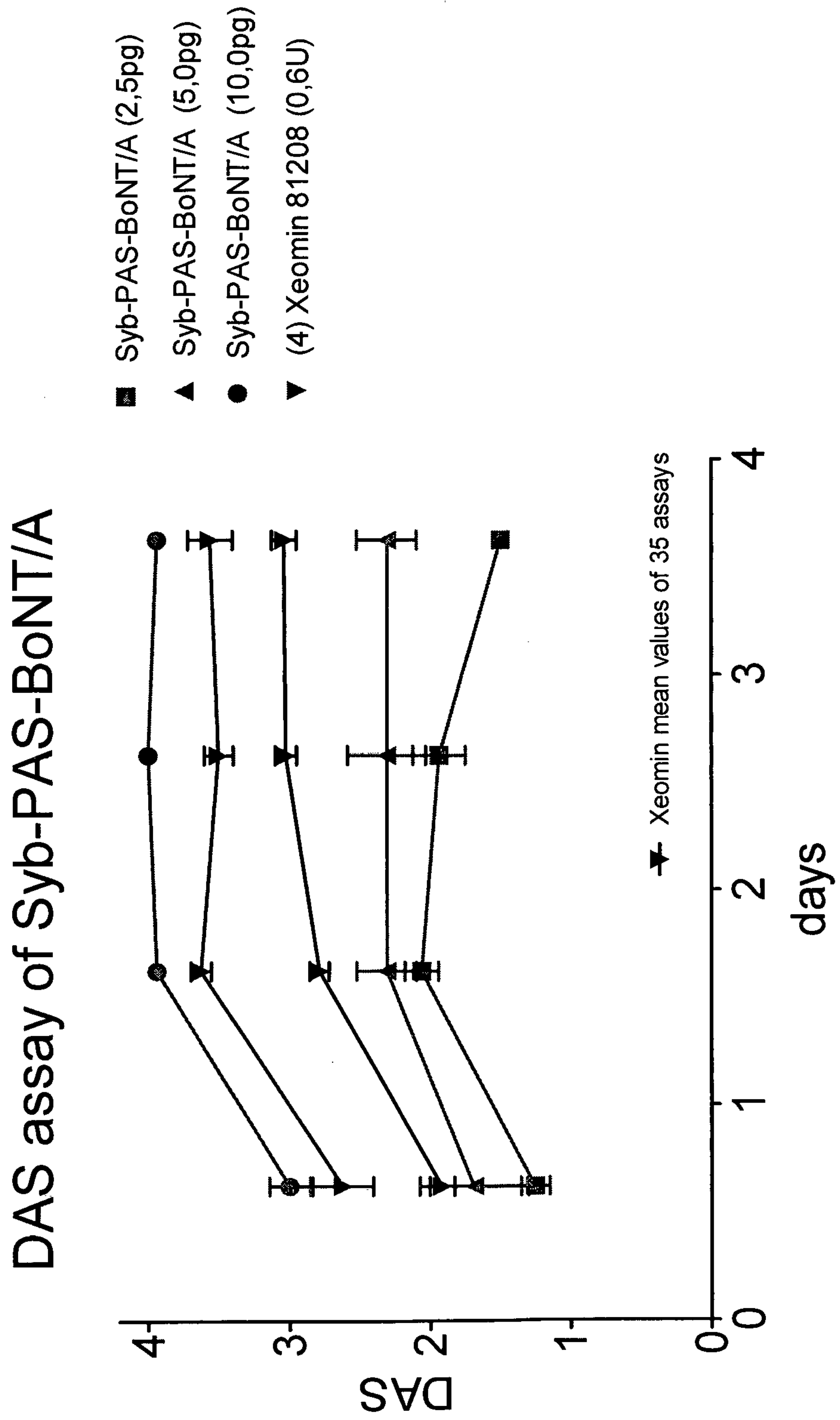
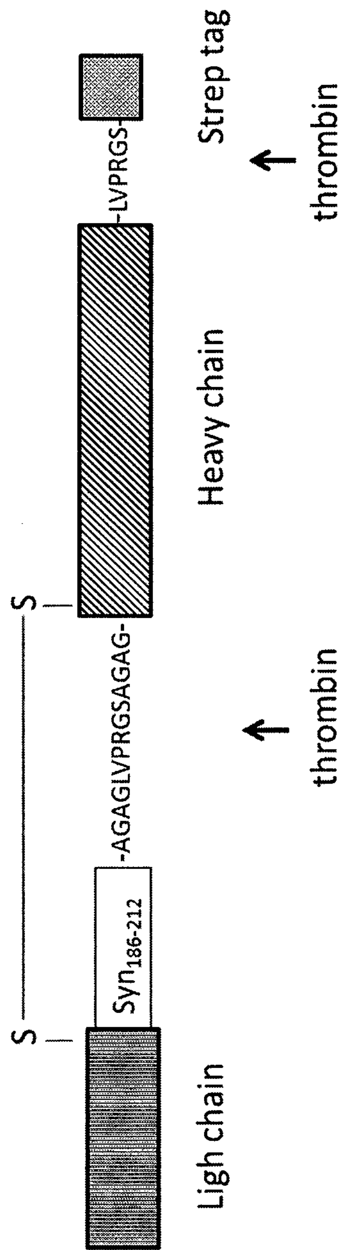


