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(56) Fremdragne publikationer:
WO-A2-2005/038029
US-B1- 6 890 745
LENG J ET AL: "CLEAVALITETM: A NOVEL BIOLUMINESCENT CASPASE ACTIVITY ASSAY", ABSTRACTS OF THE ANNUAL MEETING OF THE SOCIETY FOR NEUROSCI, SOCIETY FOR NEUROSCIENCE, US, vol. 27, no. 2, 1 January 2001 (2001-01-01), XP008005347, ISSN: 0190-5295
WAUD J P ET AL: "Engineering the C-terminus of firefly luciferase as an indicator of covalent modification of proteins", BIOCHIMICA ET BIOPHYSICA ACTA, ELSEVIER, NL, vol. 1292, no. 1, 1 January 1996 (1996-01-01), pages 89-98, XP002325291, ISSN: 0006-3002
TURTON K ET AL: "Botulinum and tetanus neurotoxins: structure, function and therapeutic utility", TRENDS IN BIOCHEMICAL SCIE, ELSEVIER, AMSTERDAM, NL, vol. 27, no. 11, 1 November 2002 (2002-11-01), pages 552-558, XP004390740, ISSN: 0968-0004, DOI: 10.1016/S0968-0004(02)02177-1
WAUD J P ET AL: "Engineering the C-terminus of firefly luciferase as an indicator of covalent modification of proteins", BIOCHIMICA ET BIOPHYSICA ACTA, ELSEVIER, NL, vol. 1292, no. 1, 1 January 1996 (1996-01-01), pages 89 - 98, XP002325291, ISSN: 0006-3002
TURTON K ET AL: "Botulinum and tetanus neurotoxins: structure, function and therapeutic utility", TRENDS IN BIOCHEMICAL SCIE, ELSEVIER, AMSTERDAM, NL, vol. 27, no. 11, 1 November 2002 (2002-11-01), pages 552 - 558, XP004390740, ISSN: 0968-0004, DOI: 10.1016/S0968-0004(02)02177-1

Fortsættes ...

DESCRIPTION

SEQUENCE LISTING

[0001] The present specification makes reference to a Sequence Listing (submitted electronically as a .txt file named "0342941_0630_SL.TXT"). The .txt file was generated on October 13, 2017, and is 34,305 bytes in size.

TECHNICAL FIELD

[0002] The technology described herein relates to methods and compositions for determining the activity of neurotoxins, e.g., botulinum toxin.

BACKGROUND

[0003] Botulinum neurotoxin (BoNT) is an agent of interest both for bioterrorism concerns as well as therapeutic applications. Traditionally, detection and characterization of BoNT has been performed by administering samples to mice, causing their death at significant doses. Assays which can be performed with cells or in vitro have been explored, but suffer from a lack of sensitivity or specificity, often causing positive signals as a result of reactions with common components of clinical samples.

[0004] WO 2005/038029 A2 is concerned with modified beetle luciferases, comprising an internal insertion at a residue or in a region which is tolerant to modification, the insertion comprising a sequence which directly or indirectly interacts with a molecule of interest.

[0005] Waud et al "Engineering the C-terminus of firefly luciferase as an indicator of covalent modification of proteins." Biochim Biophys Acta. 1996 describes engineering protein kinase recognition sequences and proteinase sites into cDNA encoding firefly luciferase in order to establish whether these modified proteins could be developed as bioluminescent indicators of covalent modification of proteins.

[0006] US6890745B1, and Leng J et al: "Cleavalite™: A Novel Bioluminescent Caspase Activity Assay", Abstracts of the annual meeting of the society for neuroscience, 2001, discuss a modified luciferase polynucleotide sequence and a luciferase polypeptide containing protease recognition sequences, wherein cleavage of the recognition sequence by a protease inhibits luciferase activity.

SUMMARY

[0007] Described herein are new assays developed to detect and measure the activity of BoNTs both in vitro and in vivo. The assays use unique single chain polypeptides that feature, e.g., a split luciferase linked through substrates of BoNTs engineered to serve as a sensor for BoNT activity. In the presence of BoNT, the linker region is cleaved by the toxin resulting in a decrease in luciferase activity, which is readily detected.

[0008] In one aspect of any of the embodiments, described herein is a single chain polypeptide comprising, from N-terminal to C-terminal: a) an N-terminal fragment of a reporter protein; b) a linker comprising: (i) a *C. botulinum* neurotoxin (BoNT) cleavage site, and (ii) a binding fragment of a BoNT receptor; and c) a C-terminal fragment of the reporter protein; wherein the reporter protein is a luciferase protein and wherein the N-terminal and C-terminal fragments collectively comprise a functional luciferase protein sequence when the fragments are joined together by the linker and not when the linker has been cleaved at the cleavage site..

[0009] In some embodiments of any of the aspects, the N-terminal domain has the sequence of amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano$_{1-159}$) and the C-terminal domain has the sequence of amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano$_{160-170}$).

[0010] In some embodiments of any of the aspects, the linker further comprises one or more spacers located between the BoNT cleavage site and the N-terminal fragment or domain , between the neurotoxin cleavage site and the C-terminal fragment of domain, or a combination thereof. In some embodiments of any of the aspects, the binding fragment of a BoNT receptor is located between the N-terminal fragment or domain and the BoNT cleavage site. In some embodiments of any of the aspects, the linker further comprises a spacer located between the binding fragment and the BoNT cleavage site. In some embodiments of any of the aspects, at least one spacer is comprised of glycine and serine. In some embodiments of any of the aspects, at least one spacer is 5 to 15 amino acids. In some embodiments of any of the aspects, at least one spacer is selected from SEQ ID NOs: 4-9.

[0011] In some embodiments of any of the aspects, the BoNT is A, E, C, B, D, F or G. In some embodiments of any of the aspects, the BoNT cleavage site is from a SNARE protein. In some embodiments of any of the aspects, the SNARE protein is SNAP-25, synaptobrevin (VAMP), or syntaxin. In some embodiments of any of the aspects, the BoNT cleavage site is amino acids 141-206 of human SNAP-25b. In some embodiments of any of the aspects, the BoNT cleavage site is amino acid 35-96 of human VAMP1. In some embodiments of any of the aspects, the receptor is human SV2C. In some embodiments of any of the aspects, the binding fragment is amino acids 529-566 of human SV2C, or a sequence having at least 90% identity with amino acids 529-566 of human SV2C, or a sequence with no more than 10 amino acid residues substitutions, deletions, or additions relative to amino acids 529-566 of human SV2C.

[0012] In some embodiments of any of the aspects, the polypeptide further comprises a

polyhistidine affinity tag. In some embodiments of any of the aspects, the polyhistidine affinity tag is located at the C-terminus of the polypeptide.

[0013] In some embodiments of any of the aspects, the linker further comprises an intact second luciferase polypeptide located between the N-terminal fragment or domain and the BoNT cleavage site. In some embodiments of any of the aspects, the second luciferase polypeptide is firefly luciferase (e.g., *Photinus pyralis*), bacterial luciferase (e.g., *Vibrio fischeri, Vibrio harveyi*), sea pansy luciferase (*Renilla reniformis*), dinoflagellate luciferase, *Gaussia* luciferase, or copepod luciferase. In some embodiments of any of the aspects, the linker further comprises a spacer located between the second luciferase polypeptide and the BoNT cleavage site. In some embodiments of any of the aspects, the spacer is from 5 to 15 amino acids. In some embodiments of any of the aspects, the spacer is GSSGGGGSGGGGSSG (SEQ ID NO: 4), GSSGGGGSGGGSSG (SEQ ID NO: 5), or GGGGS (SEQ ID NO: 6).

[0014] In one aspect of any of the embodiments, described herein is a single chain polypeptide comprising: a) amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano$_{1-159}$); b) a linker located C-terminal to the N-nano$_{1-159}$ comprising: i) a first spacer of 5 to 15 amino acids; ii) a binding fragment comprised of amino acids 529-566 of SV2C located C-terminal to the first spacer; iii) a second spacer located C-terminal to the binding fragment; iv) a BoNT cleavage site comprising amino acids 141-206 of human SNAP25b located C-terminal to the second spacer; v) a third spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site; and c) amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano$_{160-170}$) located C-terminal to the linker; wherein the linker functionally joins the N-nano$_{1-159}$ and the C-nano$_{160-170}$ to generate a functional luciferase fusion protein.

[0015] In some embodiments of any of the aspects, one or more spacers are GSSGGGGSGGGGSSG (SEQ ID NO: 4), GSSGGGGSGGGSSG (SEQ ID NO: 5), or GGGGS (SEQ ID NO: 6). In some embodiments of any of the aspects, the polypeptide further comprises a His6 sequence (SEQ ID NO: 12) located at the C-terminus.

[0016] In one aspect of any of the embodiments, described herein is a nucleic acid comprising a nucleotide sequence that encodes a single chain polypeptide described herein. In one aspect of any of the embodiments, described herein is a nucleic acid vector comprising a nucleic acid comprising a nucleotide sequence that encodes a single chain polypeptide described herein. In some embodiments of any of the aspects, the vector is an expression vector and comprises the nucleic acid sequence in expressible form. In some embodiments of any of the aspects, the vector is selected from the group consisting of a viral expression vector, a prokaryotic expression vector, a yeast expression vector, an insect expression vector, or a mammalian expression vector.

[0017] In one aspect of any of the embodiments, described herein is a cell comprising a nucleic acid or vector of the foregoing paragraph. In some embodiments of any of the aspects, the cell expresses a single chain polypeptide as described herein. In some embodiments of

any of the aspects, the cell is a prokaryotic cell, a yeast cell, an insect cell, or an animal cell.

[0018] In one aspect of any of the embodiments, described herein is an *in vitro* method for determining the potency of a botulinum neurotoxin, comprising: contacting the neurotoxin to a single chain polypeptide as described herein under conditions appropriate for BoNT activity; and determining the luciferase activity of the polypeptide, as compared to a reference, thereby determining the potency.

[0019] In one aspect of any of the embodiments, described herein is a method for detecting C. botulinum neurotoxin (BoNT) activity in a sample, comprising: contacting the sample to a single chain polypeptide as described herein under conditions appropriate for BoNT activity; and determining luciferase activity of the polypeptide, as compared to luciferase activity of the polypeptide in the absence of the sample, wherein a decrease of luciferase activity indicates BoNT activity in the sample.

[0020] In some embodiments of any of the aspects, the method is performed *in vitro.* In some embodiments of any of the aspects, contacting occurs in a buffer of 50mM HEPES, 20 µM $ZnCl_2$, 2 mM DTT, 1 mg/ml BSA, pH 7.1. In some embodiments of any of the aspects, the concentration of the single chain polypeptide contacted to the sample is from about 30 nM to 300 nM. In some embodiments of any of the aspects, the concentration of single chain polypeptide contacted to the sample is about 30 nM.