Figure 4:



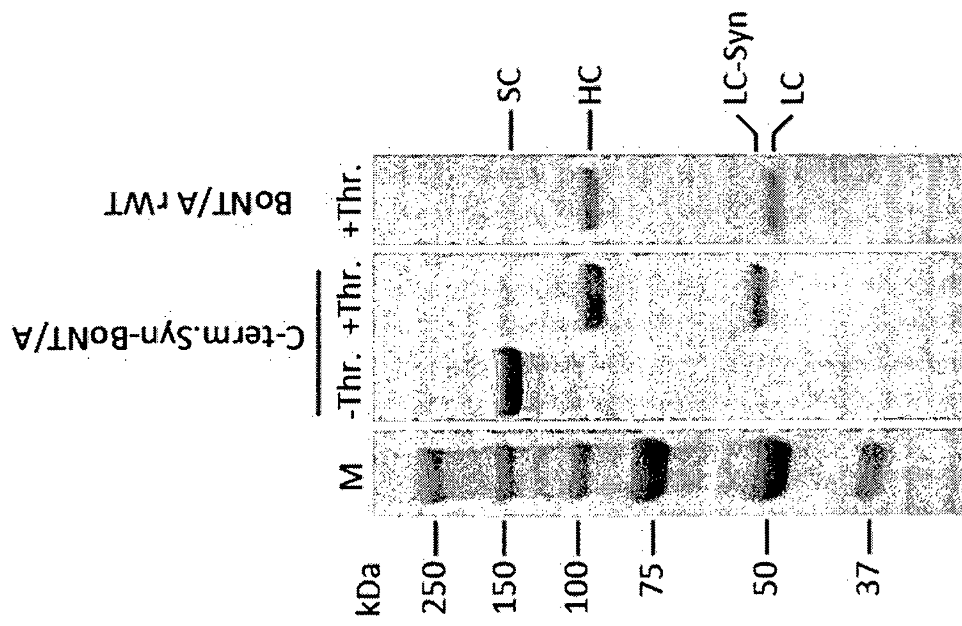
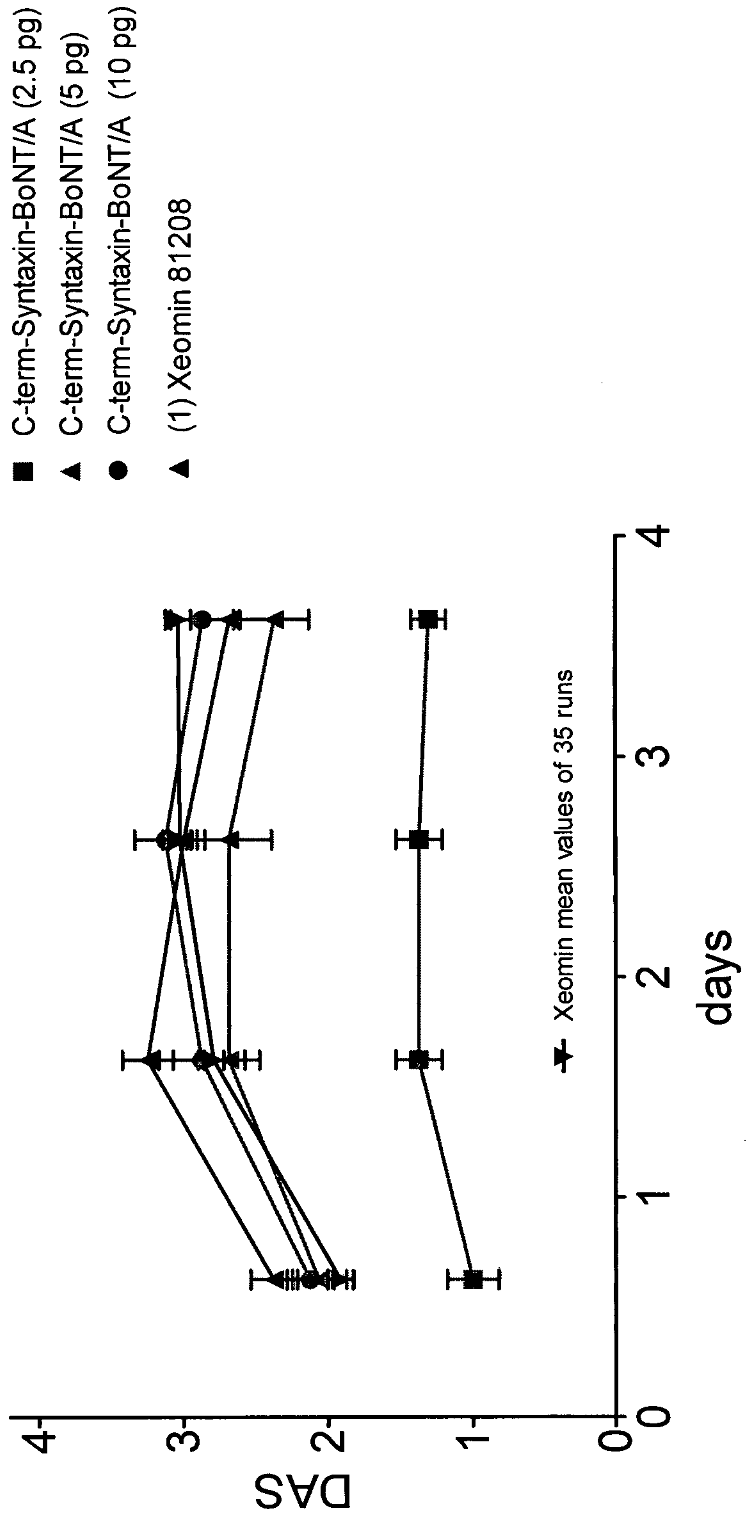


Figure 5:

Figure 6:



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/000772

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/33
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)
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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/068472 A1 (MERZ PHARMA GMBH & CO KGAA [DE]; EISELE KARL-HEINZ [DE]) 16 May 2013 (2013-05-16) the whole document	1-18
A	WO 2014/086494 A1 (MERZ PHARMA GMBH & CO KGAA [DE]) 12 June 2014 (2014-06-12) the whole document	1-18
A	US 2002/127247 A1 (STEWARD LANCE E [US] ET AL) 12 September 2002 (2002-09-12) cited in the application the whole document	1-18
A	WO 2010/022979 A1 (MERZ PHARMA GMBH & CO KGAA [DE]; FREVERT JUERGEN [DE]) 4 March 2010 (2010-03-04) the whole document	1-18

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 July 2016	14/07/2016

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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