[0021] In some embodiments of any of the aspects, the luciferase activity is determined by addition of luciferase substrate to the single chain polypeptide and quantitative measurement of a luminescent signal produced. In some embodiments of any of the aspects, the conditions comprise incubation at about 37°C for a period of from about 1 hour to about 36 hours. In some embodiments of any of the aspects, the conditions comprise incubation at about 37°C for a period of from about 1 hour to about 24 hours. In some embodiments of any of the aspects, the conditions comprise incubation at about 37°C for a period of from about 4 hours to about 24 hours.

[0022] In some embodiments of any of the aspects, the linker comprises a first spacer of 5 to 15 amino acids, a binding fragment of amino acids 529-566 of human SV2C located C-terminal to the first spacer and N-terminal to the BoNT cleavage site, and a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acids 141-206 of human SNAP25. In some embodiments of any of the aspects, the linker comprises a first spacer of 5 to 15 amino acids located N-terminal to the BoNT cleavage site, and a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acids 141-206 of human SNAP25. In some embodiments of any of the aspects, the linker comprises a first spacer of 5 to 15 amino acids located N-terminal to the BoNT cleavage site, a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acids 35-96 of human VAMP1.

[0023] In some embodiments of any of the aspects, the single chain polypeptide is expressed by a neuronal cell and the method further comprises, after the contacting step: incubating the neuronal cells for a period of from about 12 hours to about 60 hours and harvesting lysate from the neuronal cells.

[0024] In some embodiments of any of the aspects, the linker further comprises an intact second luciferase polypeptide located between the N-nano$_{1-159}$ and the cleavage site. In some embodiments of any of the aspects, the second luciferase polypeptide firefly luciferase (photinus pyralis), bacterial luciferase (vibrio fischiri, vibrio harveyi), sea pansy luciferase (renilla reniformis), dinoflagellate luciferase, gaussia luciferase, or copepod luciferase. In some embodiments of any of the aspects, the luciferase activity of the second luciferase polypeptide in the sample is determined and used as an indicator of total single chain polypeptide present in the harvested lysate. In some embodiments of any of the aspects, the neuronal cells express the single chain polypeptide from a viral expression vector. In some embodiments of any of the aspects, the single chain polypeptide is expressed for 6 days prior to step a). In some embodiments of any of the aspects, the viral expression system is a lentivirus expression system.

[0025] In some embodiments of any of the aspects, the incubating step is about 48 hours. In some embodiments of any of the aspects, the harvesting step is by addition of a lysis buffer.

[0026] In some embodiments of any of the aspects, the BoNT cleavage site is from SNAP-25, synaptobrevin (VAMP), or syntaxin. In some embodiments of any of the aspects, the BoNT cleavage site is specifically recognized by BoNT A, E and C, or is specifically recognized by BoNT B, D, F and G. In some embodiments of any of the aspects, a combination of single chain polypeptides having different BoNT cleavage sites are used. In some embodiments of any of the aspects, the BoNT cleavage site is a.a. 141-206 of human SNAP-25, or a.a. 35-96 of human VAMP1, a.a. 1-206 of human SNAP35, or a.a. 1-96 of VAMP1. In these aspects, the linker comprises a binding fragment of a BoNT receptor. In some embodiments of any of the aspects, the receptor is SV2C. In some embodiments of any of the aspects, the binding fragment comprises amino acids 529-566 of human SV2C.

[0027] In some embodiments of any of the aspects, the linker comprises a second luciferase polypeptide, a first spacer of 5 to 15 amino acids located C-terminal of the second luciferase polypeptide and N-terminal of the BoNT cleavage site, a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acid 1-206 of human SNAP25 and is located C-terminal to the first spacer.

[0028] In some embodiments of any of the aspects, the measurement of luciferase activity is accomplished by addition of a substrate specific for the luciferase and quantitative detection of the resulting luminescent signal. In some embodiments of any of the aspects, the substrate for the luciferase of SEQ ID NO: 1 is furimazine (2-furanylmethyl-deoxy-coelenterazine).

[0029] In one aspect, described herein is a kit comprising: a) one or more single chain

polypeptides as described herein, with each or a combination of the single chain polypeptides packaged into a separate container; b) one or more nucleic acids or nucleic acid vectors as described herein, with each or a combination of the nucleic acids or nucleic acid vectors packaged into a separate container; and/or c) one or more cells as described herein with each or a combination of the cells packaged into a separate container. In some embodiments of any of the aspects, the kit further comprises a luciferase substrate packaged into a separate container.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030]

FIG. 1 depicts a schematic drawing of split luciferase based toxin sensors. *Left panel*: NANOLUC™ luciferase is separated into two complementary fragments, N-nano and C-nano. These two fragments are linked together with a linker that is based on toxin substrates (SNAP-25 (also known as p25) or VAMP1/2/3 (e.g., synaptobrevins)). *Right panel:* Cleavage of the linker region by BoNTs separates the two parts of NANOLUC™ luciferase and abolishes the luminescent signals.

FIG. 2A-FIG. 2B demonstrate an in vitro detection assay of BoNT/A. Two sensor proteins were designed and investigated for BoNT/A toxin detection. One is N_{Nano}-SV2C-L4-p25-C_{Nano} whereas the other one is N_{Nano}-p25-C_{Nano}. They were prepared at 30 nM in 30 ul volume while the BoNT/A was diluted from 1 nM to 1 fM with factor 10. The negative control was BoNT/B added in the sensor protein solution. The curve (FIG. 2A) was plotted based on the percentage of luminescence of NANOLUC™ luciferase. EC50 of using N_{Nano}-SV2C-p25-C_{Nano} for BoNT/A is 9.73 pM after 4h and 7.86 pM after 24h, meanwhile EC50 of using N_{Nano}-p25-C_{Nano} is 48.26 pM after 4h and 35.87 pM after 24h at 37°C. The sensor protein containing SV2C-L4 which is BoNT/A binding domain showed approximate 5-fold increase of EC50 (FIG. 2B).

FIG. 3A-FIG. 3B demonstrate an in vitro detection assay of BoNT/B after 4h and 24h. The sensor protein N_{Nano}-VAMP1-C_{Nano} was prepared at 30 nM in 30 ul volume while the BoNTB was diluted from 10 nM to 1 fM with factor 10. The negative control was BoNT/A added in the sensor protein solution. The curve (FIG. 3A) was plotted based on the percentage of luminescence of NANOLUC™ luciferase. EC 50 for BoNTB is 75.7 pM after 4h and 3.9 pM after 24h at 37°C (FIG. 3B).

FIG. 4 demonstrates an in vivo detection assay of BoNT/A using virus infected neurons cells. For each neuron cell lysate sample, both Firefly and NANOLUC™ luciferase signals were measured and the ratio (NANOLUC™ /Firefly) was calculated. Afterwards, the percentage of each sample's ratio was generated using no toxin exposure as reference control. The EC50= 2.9 pM equal to 30 fold of LD50 after 48h challenged with toxin proteins.

DETAILED DESCRIPTION

[0031] Described herein are compositions and methods relating to measuring and/or detecting enzymatic activity. In particular, the compositions and methods relate to the detection and/or measurement of neurotoxins, e.g., *C. botulinum* neurotoxin (BoNT).

[0032] As used herein, "*C. botulinum* neurotoxin" or "BoNT" refers to any polypeptide that can execute the overall cellular mechanism whereby a *C. botulinum* toxin enters a neuron and inhibits neurotransmitter release and encompasses the binding of a *C. botulinum* toxin to a low or high affinity receptor complex, the internalization of the toxin, the translocation of the toxin light chain into the cytoplasm and the enzymatic modification of a *C. botulinum* toxin substrate.

[0033] Strains of *Clostridium botulinum* produce seven antigenically-distinct types of Botulinum toxins (BoNTs), which have been identified by investigating botulism outbreaks in man (BoNT/A, /B, /E and /F), animals (BoNT/C1 and /D), or isolation from soil (BoNT/G). While all seven BoNT serotypes have similar structure and pharmacological properties, each also displays heterogeneous bacteriological characteristics. The genetic diversity of the *C. botulinum* strains is described in detail in Hill et al. (Journal of Bacteriology, Vol. 189, No. 3, p. 818-832 (2007)). In some embodiments of any of the aspects described herein, the BoNT is of strain A, E, C, B, D, F or G. Various non-naturally occurring *C. botulinum* neurotoxins are also known in the art and described, e.g., in International Patent Publications WO95/32738, WO96/33273, WO98/07864 and WO99/17806.

[0034] Toxins from the various *C. botulinum* strains share the same functional domain organization and overall structural architecture. *C. botulinum* toxins are each translated as a single chain polypeptide of approximately 150 kDa that is subsequently cleaved by proteolytic scission within a disulfide loop by a naturally-occurring proteases, such as, e.g., an endogenous *C. botulinum* toxin protease or a naturally-occurring proteases produced in the environment. This posttranslational processing yields a di-chain molecule comprising an approximately 50 kDa light chain (LC) and an approximately 100 kDa heavy chain (HC) held together by a single disulfide bond and noncovalent interactions. Each mature di-chain molecule comprises three functionally distinct domains: 1) a proteolytic domain located in the LC that includes a metalloprotease region containing a zinc-dependent endopeptidase activity which specifically targets core components of the neurotransmitter release apparatus; 2) a translocation domain contained within the amino-terminal half of the HC (H_N) that facilitates release of the LC from intracellular vesicles into the cytoplasm of the target cell; and 3) a binding domain found within the carboxyl-terminal half of the HC that determines the binding activity and binding specificity of the toxin to the receptor complex located at the surface of the target cell.

[0035] The binding, translocation and protease activity of these three functional domains are

all necessary for toxicity. The overall cellular intoxication mechanism whereby *C. botulinum* toxins enter a neuron and inhibit neurotransmitter release is similar, regardless of serotype or subtype. Without wishing to be bound by theory, the intoxication mechanism involves at least four steps: 1) receptor binding, 2) complex internalization, 3) light chain translocation, and 4) protease target modification. The process is initiated when the He domain of a *C. botulinum* toxin binds to a toxin-specific receptor located on the plasma membrane surface of a target cell. The binding specificity of a receptor complex is thought to be achieved, in part, by specific combinations of gangliosides and protein receptors. Once bound, the toxin/receptor complexes are internalized by endocytosis and the internalized vesicles are sorted to specific intracellular routes. The translocation step is triggered by the acidification of the vesicle compartment. Once translocated, light chain endopeptidase of the toxin is released from the intracellular vesicle into the cytosol where it specifically targets one of three proteins known as the core components of the neurotransmitter release apparatus (vesicle-associated membrane protein (VAMP)/synaptobrevin, synaptosomal-associated protein of 25 kDa (SNAP-25) and Syntaxin).

[0036] These core components are necessary for synaptic vesicle docking and fusion at the nerve terminal and constitute members of the soluble N-ethylmaleimide-sensitive factor-attachment protein-receptor (SNARE) family. BoNT/A and BoNT/E cleave SNAP-25 in the carboxyl-terminal region, releasing a nine or twenty-six amino acid segment, respectively, and BoNT/C1 also cleaves SNAP-25 near the carboxyl-terminus. The botulinum serotypes BoNT/B, BoNT/D, BoNT/F and BoNT/G, and tetanus toxin, act on the conserved central portion of VAMP, and release the amino-terminal portion of VAMP into the cytosol. BoNT/C1 cleaves syntaxin at a single site near the cytosolic plasma membrane surface. The selective proteolysis of synaptic SNAREs accounts for the block of neurotransmitter release caused by *C. botulinum* toxins in vivo. The SNARE protein targets of *C. botulinum* toxins are common to exocytosis in a variety of non-neuronal types; in these cells, as in neurons, light chain peptidase activity inhibits exocytosis, see, e.g., Yann Humeau et al., How Botulinum and Tetanus Neurotoxins Block Neurotransmitter Release, 82(5) Biochimie. 427-446 (2000); Kathryn Turton et al., Botulinum and Tetanus Neurotoxins: Structure, Function and Therapeutic Utility, 27(11) Trends Biochem. Sci. 552-558. (2002); Giovanna Lalli et al., The Journey of Tetanus and Botulinum Neurotoxins in Neurons, 11(9) Trends Microbiol. 431-437, (2003).

[0037] Described herein are single chain polypeptides in which a reporter protein is split or interrupted in its sequence by a linker segment. The split reporter protein is functional.

[0038] As used herein, the term "single chain" when referring to a polypeptide refers to a single polypeptide molecule having a series of amino acid residues, connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. That is, each recited element of a single chain polypeptide is connected to the other element(s) by means of a peptide bond. This is in contrast to multi-chain polypeptides which comprise multiple single chain polypeptides which can bind to each other via, e.g., hydrogen bonding or disulfide bonds to form a polypeptide complex.

[0039] The single chain polypeptides described herein can comprise luciferase and/or portions

thereof, e.g., the reporter protein can be luciferase. As used herein, "luciferase" refers to an enzyme that catalyzes a bioluminescent reaction, e.g., by catalyzing the oxidation of luciferin, emitting light and releasing oxyluciferin. A luciferase can be naturally-occurring or engineered. As used herein, a "functional" luciferase is a luciferase that is capable of catalyzing a reaction in the presence of a suitable substrate. Numerous variations and embodiments of luciferase are available, including, e.g., firefly luciferase (*Photinus pyralis*) (see, e.g, SEQ ID NO: 13), bacterial luciferase (*Vibriofischeri, Vibrio harveyi),* sea pansy luciferase (*Renilla reniformis*), dinoflagellate luciferase, *Cypridina* luciferase, *Coleoptera* luciferase, *Gaussia* luciferase (see, e.g., Heise, K., et al, "Dual luciferase assay for secreted luciferase based on Gaussia and NANOLUC" Assay Drug Dev. Technol.(2013)), copepod luciferase (see, e.g., Takenaka, Y., et al, "Computational analysis and functional expression of ancestral copepod luciferase" Gene (2013) and NANOLUC™ luciferase.

[0040] In some embodiments of any of the aspects described herein, the luciferase can be NANOLUC™ luciferase. As used herein, "NANOLUC luciferase" refers to a 19.1 kDa luciferase derived from *Oplophorus gracilirostris* luciferase that can utilize furimazine or coelenterazine as a substrate and which has the sequence of SEQ ID NO: 1. Variants of NANOLUC™ luciferase can also be used in any of the aspects and embodiments of the methods and compositions herein and are described, e.g., in US Patent No. 8,557,970.

[0041] In one aspect of any of the embodiments, described herein is a single chain polypeptide comprising, from N-terminal to C-terminal: a N-terminal fragment of a reporter protein; a linker comprising a neurotoxin cleavage site; and a C-terminal fragment of the reporter protein. As used herein, "fragment" refers to a part or portion of a molecule, e.g., a part or portion of a polypeptide. In the single chain polypeptides described herein, the N-terminal fragment and C-terminal fragments of a reporter protein collectively comprise a functional reporter protein sequence but do not individually comprise a functional reporter protein sequence. When present in the same single chain polypeptide, e.g., joined functionally by the linker sequence, the N-terminal and C-terminal fragments of a reporter protein generate a functional reporter fusion protein. In embodiments of the invention, the reporter protein is a luciferase protein. In some embodiments of any of the aspects, the fragments can be variants or derivatives of naturally-occurring sequences, reporter protein sequences described herein, and/or reporter protein sequences known in the art, e.g., they can have at least 90% sequence identity to a reporter protein sequence or have no more than 10 amino acid residue substitutions, deletions, or additions relative to a reporter protein sequence.

[0042] Also described herein is a single chain polypeptide comprising a N-terminal domain comprising a sequence with at least 90% sequence identity to amino acids 1-159 of the luciferase of SEQ ID NO 1 (N-nano$_{1-159}$); a linker comprising a neurotoxin cleavage site located C-terminal to the N-nano$_{1-159;}$ and a C-terminal domain having a sequence with at least 90% sequence identity amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano$_{160-170}$) located C-terminal to the linker; wherein the linker functionally joins the N-nano$_{1-159}$ and the C-nano$_{160-170}$ to generate a functional luciferase fusion protein.

[0043] In some embodiments of any of the aspects, a domain or fragment of a reporter protein can have at least at least 90%, at least 95%, or at least 98% sequence identity with a reporter protein sequence, e.g., with amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano$_{1-159}$) or amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano$_{160-170}$). In some embodiments of any of the aspects, the variant amino acids can be conservative substitution variations.

[0044] Also described herein is a single chain polypeptide comprising: a N-terminal domain comprising a sequence having less than 10 amino acid residues substitutions, deletions, or additions relative to amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano$_{1-159}$); a linker comprising a neurotoxin cleavage site located C-terminal to the N-nano$_{1-159;}$ and a C-terminal domain having a sequence having less than 3 amino acid residues substitutions, deletions, or additions relative to amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano$_{160-170}$) located C-terminal to the linker; wherein the linker functionally joins the N-nano$_{1-159}$ and the C-nano$_{160-170}$ to generate a functional luciferase fusion protein.

[0045] In some embodiments of any of the aspects, a domain or fragment of a reporter protein can be a sequence with 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, 2 or less, or 1 deletions, insertions, or substitutions relative to a reporter protein sequence, e.g., to amino acids 1-159 of the luciferase of SEQ ID NO: 1 (N-nano$_{1-159}$) or amino acids 160-170 of the luciferase of SEQ ID NO: 1 (C-nano$_{160-170}$). In some embodiments of any of the aspects, the substituted amino acids can be conservative substitution variations. In one aspect of any of the embodiments, described herein is a single chain polypeptide comprising: a) amino acids 1-159 of NANOLUC™ luciferase (N-nano$_{1-159}$); b) a linker comprising a neurotoxin cleavage site located C-terminal to the N-nano$_{1-159;}$ and c) amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$) located C-terminal to the linker; wherein the linker functionally joins the N-nano$_{1-159}$ and the C-nano$_{160-170}$ to generate a functional luciferase fusion protein.

[0046] As demonstrated herein, a luciferase protein can be interrupted by a linker sequence while still providing a functional luciferase. In some embodiments of any of the aspects described herein, a linker is provided between residues corresponding to residues 159 and 160 of SEQ ID NO: 1. In some embodiments of any of the aspects described herein, a linker is provided between residues 159 and 160 of SEQ ID NO: 1.

[0047] In some embodiments of any of the aspects described herein, a single chain polypeptide as described herein can comprise an N-terminal domain comprising a sequence corresponding to residues 1-159 of SEQ ID NO: 1 and a C-terminal domain comprising a sequence corresponding to residues 160-170 of SEQ ID NO: 1. In some embodiments of any of the aspects described herein, a single chain polypeptide as described herein can comprise

an N-terminal domain consisting essentially of a sequence corresponding to residues 1-159 of SEQ ID NO: 1 and a C-terminal domain consisting essentially of a sequence corresponding to residues 160-170 of SEQ ID NO: 1.

[0048] In some embodiments of any of the aspects described herein, a single chain polypeptide as described herein can comprise an N-terminal domain comprising residues 1-159 of SEQ ID NO: 1 and a C-terminal domain comprising residues 160-170 of SEQ ID NO: 1. In some embodiments of any of the aspects described herein, a single chain polypeptide as described herein can comprise an N-terminal domain consisting essentially of residues 1-159 of SEQ ID NO: 1 and a C-terminal domain consisting essentially of residues 160-170 of SEQ ID NO: 1. In some embodiments of any of the aspects described herein, a single chain polypeptide as described herein can comprise an N-terminal domain consisting of residues 1-159 of SEQ ID NO: 1 and a C-terminal domain consisting of residues 160-170 of SEQ ID NO: 1.

[0049] In the single chain polypeptides described herein, a linker domain is found between the N-terminal and C-terminal domains. As used herein, "linker" refers to a sequence exogenous to a reporter protein (e.g. a luciferase enzyme) and which is engineered to functionally join the N-terminal and C-terminal domains of the reporter protein. A linker can comprise, e.g., one or more cleavage sites, one or more spacers, one or more binding fragments, and one or more additional luciferase enzymes.

[0050] As used herein, "functionally join" refers to joining the two fragments of a reporter protein, e.g., a luciferase in such a way that the intervening sequence does not prevent the two fragments from forming an active and functional tertiary structure. Two fragments which are functionally joined will exhibit the activity which characterizes the protein in the absence of the linker sequence.

[0051] In some embodiments of any of the aspects described herein, a linker can comprise one or more cleavage sites, e.g., an amino acid sequence which is specifically cleaved by the action of one or more enzymes. In some embodiments of any of the aspects described herein, the cleavage site can be a neurotoxin cleavage site. Accordingly, a neurotoxin cleavage site comprises a sequence which is specifically cleaved by at least one neurotoxin. Neurotoxins are compounds which inhibit the function of and/or kill nervous tissue. In some embodiments of any of the aspects described herein, the neurotoxin can be an inhibitor of synaptic vesicle release. Exemplary non-limiting neurotoxin cleavage sites can include a *C. botulinum* neurotoxin (BoNT) and/or a Tetanus neurotoxin cleavage site. In embodiments of the invention, the linker comprises a *C. botulinum* neurotoxin (BoNT) cleavage site. In some embodiments of any of the aspects described herein, a linker can comprise multiple neurotoxin cleavage sites, e.g., it can be cleaved by multiple neurotoxins, thereby permitting the detection and/or measurement of multiple neurotoxins.

[0052] BoNT acts by cleaving SNARE proteins. Accordingly, a BoNT cleavage site can be from a SNARE protein. As used herein, "SNARE protein" refers to a superfamily of proteins that

mediate vesicle fusion, including fusion of synaptic vesicles of the presynaptic membrane in neurons. SNARE proteins include, by way of non-limiting examples, SNAP-25 (e.g., NCBI Gene ID: 6616, see also NCBI Reference Sequence: NP_570824.1 for human SNAP25b and NCBI Reference Sequence: NP_112253.1 for rat SNAP25b), synaptobrevins (VAMPs)(e.g., NCBI Gene ID Nos: 6843; 6844; 6845; 8673; 8674; 9341; 9554; 10652; 10791; and 26984) and syntaxins (e.g., NCBI Gene ID Nos. 2054; 6804; 6809; 6810; 6811; 8417; 8675; 8676; 8677; 9482; 10228; 23673; 53407; 55014; 112755; and 415177). SNARE scissile bonds cleaved by clostridial neurotoxins are described for example in Binz, Th. et al. "Clostridial neurotoxins: mechanism of SNARE cleavage and outlook on potential substrate specificity reengineering." Toxins 2.4 (2010): 665-682. Preferably, the BoNT cleavage site is from a human SNARE protein. Examples of human SNARE scissile bonds are provided in Table 1.

Table 1 - human SNARE scissile bonds cleaved by clostridial neurotoxins

Neurotoxin	SNARE	Scissile bond
BoNT/A	SNAP25 (SEQ ID NO: 23).	Gln197-Arg198
BoNTB	VAMP-1 (SEQ ID NO:24).	Gln78-Phe79
	VAMP-2 (SEQ ID NO:25).	Gln76-Phe77
	VAMP-3 (SEQ ID NO:26).	Gln59-Phe60
BoNT/C1	SNAP25 (SEQ ID NO: 23).	Arg198-Ala199
	Syntaxin 1A (SEQ ID NO: 27).	Lys253-Ala254
	Syntaxin 1B (SEQ ID NO: 28).	Lys252-Ala253
BoNT/D	VAMP-1 (SEQ ID NO:24).	Lys61-Leu62
	VAMP-2 (SEQ ID NO: 25).	Lys59-Leu60
	VAMP-3 (SEQ ID NO: 26).	Lys42-Leu43
BoNT/E	SNAP25 (SEQ ID NO: 23).	Arg180-Ile181
BoNT/F	VAMP-1 (SEQ ID NO: 24).	Gln60-Lys61
	VAMP-2 (SEQ ID NO: 25).	Gln58-Lys59
	VAMP-3 (SEQ ID NO: 26).	Gln41-Lys42
BoNT/G	VAMP-1 (SEQ ID NO: 24).	Ala83-Ala84
	VAMP-2 (SEQ ID NO: 25).	Ala81-Ala82
	VAMP-3 (SEQ ID NO: 26).	Ala64-Ala65
TeNT	VAMP-1 (SEQ ID NO: 24).	Gln78-Phe79
	VAMP-2 (SEQ ID NO: 25).	Gln76-Phe77
	VAMP-3 (SEQ ID NO: 26).	Gln59-Phe60

[0053] In one embodiment, the linker comprises at least one clostridial neurotoxin cleavage site, preferably a human neurotoxin cleavage site. A clostridial neurotoxin cleavage site may be defined as a sequence comprising at least amino acid residues P3P2P1P1'P2'P3' of a SNARE, preferably a human SNARE, wherein P1 and P1' are the residues located on either side of the

scissile peptide bond cleaved by the clostridial neurotoxin, for example Gln197 and Arg198 in human SNAP25 for BoNT/A.

[0054] In a preferred embodiment, a clostridial neurotoxin cleavage site comprises at least amino acid residues P4P3P2P1P1'P2'P3'P4' of the SNARE. In a more preferred embodiment, a clostridial neurotoxin cleavage site comprises at least amino acid residues P5P4P3P2P1P1'P2'P3'P4'P5' of the SNARE, more preferably at least amino acid residues P6P5P4P3P2P1P1'P2'P3'P4'P5'P6'.

[0055] In one embodiment, the linker comprises at least one BoNT/A cleavage site wherein said BoNT/A cleavage site comprises residues 195 to 200, preferably 194 to 201, 193 to 202 or 192 to 203 of human SNAP25 (SEQ ID NO: 23).

[0056] In one embodiment, the linker comprises at least one BoNT/B cleavage site wherein said BoNT/B cleavage site comprises residues 76 to 81, preferably 75 to 82, 74 to 83 or 73 to 84 of human VAMP-1 (SEQ ID NO: 24).

[0057] In one embodiment, the linker comprises at least one BoNT/B cleavage site wherein said BoNT/B cleavage site comprises residues 74 to 79, preferably 73 to 80, 72 to 81 or 71 to 82 of human VAMP-2 (SEQ ID NO: 25).

[0058] In one embodiment, the linker comprises at least one BoNT/B cleavage site wherein said BoNT/B cleavage site comprises residues 57 to 62, preferably 56 to 63, 55 to 64 or 54 to 65 of human VAMP-3 (SEQ ID NO: 26).

[0059] In one embodiment, the linker comprises at least one BoNT/C1 cleavage site wherein said BoNT/C1 cleavage site comprises residues 196 to 201, preferably 195 to 202, 194 to 203 or 193 to 204 of human SNAP25 (SEQ ID NO: 23).

[0060] In one embodiment, the linker comprises at least one BoNT/C1 cleavage site wherein said BoNT/C1 cleavage site comprises residues 251 to 256, preferably 250 to 257, 249 to 258 or 148 to 259 of human Syntaxin 1A (SEQ ID NO: 27).

[0061] In one embodiment, the linker comprises at least one BoNT/C1 cleavage site wherein said BoNT/C1 cleavage site comprises residues 252 to 257, preferably 251 to 258, 249 to 259 or 149 to 260 of human Syntaxin 1B (SEQ ID NO: 28).

[0062] In one embodiment, the linker comprises at least one BoNT/D cleavage site wherein said BoNT/D cleavage site comprises residues 59 to 64, preferably 58 to 65, 57 to 66 or 56 to 67 of human VAMP-1 (SEQ ID NO: 24).

[0063] In one embodiment, the linker comprises at least one BoNT/D cleavage site wherein said BoNT/D cleavage site comprises residues 57 to 62, preferably 56 to 63, 55 to 64 or 54 to 65 of human VAMP-2 (SEQ ID NO: 25).

[0064] In one embodiment, the linker comprises at least one BoNT/D cleavage site wherein said BoNT/D cleavage site comprises residues 40 to 45, preferably 39 to 46, 38 to 47 or 37 to 48 of human VAMP-3 (SEQ ID NO: 26).

[0065] In one embodiment, the linker comprises at least one BoNT/E cleavage site wherein said BoNT/E cleavage site comprises residues 178 to 183, preferably 177 to 184, 176 to 185 or 175 to 186 of human SNAP25 (SEQ ID NO: 23).

[0066] In one embodiment, the linker comprises at least one BoNT/F cleavage site wherein said BoNT/F cleavage site comprises residues 58 to 63, preferably 57 to 64, 56 to 65 or 55 to 66 of human VAMP-1 (SEQ ID NO: 24).

[0067] In one embodiment, the linker comprises at least one BoNT/F cleavage site wherein said BoNT/F cleavage site comprises residues 56 to 61, preferably 55 to 62, 54 to 63 or 53 to 64 of human VAMP-2 (SEQ ID NO: 25).

[0068] In one embodiment, the linker comprises at least one BoNT/F cleavage site wherein said BoNT/F cleavage site comprises residues 39 to 44, preferably 38 to 45, 37 to 46 or 36 to 47 of human VAMP-3 (SEQ ID NO: 26).

[0069] In one embodiment, the linker comprises at least one BoNT/G cleavage site wherein said BoNT/G cleavage site comprises residues 81 to 86, preferably 80 to 87, 79 to 88 or 78 to 89 of human VAMP-1 (SEQ ID NO: 24).

[0070] In one embodiment, the linker comprises at least one BoNT/G cleavage site wherein said BoNT/G cleavage site comprises residues 79 to 84, preferably 78 to 85, 77 to 86 or 76 to 87 of human VAMP-2 (SEQ ID NO: 25).

[0071] In one embodiment, the linker comprises at least one BoNT/G cleavage site wherein said BoNT/G cleavage site comprises residues 62 to 67, preferably 61 to 68, 60 to 69 or 59 to 70 of human VAMP-3 (SEQ ID NO: 26).

[0072] In one embodiment, the linker comprises at least one BoNT/B cleavage site wherein said TeNT cleavage site comprises residues 76 to 81, preferably 75 to 82, 74 to 83 or 73 to 84 of human VAMP-1 (SEQ ID NO: 24).

[0073] In one embodiment, the linker comprises at least one TeNT cleavage site wherein said TeNT cleavage site comprises residues 74 to 79, preferably 73 to 80, 72 to 81 or 71 to 82 of human VAMP-2 (SEQ ID NO: 25).

[0074] In one embodiment, the linker comprises at least one TeNT cleavage site wherein said BoNT/B cleavage site comprises residues 57 to 62, preferably 56 to 63, 55 to 64 or 54 to 65 of human VAMP-3 (SEQ ID NO: 26).

[0075] An example of suitable BoNT cleavage sites in SNARE proteins include, by way of non-limiting example, amino acids 141-206 of human SNAP-25b (e.g., SEQ ID NO: 10); and amino acid 35-96 of human VAMP1 (e.g., NCBI Gene ID: 6843). In some embodiments of any of the aspects described herein, amino acids of 35-96 of human VAMP1 can be amino acids 35-96 of SEQ ID NO: 16. Further non-limiting examples of BoNT cleavage sites in SNARE proteins include amino acids 60-87 of human VAMP2, amino acids 43-70 of human VAMP 3, and amino acids 62-89 of human VAMP1. In some embodiments of any of the aspects, amino acids 60-87 of human VAMP2 can be the sequence of SEQ ID NO: 19. In some embodiments of any of the aspects, amino acids 43-70 of human VAMP3 can be the sequence of SEQ ID NO: 20. In some embodiments of any of the aspects, amino acids 62-89 of human VAMP1 can be the sequence of SEQ ID NO: 21

[0076] In some embodiments of any of the aspects, a BoNT cleavage site can be a variant of any of the specific sequences recited herein, e.g., a variant of a native BoNT cleavage site found in a SNARE protein. In some embodiments of any of the aspects, a BoNT cleavage site can be a variant of amino acids 141-206 of SEQ ID NO: 10 or amino acids 35-96 of SEQ ID NO: 16. In some embodiments of any of the aspects, a BoNT cleavage site can have at least 80%, at least 85%, at least 90%, at least 95%, or at least 98% sequence identity with a native BoNT cleavage site found in a SNARE protein In some embodiments of any of the aspects, a BoNT cleavage site can have at least 80%, at least 85%, at least 90%, at least 95%, or at least 98% sequence identity with amino acids 141-206 of SEQ ID NO: 10 or amino acids 35-96 of SEQ ID NO: 16. In some embodiments of any of the aspects, the variant can be a conservative substitution variant.

[0077] In some embodiments of any of the aspects, a BoNT cleavage site can be a sequence with 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, 2 or less, or 1 deletions, insertions, or substitutions relative to a native BoNT cleavage site found in a SNARE protein. In some embodiments of any of the aspects, a BoNT cleavage site can be a sequence with 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, 2 or less, or 1 residues which vary relative to a native BoNT cleavage site found in a SNARE protein. In some embodiments of any of the aspects, a BoNT cleavage site can be a sequence with 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, 2 or less, or 1 deletion, insertion, or substitution relative to amino acids 141-206 of SEQ ID NO: 10 or amino acids 35-96 of SEQ ID NO: 16. In some embodiments of any of the aspects, a BoNT cleavage site can be a sequence with 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, 2 or less, or 1 residues which vary relative to with amino acids 141-206 of SEQ ID NO: 10 or amino acids 35-96 of SEQ ID NO: 16.

[0078] In some embodiments of any of the aspects described herein, a BoNT cleavage site can comprise amino acids 141-206 of human SNAP-25b (e.g., SEQ ID NO: 10); amino acid 35-96 of human VAMP1; amino acids 1-96 of VAMP1; human SNAP25; and/or amino acids 1-206 of human SNAP25b (SEQ ID NO: 14).

[0079] In some embodiments of any of the aspects described herein, a BoNT cleavage site

can be specifically recognized (e.g. cleaved) by BoNT A, B, C, D, E, F, and/or G. In some embodiments of any of the aspects described herein, a BoNT cleavage site can be specifically recognized (e.g. cleaved) by BoNT A, E and C. In some embodiments of any of the aspects described herein, a BoNT cleavage site can be specifically recognized (e.g. cleaved) by BoNT B, D, F and G.

[0080] In some embodiments of any of the aspects described herein, a linker can comprise one or more spacers. As used herein, "spacer" refers to an amino acid sequence that serves the structural purpose of separating two other sequences in the same peptide chain. In some embodiments of any of the aspects described herein, the spacer sequence can be a flexible peptide sequence. In some embodiments of any of the aspects described herein, a spacer can comprise glycine and serine residues. In some embodiments of any of the aspects described herein, a spacer can consist essentially of glycine and serine residues. In some embodiments of any of the aspects described herein, a spacer can consist of glycine and serine residues.

[0081] In some embodiments of any of the aspects described herein, a spacer can be from about 2 to about 30 amino acids in length. In some embodiments of any of the aspects described herein, a spacer can be from 2 to 30 amino acids in length. In some embodiments of any of the aspects described herein, a spacer can be from about 3 to about 20 amino acids in length. In some embodiments of any of the aspects described herein, a spacer can be from 3 to 20 amino acids in length. In some embodiments of any of the aspects described herein, a spacer can be from about 5 to about 15 amino acids in length. In some embodiments of any of the aspects described herein, a spacer can be from 5 to 15 amino acids in length.

[0082] Exemplary, non-limiting spacers can include GSSGGGGSGGGGSSG (SEQ ID NO: 4); GSSGGGGSGGGSSG (SEQ ID NO: 5); GGGGS (SEQ ID NO: 6); (GGGGS)n (n=1-3) (SEQ ID NO: 7); KESGSVSSEQLAQFRSLD (SEQ ID NO: 8); and EGKSSGSGSESKST (SEQ ID NO: 9). Linker design, selection, and further exemplary linkers are well-known in the art and described, e.g., in Chen, X., et al, "Fusion protein linkers: proterty, design and functionality" Adv. Drug Deliv. Rev. (2013).

[0083] In some embodiments of any of the aspects described herein, a linker can comprise a neurotoxin binding fragment, e.g., a sequence that is bound by and/or binds to a neurotoxin and which is not cleaved by the neurotoxin. In some embodiments of any of the aspects described herein, a neurotoxin binding fragment can be a fragment of a neurotoxin receptor, e.g., a receptor for the BoNT and/or a tetanus neurotoxin. In embodiments of the invention, the linker comprises a binding fragment of a BoNT receptor.

[0084] In some embodiments of any of the aspects described herein, the neurotoxin receptor can be a human, mouse, rat, or primate neurotoxin receptor. In a preferred embodiment, the neurotoxin receptor is a human neurotoxin receptor. In some embodiments of any of the aspects described herein, the neurotoxin receptor can be SV2C, e.g., human SV2C (NCBI Gene ID: 22987; GenBank: AAI00828.1). In some embodiments of any of the aspects described herein, the neurotoxin binding fragment of a receptor can comprise a sequence

corresponding to amino acids 529-566 of human SV2C (SV2C-L4, e.g., SEQ ID NO: 11). In some embodiments of any of the aspects described herein, the neurotoxin binding fragment can comprise amino acids 529-566 of human SV2C (SV2C-L4, e.g., SEQ ID NO: 11). In some embodiments of any of the aspects described herein, the neurotoxin receptor can be SYTI (NCBI Gene ID: 6857) or SYT2 (NCBI Gene ID: 127833). In some embodiments of any of the aspects described herein, the neurotoxin binding fragment of a receptor can comprise a sequence corresponding to amino acids 32-52 of SYTI (e.g., SEQ ID NO: 17) or amino acids 40-60 of SYT2 (SEQ ID NO: 18).

[0085] In some embodiments of any of the aspects described herein, a linker can comprise an intact second luciferase polypeptide. In some embodiments of any of the aspects described herein, the second luciferase is active before and/or after cleavage of the single chain polypeptide, e.g. by a neurotoxin. In some embodiments of any of the aspects described herein, the second luciferase is distinguishable from the first luciferase, e.g. it acts on a different substrate and/or produces a different wavelength of light. For example, if the first luciferase is NANOLUC, the second luciferase can be firefly luciferase (e.g., *Photinus pyralis*), bacterial luciferase (e.g., *Vibrio fischieri, Vibrio harveyi*), sea pansy luciferase (e.g., *Renilla reniformis*), dinoflagellate luciferase, or *Gaussia* luciferase. In some embodiments of any of the aspects described herein, the second luciferase can comprise a sequence corresponding to SEQ ID NO: 13. In some embodiments of any of the aspects described herein, the second luciferase can comprise SEQ ID NO: 13.

[0086] In some embodiments of any of the aspects described herein, a linker can comprise an intact second luciferase polypeptide located between the N-terminal reporter protein domain and at least one cleavage site. In some embodiments of any of the aspects described herein, a linker can comprise an intact second luciferase polypeptide located between the N-nano$_{1-159}$ and the BoNT cleavage site.

[0087] In some embodiments of any of the aspects described herein, the binding fragment can be located N-terminal of at least one cleavage site. In some embodiments of any of the aspects described herein, the binding fragment can be located between the N-nano$_{1-159}$ and the BoNT cleavage site. In some embodiments of any of the aspects described herein, the linker can comprise, from N-terminal to C-terminal, at least one binding fragment, at least one spacer, and at least one cleavage site. In some embodiments of any of the aspects described herein, the linker can comprise, from N-terminal to C-terminal, at least one binding fragment, at least one spacer, and at least one BoNT cleavage site.

[0088] In some embodiments of any of the aspects described herein, a linker can comprise, from N-terminus to C-terminus, one or more spacers and at least one cleavage site. In some embodiments of any of the aspects described herein, a linker can comprise, from N-terminus to C-terminus, one or more spacers, at least one cleavage site, and one or more spacers. In some embodiments of any of the aspects described herein, a linker can comprise, from N-terminus to C-terminus, at least one cleavage site, and one or more spacers. In some embodiments of any of the aspects described herein, a linker can comprise one or more

spacers located between the BoNT cleavage site and the N-nano$_{1-159}$, between the neurotoxin cleavage site and the C-nano$_{160-170}$, or a combination thereof.

[0089] In some embodiments of any of the aspects described herein, the linker can comprise a spacer located between a second luciferase polypeptide and a cleavage site. In some embodiments of any of the aspects described herein, the linker can comprise, from N-terminal to C-terminal, a second luciferase polypeptide, at least one spacer, and at least one cleavage site. In some embodiments of any of the aspects described herein, the linker can comprise a spacer located between the second luciferase polypeptide and the BoNT cleavage site.

[0090] In some embodiments of any of the aspects described herein, the single chain polypeptide can further comprise a polyhistidine affinity tag, e.g., His6 (SEQ ID NO: 12). In some embodiments of any of the aspects described herein, the polyhistidine affinity tag is located at the C-terminus of the polypeptide, e.g., C-terminal of the reporter protein C-terminal domain.

[0091] In one aspect of any of the embodiments, described herein is a single chain polypeptide comprising: a) amino acids 1-159 of NANOLUC™ luciferase (N-nano$_{1-159}$); b) a linker located C-terminal to the N-nano$_{1-159}$ comprising: i) a first spacer of 5 to 15 amino acids; ii) a binding fragment comprised of amino acids 529-566 of SV2C located C-terminal to the first spacer; iii) a second spacer located C-terminal to the binding fragment; iv) a BoNT cleavage site comprising amino acids 141-206 of human SNAP25b located C-terminal to the second spacer; iv) a third spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site; and c) amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$) located C-terminal to the linker; wherein the linker functionally joins the N-nano$_{1-159}$ and the C-nano$_{160-170}$ to generate a functional luciferase fusion protein. In some embodiments of any of the aspects described herein, the polypeptide further comprises a polyhistidine affinity tag C-terminal of the amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$).

[0092] In one aspect of any of the embodiments, described herein is a single chain polypeptide comprising: a) amino acids 1-159 of NANOLUC™ luciferase (N-nano$_{1-159}$); b) a linker located C-terminal to the N-nano$_{1-159}$ comprising: i) a first spacer from 5 to 15 amino acids; ii) a BoNT cleavage site comprising amino acids 141-206 of human SNAP25b located C-terminal to the first spacer; iii) a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site; and c) amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$) located C-terminal to the linker; wherein the linker functionally joins the N-nano$_{1-159}$ and the C-nano$_{160-170}$ to generate a functional luciferase fusion protein. In some embodiments of any of the aspects described herein, the polypeptide further comprises a polyhistidine affinity tag C-terminal of the amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$).

[0093] In one aspect of any of the embodiments, described herein is a single chain polypeptide

comprising: a) amino acids 1-159 of NANOLUC™ luciferase (N-nano$_{1-159}$); b) a linker located C-terminal to the N-nano$_{1-159}$ comprising: i) a first spacer of 5 to 15 amino acids; ii) a BoNT cleavage site comprising amino acids 35-96 of human VAMP1 located C-terminal to the first spacer; iii) a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site; and c) amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$) located C-terminal to the linker; wherein the linker functionally joins the N-nano$_{1-159}$ and the C-nano$_{160-170}$ to generate a functional luciferase fusion protein. In some embodiments of any of the aspects described herein, the polypeptide further comprises a polyhistidine affinity tag C-terminal of the amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$).

[0094] In one aspect of any of the embodiments, described herein is a single chain polypeptide comprising: a) amino acids 1-159 of NANOLUC™ luciferase (N-nano$_{1-159}$); b) a linker located C-terminal to the N-nano$_{1-159}$ comprising: i) a second functional luciferase polypeptide; ii) a first spacer of 5 to 15 amino acids located C-terminal of the second luciferase polypeptide; iii) a BoNT cleavage site comprising amino acids 1-206 of human SNAP25b (e.g. SEQ ID NO: 14) located C-terminal to the first spacer; iv) a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site; and c) amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$) located C-terminal to the linker; wherein the linker functionally joins the N-nano$_{1-159}$ and the C-nano$_{160-170}$ to generate a functional luciferase fusion protein.

[0095] In some embodiments of any of the aspects described herein, the polypeptide further comprises a polyhistidine affinity tag C-terminal of the amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$).

Table 2

Polypeptide Description	Sequence	SEQ ID NO:
NANOLUC TM Luciferase	MVFTLEDFVGDWEQTAAYNLDQVLEQGGVSSLLQNLAVSVTPI QRIVRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDH HFKVILPYGTLVIDGVTPNMLNYFGRPYEGIAVFDGKKITVTGTL WNGNKIIDERLITPDGSMLFRVTINSVTGYRLFEEIL	1
N-nano1-159	MVFTLEDFVGDWEQTAAYNLDQVLEQGGVSSLLQNLAVSVTPI QRIVRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDH HFKVILPYGTLVIDGVTPNMLNYFGRPYEGIAVFDGKKITVTGTL WNGNKIIDERLITPDGSMLFRVTINS	2
C-nano$_{160-170}$	VTGYRLFEEIL	3
141-206 of SNAP25b	ARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEK	10

Polypeptide Description	Sequence	SEQ ID NO:
(human and rat)	ADSNKTRIDEANQRATKMLGSG	
529-566 of human SV2C	NTYFKNCTFIDTVFDNTDFEPYKFIDSEFKNCSFFHNK	11
Firefly luciferase	MEDAKNIKKGPAPFYPLEDGTAGEQLHKAMKRYALVPGTIAFT DAHIEVDITYAEYFEMSVRLAEAMKRYGLNTNHRIVVCSENSLQ FFMPVLGALFIGVAVAPANDIYNERELLNSMGISQPTVVFVSKK GLQKILNVQKKLPIIQKIIIMDSKTDYQGFQSMYTFVTSHLPPGFN EYDFVPESFDRDKTIALIMNSSGSTGLPKGVALPHRTACVRFSHA RDPIFGNQIIPDTAILSVVPFHHGFGMFTTLGYLICGFRVVLMYRF EEELFLRSLQDYKIQSALLVPTLFSFFAKSTLIDKYDLSNLHEIAS GGAPLSKEVGEAVAKRFHLPGIRQGYGLTETTSAILITPEGDDKP GAVGKVVPFFEAKVVDLDTGKTLGVNQRGELCVRGPMIMSGY VNNPEATNALIDKDGWLHSGDIAYWDEDEHFFIVDRLKSLIKYK GYQVAPAELESILLQHPNIFDAGVAGLPDDDAGELPAAVVVLEH	13
	GKTMTEKEIVDYVASQVTTAKKLRGGVVFVDEVPKGLTGKLDA RKIREILIKAKKGGKIAV	
Human SNAP25b (NCBI Reference Sequence: NP_570824.1)	MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDA GIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFC GLCVCPCNKLKSSDAYKKAWGNNQDGVVASQPARVVDEREQ MAISGGFIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGN EIDTQNRQIDRIMEKADSNKTRIDEANQRATKMLGSG	14
Sensor 1 DNA sequence	ATGGTCTTCACACTCGAAGATTTCGTTGGGGACTGGGAACAG ACAGCCGCCTACAACCTGGACCAAGTCCTTGAACAGGGAGGT GTGTCCAGTTTGCTGCAGAATCTCGCCGTGTCCGTAACTCCGA TCCAAAGGATTGTCCGGAGCGGTGAAAATGCCCTGAAGATCG ACATCCATGTCATCATCCCGTATGAAGGTCTGAGCGCCGACC AAATGGCCCAGATCGAAGAGGTGTTTAAGGTGGTGTACCCTG TGGATGATCATCACTTTAAGGTGATCCTGCCCTATGGCACACT GGTAATCGACGGGGTTACGCCGAACATGCTGAACTATTTCGG ACGGCCGTATGAAGGCATCGCCGTGTTCGACGGCAAAAAGAT CACTGTAACAGGGACCCTGTGGAACGGCAACAAAATTATCGA CGAGCGCCTGATCACCCCCGACGGCTCCATGCTGTTCCGAGT AACCATCAACAGtgggagttccGGTGGTGGCGGGAGCGGAGGTGG AGGctcgAGCGGTgGAGCTCagAACACCTACTTCAAGAACTGCA CATTTATTGACACTGTTTTTGACAACACAGATTTTGAGCCATA TAAATTCATTGACAGTGAATTTAAAAACTGCTCGTTTTTTCAC AACAAGGGGGGCGGAGGTTCCGCCCGGGAAAATGAAATGGA TGAAAACCTAGAGCAGGTGAGCGGCATCATCGGAAACCTCCG TCATATGGCCCTAGACATGGGCAATGAGATTGACACCCAGAA TCGCCAGATTGACAGGATCATGGAGAAGGCTGACTCCAACAA AACCAGAATTGATGAAGCCAACCAACGTGCAACAAAGATGC TGGGAAGTGGTggGAATTCtggcTCGAGcGGTGGTGGCGGGAGC	15

Polypeptide Description	Sequence	SEQ ID NO:
	GGAGGTGGAGGGtcgtcaGGTGTGACCGGCTACCGGCTGTTCGA GGAGATTCTGGCGGCCGCACTCGAGCACCACCACCACCACCA CTGA	
Human VAMP 1	MSAPAQPPAE GTEGTAPGGG PPGPPPNMTS NRRLQQTQAQ VEEVVDIIRV NVDKVLERDQ KLSELDDRAD ALQAGASQFE SSAAKLKRKY WWKNCKMMIM LGAICAIIVV VIVIYFFT	16
Amino acids 32-52 of SYT1	GEGKEDAFSKLKQKFMNELHK	17
Amino acids 40-60 of SYT2	GESQEDMFAKLKEKFFNEINK	18
Amino acids 60-87 of	LSELDDRADAL QAGASQFETS AAKLKRK	19
human VAMP2		
Amino acids 43-70 of human VAMP3	LSELDDRA DALQAGASQFETSAAKLKRK	20
Amino acids 62-89 of human VAMP1	LSELDDRAD ALQAGASQFE SSAAKLKRK	21
Sensor 2 DNA sequences	ATGGTCTTCACACTCGAAGATTTCGTTGGGGACTGGGAACAG ACAGCCGCCTACAACCTGGACCAAGTCCTTGAACAGGGAGGT GTGTCCAGTTTGCTGCAGAATCTCGCCGTGTCCGTAACTCCGA TCCAAAGGATTGTCCGGAGCGGTGAAAATGCCCTGAAGATCG ACATCCATGTCATCATCCCGTATGAAGGTCTGAGCGCCGACC AAATGGCCCAGATCGAAGAGGTGTTTAAGGTGGTGTACCCTG TGGATGATCATCACTTTAAGGTGATCCTGCCCTATGGCACACT GGTAATCGACGGGGTTACGCCGAACATGCTGAACTATTTCGG ACGGCCGTATGAAGGCATCGCCGTGTTCGACGGCAAAAAGAT CACTGTAACAGGGACCCTGTGGAACGGCAACAAAATTATCGA CGAGCGCCTGATCACCCCCGACGGCTCCATGCTGTTCCGAGT AACCATCAACAGtgggagttccGGTGGTGGCGGGAGCGGAGGTGG AGGctcgAGCGGTgGAGCTCagGCCCGGGAAAATGAAATGGATG AAAACCTAGAGCAGGTGAGCGGCATCATCGGAAACCTCCGTC ATATGGCCCTAGACATGGGCAATGAGATTGACACCCAGAATC GCCAGATTGACAGGATCATGGAGAAGGCTGACTCCAACAAA ACCAGAATTGATGAAGCCAACCAACGTGCAACAAAGATGCT GGGAAGTGGTggGAATTCtggcTCGAGcGGTGGTGGCGGGAGCG GAGGTGGAGGGtcgtcaGGTGTGACCGGCTACCGGCTGTTCGAG GAGATTCTGGCGGCCGCACTCGAGCACCACCACCACCACCAC TGA	29

Polypeptide Description	Sequence	SEQ ID NO:
	TGA	
Sensor 3 DNA sequences	ATGGTCTTCACACTCGAAGATTTCGTTGGGGACTGGGAACAG ACAGCCGCCTACAACCTGGACCAAGTCCTTGAACAGGGAGGT GTGTCCAGTTTGCTGCAGAATCTCGCCGTGTCCGTAACTCCGA TCCAAAGGATTGTCCGGAGCGGTGAAAATGCCCTGAAGATCG ACATCCATGTCATCATCCCGTATGAAGGTCTGAGCGCCGACC AAATGGCCCAGATCGAAGAGGTGTTTAAGGTGGTGTACCCTG TGGATGATCATCACTTTAAGGTGATCCTGCCCTATGGCACACT GGTAATCGACGGGGTTACGCCGAACATGCTGAACTATTTCGG ACGGCCGTATGAAGGCATCGCCGTGTTCGACGGCAAAAAGAT CACTGTAACAGGGACCCTGTGGAACGGCAACAAAATTATCGA CGAGCGCCTGATCACCCCCGACGGCTCCATGCTGTTCCGAGT AACCATCAACAGtgggagttccGGTGGTGGCGGGAGCGGAGGTGG AGGctcgAGCGGTgGAGCTCagCAGCAAACCCAGGCACAAGTGG AGGAGGTGGTGGACATCATACGTGTGAACGTGGACAAGGTCC TGGAGAGGGACCAGAAGCTGTCAGAGCTGGATGACCGAGCT	30
	GATGCCTTGCAGGCAGGAGCATCACAATTTGAGAGCAGTGCT GCCAAGCTAAAGAGGAAGTATTGGTGGAAAAACTGCAAGggG AATTCtggcTCGAGcGGTGGTGGCGGGAGCGGAGGTGGAGGGtc gtcaGGTGTGACCGGCTACCGGCTGTTCGAGGAGATTCTGGCGG CCGCACTCGAGCACCACCACCACCACCACTGA	
In vivo construct DNA sequences	ATGGTCTTCACACTCGAAGATTTCGTTGGGGACTGGGAACAG ACAGCCGCCTACAACCTGGACCAAGTCCTTGAACAGGGAGGT GTGTCCAGTTTGCTGCAGAATCTCGCCGTGTCCGTAACTCCGA TCCAAAGGATTGTCCGGAGCGGTGAAAATGCCCTGAAGATCG ACATCCATGTCATCATCCCGTATGAAGGTCTGAGCGCCGACC AAATGGCCCAGATCGAAGAGGTGTTTAAGGTGGTGTACCCTG TGGATGATCATCACTTTAAGGTGATCCTGCCCTATGGCACACT GGTAATCGACGGGGTTACGCCGAACATGCTGAACTATTTCGG ACGGCCGTATGAAGGCATCGCCGTGTTCGACGGCAAAAAGAT CACTGTAACAGGGACCCTGTGGAACGGCAACAAAATTATCGA CGAGCGCCTGATCACCCCCGACGGCTCCATGCTGTTCCGAGT AACCATCAACAGtgggagttccGGTGGTGGCGGGAGCGGAGGTGG AGGctcgAGCGGTgGAGCTCagGAAGATGCCAAAAACATTAAGA AGGGCCCAGCGCCATTCTACCCACTCGAAGACGGGACCGCCG GCGAGCAGCTGCACAAAGCCATGAAGCGCTACGCCCTGGTGC CCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACA TTACCTACGCCGAGTACTTCGAGATGAGCGTTCGGCTGGCAG AAGCTATGAAGCGCTATGGGCTGAATACAAACCATCGGATCG TGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTT GGGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGA CATCTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCAG CCAGCCCACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAA GATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAAGAT CATCATCATGGATAGCAAGACCGACTACCAGGGCTTCCAAAG CATGTACACCTTCGTGACTTCCCATTTGCCACCCGGCTTCAAC GAGTACGACTTCGTGCCCGAGAGCTTCGACCGGGACAAAACC ATCGCCCTGATCATGAACAGTAGTGGCAGTACCGGATTGCCC	31

Polypeptide Description	Sequence	SEQ ID NO:
	AAGGGCGTAGCCCTACCGCACCGCACCGCTTGTGTCCGATTC AGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATCCCC GACACCGCTATCCTCAGCGTGGTGCCATTTCACCACGGCTTCG GCATGTTCACCACGCTGGGCTACTTGATCTGCGGCTTTCGGGT CGTGCTCATGTACCGCTTCGAGGAGGAGCTATTCTTGCGCAG CTTGCAAGACTATAAGATTCAATCTGCCCTGCTGGTGCCCAC ACTATTTAGCTTCTTCGCTAAGAGCACTCTCATCGACAAGTAC GACCTAAGCAACTTGCACGAGATCGCCAGCGGCGGGGCGCC GCTCAGCAAGGAGGTAGGTGAGGCCGTGGCCAAACGCTTCCA CCTACCAGGCATCCGCCAGGGCTACGGCCTGACAGAAACAAC CAGCGCCATTCTGATCACCCCCGAAGGGGACGACAAGCCTGG CGCAGTAGGCAAGGTGGTGCCCTTCTTCGAGGCTAAGGTGGT GGACTTGGACACCGGTAAGACACTGGGTGTGAACCAGCGCG GCGAGCTGTGCGTCCGTGGCCCCATGATCATGAGCGGCTACG	
	TTAACAACCCCGAGGCTACAAACGCTCTCATCGACAAGGACG GCTGGCTGCACAGCGGCGACATCGCCTACTGGGACGAGGACG AGCACTTCTTCATCGTGGACCGGCTGAAGAGCCTGATCAAAT ACAAGGGCTACCAGGTAGCCCCAGCCGAACTGGAGAGCATC CTGCTGCAACACCCCAACATCTTCGACGCCGGGGTCGCCGGC CTGCCCGACGACGATGCCGGCGAGCTGCCCGCCGCAGTCGTC GTGCTGGAACACGGTAAAACCATGACCGAGAAGGAGATCGT GGACTATGTGGCCAGCCAGGTTACAACCGCCAAGAAGCTGCG CGGTGGTGTTGTGTTCGTGGACGAGGTGCCTAAAGGACTGAC CGGCAAGTTGGACGCCCGCAAGATCCGCGAGATTCTCATTAA GGCCAAGAAGGGCGGCAAGATCGCCGTGGGGGGCGGAGGTT CCGCCGAGGACGCAGACATGCGTAATGAACTGGAGGAGATG CAGAGGAGGGCTGACCAGCTGGCTGATGAGTCCCTGGAAAG CACCCGTCGCATGCTGCAGCTGGTCGAAGAGAGTAAAGATGC TGGCATCAGGACTTTGGTTATGTTGGATGAGCAAGGCGAACA ACTGGAACGCATTGAGGAAGGGATGGACCAAATCAATAAGG ATATGAAAGAAGCAGAAAAGAATTTGACGGACCTAGGAAAA TTCTGCGGGCTTTGTGTGTGTCCCTGTAACAAGCTTAAATCCA GTGATGCTTACAAAAAAGCCTGGGGCAATAATCAGGATGGA GTAGTGGCCAGCCAGCCTGCCCGTGTGGTGGATGAACGGGAG CAGATGGCCATCAGTGGTGGCTTCATCCGCAGGGTAACAAAC GATGCCCGGGAAAATGAAATGGATGAAAACCTAGAGCAGGT GAGCGGCATCATCGGAAACCTCCGTCATATGGCCCTAGACAT GGGCAATGAGATTGACACCCAGAATCGCCAGATTGACAGGAT CATGGAGAAGGCTGACTCCAACAAAACCAGAATTGATGAAG CCAACCAACGTGCAACAAAGATGCTGGGAAGTGGTggGAATT CtggcTCGAGcGGTGGTGGCGGGAGCGGAGGTGGAGGGtcgtcaG GTGTGACCGGCTACCGGCTGTTCGAGGAGATTCTGTAA	

[0096] In one aspect of any of the embodiments, described herein is a nucleic acid comprising a nucleotide sequence that encodes a single chain polypeptide as described herein. In some embodiments of any of the aspects described herein, the nucleic acid can comprise SEQ ID NO: 15, 29, 30, or 31.

[0097] Another aspect of the invention relates to a nucleic acid vector comprising the nucleic acid molecule described herein. In one embodiment the vector is an expression vector. Such an expression vector is referred to herein as an expression construct, and comprises a nucleic acid molecule disclosed herein operably-linked to the expression vector useful for expressing the nucleic acid molecule in a cell or cell-free extract. A wide variety of expression vectors can be employed for expressing a nucleic acid molecule encoding a single chain polypeptide of the present invention including, without limitation, a viral expression vector; a prokaryotic expression vector; eukaryotic expression vectors, such as, e.g., a yeast expression vector, an insect expression vector and a mammalian expression vector; and a cell-free extract expression vector. It is further understood that expression vectors useful to practice aspects of these methods may include those which express the single chain polypeptide under control of a constitutive, tissue-specific, cell-specific or inducible promoter element, enhancer element or both. Non-limiting examples of expression vectors, along with well-established reagents and conditions for making and using an expression construct from such expression vectors are readily available from commercial vendors that include, without limitation, BD Biosciences-Clontech, Palo Alto, Calif; BD Biosciences Pharmingen, San Diego, Calif; Invitrogen, Inc, Carlsbad, Calif.; EMD Biosciences-Novagen, Madison, Wis.; QIAGEN, Inc., Valencia, Calif; and Stratagene, La Jolla, Calif. The selection, making and use of an appropriate expression vector are routine procedures well within the scope of one skilled in the art and from the teachings herein.

[0098] In one aspect of any of the embodiments, described herein is a nucleic acid vector comprising a nucleic acid comprising a nucleotide sequence that encodes a single chain polypeptide as described herein. In some embodiments of any of the aspects described herein, the nucleic acid can comprise SEQ ID NO: 15. In some embodiments of any of the aspects described herein, the vector can be an expression vector and comprise the nucleic acid sequence in expressible form. In some embodiments of any of the aspects described herein, the vector can be a viral expression vector, a prokaryotic expression vector, a yeast expression vector, an insect expression vector, or a mammalian expression vector.

[0099] The term "vector", as used herein, refers to a nucleic acid construct designed for delivery to a cell or transfer between different cells. Many vectors useful for transferring exogenous genes into target cells are available, e.g. the vectors may be episomal, e.g., plasmids, virus derived vectors or may be integrated into the target cell genome, through homologous recombination or random integration. In some embodiments of any of the aspects described herein, a vector can be an expression vector. As used herein, the term "expression vector" refers to a vector that has the ability to incorporate and express heterologous nucleic acid fragments in a cell. An expression vector may comprise additional elements. The nucleic acid incorporated into the vector can be operatively linked to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that polynucleotide sequence.

[0100] Another aspect of the invention relates to a cell comprising the nucleic acid molecule or expression construct described herein. The cell can be for propagation of the nucleic acid or

for expression of the nucleic acid, or both. In one aspect of any of the embodiments, described herein is a cell comprising a nucleic acid and/or vector as described herein. In some embodiments of any of the aspects described herein, the cell expresses a single chain polypeptide as described herein. In some embodiments of any of the aspects described herein, the cell can be a prokaryotic cell, 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. In some embodiments of any of the aspects, the cell is a neuronal cell, in particular, cells with a high sensitivity to BoNT.

[0101] 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 A, 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 of a BoNT, about 90fM or less of a BoNT, about 80fM or less of a BoNT, about 70fM or less of a BoNT, about 60fM or less of a BoNT, about 50fM or less of a BoNT, about 40fM or less of a BoNT, about 30fM or less of a BoNT, about 20fM or less of a BoNT, or about 10fM or less of a BoNT.

[0102] In some embodiments, the cell is a primary neuronal cell with a high sensitivity to BoNT, e.g., cortical neurons, hippocampal neurons, and/or spinal cord neurons.

[0103] In some embodiments, the cell is from a neuronal cell line with a high sensitivity to BoNT, e.g. BE(2)-M17, Kelly, LA1-SSn, N1 E-115, N4TG3, N18, Neuro-2a, NG108-15, PC12, SH-SY5Y, SiMa, and/or SK-N-BE(2)-C.

[0104] In some embodiments, 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).

[0105] In some embodiments of any of the aspects, the cell can be a prokaryotic cell, including, without limitation, strains of aerobic, microaerophilic, capnophilic, facultative, anaerobic, gram-negative and gram-positive bacterial cells such as those derived from, e.g., *Escherichia coli, Bacillus subtilis, Bacillus licheniformis, Bacteroides fragilis, Clostridium perfringens, Clostridium difficile, Caulobacter crescentus, Lactococcus lactis, Methylobacterium extorquens, Neisseria meningirulls, Neisseria meningitidis, Pseudomonas fluorescens* and *Salmonella typhimurium*; and a eukaryotic cell including, without limitation, yeast strains, such

as, e.g., those derived from *Pichia pastoris, Pichia methanolica, Pichia angusta, Schizosaccharomyces pombe, Saccharomyces cerevisiae* and *Yarrowia lipolytica;* a insect cell or cell line derived from insects, such as, e.g., those derived from *Spodoptera frugiperda, Trichoplusia ni, Drosophila melanogaster* and *Manduca sexta*; or a mammalian cell or cell line derived from mammalian cells, such as, e.g., those derived from mouse, rat, hamster, porcine, bovine, equine, primate and human. Cell lines may be obtained from the American Type Culture Collection, European Collection of Cell Cultures and the German Collection of Microorganisms and Cell Cultures. Non-limiting examples of specific protocols for selecting, making and using an appropriate cell line are described in e.g., INSECT CELL CULTURE ENGINEERING (Mattheus F. A. Goosen et al. eds., Marcel Dekker, 1993); INSECT CELL CULTURES: FUNDAMENTAL AND APPLIED ASPECTS (J. M. Vlak et al. eds., Kluwer Academic Publishers, 1996); Maureen A. Harrison & Ian F. Rae, GENERAL TECHNIQLTES OF CELL CULTURE (Cambridge University Press, 1997); CELL AND TISSUE CULTURE: LABORATORY PROCEDURES (Alan Doyle et al eds., John Wiley and Sons, 1998); R. Ian Freshney, CLTLTURE OF ANIMAL CELLS: A MANUAL OF BASIC TECHNIQUE (Wiley-Liss, 4.sup.th ed. 2000); ANIMAL CELL CULTURE: A PRACTICAL APPROACH (John R. W. Masters ed., Oxford University Press, 3.sup.rd ed. 2000); MOLECULAR CLONING A LABORATORY MANUAL, supra, (2001); BASIC CELL CULTURE: A PRACTICAL APPROACH (John M. Davis, Oxford Press, 2.sup.nd ed. 2002); and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, supra, (2004). These protocols are routine procedures within the scope of one skilled in the art and from the teaching herein.

[0106] The compositions described herein can be used in methods to detect and/or measure the presence, potency, and/or activity of, e.g., a neurotoxin (e.g. BoNT). In the presence of BoNT, a single chain polypeptide with a BoNT cleavage site will be cleaved in the linker sequence, disrupting the activity of the split reporter protein (e.g. luciferase). Accordingly, a decrease in the activity of the reporter protein indicates the presence of BoNT. In one aspect of any of the embodiments, described herein is a method for detecting *C. botulinum* neurotoxin (BoNT) activity in a sample, comprising: a) contacting the sample to a single chain polypeptide as described herein, under conditions appropriate for BoNT activity; and determining luciferase activity of the polypeptide, as compared to luciferase activity of the polypeptide in the absence of the sample, wherein a decrease of luciferase activity indicates BoNT activity in the sample. In one aspect of any of the embodiments, described herein is an *in vitro* method for determining the potency of a botulinum neurotoxin, comprising: a) contacting the neurotoxin to a single chain polypeptide as described herein under conditions appropriate for BoNT activity; and b) determining the luciferase activity of the polypeptide, as compared to a reference, thereby determining the potency. In some embodiments of any of the aspects, the methods are suitable for batch release, e.g. for batch release testing and/or lot release testing. In some embodiments of any of the aspects described herein, the methods described herein can be performed *in vitro.*

[0107] In one aspect of any of the embodiments, described herein is a method for detecting *C. botulinum* neurotoxin (BoNT) activity in a sample, comprising: a) contacting the sample to a single chain polypeptide as described herein, under conditions appropriate for BoNT activity;

and b) determining luciferase activity of the polypeptide, as compared to luciferase activity of the polypeptide in the absence of the sample, wherein a decrease of luciferase activity indicates BoNT activity in the sample. In one aspect of any of the embodiments, described herein is a method for detecting C. botulinum neurotoxin (BoNT) activity in a sample, comprising: a) contacting the sample to a single chain polypeptide comprising: amino acids 1-159 of NANOLUC™ luciferase (N-nano$_{1-159}$), amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$), separated and functionally joined by a linker comprising a BoNT cleavage site located C-terminal to the N-nano$_{1-159}$; and N-terminal to the C-nano$_{160-170}$, under conditions appropriate for BoNT activity; and b) determining luciferase activity of the polypeptide, as compared to luciferase activity of the polypeptide in the absence of the sample, wherein a decrease of luciferase activity indicates BoNT activity in the sample.

[0108] In one aspect of any of the embodiments, described herein is a method for detecting *C. botulinum* neurotoxin (BoNT) activity in a sample, comprising: a) contacting the sample to a single chain polypeptide comprising: amino acids 1-159 of NANOLUC™ luciferase (N-nano$_{1-159}$), amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$), separated and functionally joined by a linker comprising a BoNT cleavage site located C-terminal to the N-nano$_{1-159}$; and N-terminal to the C-nano$_{160-170}$, under conditions appropriate for BoNT activity; and b) determining luciferase activity of the polypeptide, as compared to activity of the polypeptide in the absence of the sample, wherein a decrease in the luciferase activity is an indication of BoNT activity in the sample. In some embodiments of any the aspects described herein, the linker comprises a first spacer of 5 to 15 amino acids, a binding fragment of amino acids 529-566 of human SV2C located C-terminal to the first spacer and N-terminal to the BoNT cleavage site, and a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acids 141-206 of human SNAP25. In some embodiments of any the aspects described herein, the linker comprises a first spacer of 5 to 15 amino acids located N-terminal to the BoNT cleavage site, and a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acids 141-206 of human SNAP25. In some embodiments of any the aspects described herein, the linker comprises a first spacer of 5 to 15 amino acids located N-terminal to the BoNT cleavage site, a second spacer of 5 to 15 amino acids located C-terminal to the BoNT cleavage site, wherein the BoNT cleavage site comprises amino acids 35-96 of human VAMP1.

[0109] In one aspect of any of the embodiments, described herein is a method for determining the EC50 of a BoNT, comprising: a) contacting a single chain polypeptide as described herein, under conditions appropriate for BoNT activity with the BoNT at a first concentration; and determining luciferase activity of the polypeptide, as compared to luciferase activity of the polypeptide in the absence of the sample; b) repeating step a) at a different concentration of the BoNT until the EC50 can be determined. In some embodiments of any of the aspects described herein, the different concentrations of BoNT encompass at least one order of magnitude difference.

[0110] As known in the art, the "half maximal effective concentration (EC50)" refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after some specified exposure time. It is commonly used as a measure of drug's potency. The EC50 of a graded dose response curve therefore represents the concentration of a compound where 50% of its maximal effect is observed. The EC50 of a quantal dose response curve represents the concentration of a compound where 50% of the population exhibits a response, after a specific exposure duration.

[0111] As used herein, "conditions appropriate for BoNT activity" refers to conditions (e.g. temperature, pH, cofactors, etc) under which BoNT can specifically cleave a BoNT cleavage site. In some embodiments of any of the aspects described herein, conditions appropriate for BoNT activity can comprise a buffer of HEPES, 20 µM $ZnCl_2$, 2 mM DTT, 1 mg/ml BSA, pH 7.1. In some embodiments of any of the aspects described herein, conditions appropriate for BoNT activity can comprise a buffer of HEPES, 10-30 µM $ZnCl_2$, 1-3 mM DTT, 0.5-2 mg/ml BSA, pH 6.5-7.5. In some embodiments of any of the aspects described herein, conditions appropriate for BoNT activity can comprise a buffer of HEPES, 5-50 µM $ZnCl_2$, 0.1-10 mM DTT, 0.1-10 mg/ml BSA, pH 6.5-7.5.

[0112] In some embodiments of any of the aspects described herein, the conditions can comprise incubation at about 37°C for a period of from about 1 hour to about 36 hours. In some embodiments of any of the aspects described herein, the conditions can comprise incubation at about 37°C for a period of from 1 hour to 36 hours. In some embodiments of any of the aspects described herein, the conditions can comprise incubation at about 37°C for a period of from about 1 hour to about 24 hours. In some embodiments of any of the aspects described herein, the conditions can comprise incubation at about 37°C for a period of from 1 hour to 24 hours. In some embodiments of any of the aspects described herein, the conditions can comprise incubation at about 37°C for a period of from about 4 hours to about 24 hours. In some embodiments of any of the aspects described herein, the conditions can comprise incubation at about 37°C for a period of from 4 hours to 24 hours.

[0113] In some embodiments of any of the aspects described herein, the single chain polypeptide is present at a concentration of from about 3 nM to about 300 nM. In some embodiments of any of the aspects described herein, the single chain polypeptide is present at a concentration of from 3 nM to 300 nM. In some embodiments of any of the aspects described herein, the single chain polypeptide is present at a concentration of from about 30 nM to about 300 nM. In some embodiments of any of the aspects described herein, the single chain polypeptide is present at a concentration of from 30 nM to 300 nM. In some embodiments of any of the aspects described herein, the single chain polypeptide is present at a concentration of about 30 nM. In some embodiments of any of the aspects described herein, the single chain polypeptide is present at a concentration of 30 nM.

[0114] BoNT activity can also be detected in cells, e.g., in neuronal cells. In one aspect of any of the embodiments, described herein is a method for detecting *C. botulinum* neurotoxin

(BoNT) activity in a sample, comprising: a) contacting the sample to neuronal cells expressing a single chain polypeptide as described herein; b) incubating the neuronal cells for a period of from about 12 hours to about 60 hours; c) harvesting lysate from the neuronal cells; and d) measuring NANOLUC™ luciferase activity in the lysate, as compared to NANOLUC™ luciferase activity in identically treated neuronal cells in the absence of the sample, wherein a decrease in the NANOLUC™ luciferase activity indicates BoNT activity in the sample. In one aspect of any of the embodiments, described herein is a method for detecting *C. botulinum* neurotoxin (BoNT) activity in a sample, comprising: a) contacting the sample to neuronal cells expressing a single chain polypeptide comprising: amino acids 1-159 of NANOLUC™ luciferase (N-nano$_{1-159}$), amino acids 160-170 of NANOLUC™ luciferase (C-nano$_{160-170}$), separated and functionally joined by a linker comprising a BoNT cleavage site located C-terminal to the N-nano$_{1-159}$ and N-terminal to the C-nano$_{160-170}$; b) incubating the neuronal cells for a period of from about 12 hours to about 60 hours; c) harvesting lysate from the neuronal cells; and d) measuring NANOLUC™ luciferase activity in the lysate, as compared to NANOLUC™ luciferase activity in identically treated neuronal cells in the absence of the sample, wherein a decrease in the NANOLUC™ luciferase activity indicates BoNT activity in the sample. In some embodiments of any of the aspects described herein, the linker further comprises an intact second luciferase polypeptide located between the N-nano$_{1-159}$ and the cleavage site. In some embodiments of any of the aspects described herein, the neuronal cells express the single chain polypeptide from a viral expression vector. In some embodiments of any of the aspects described herein, the neuronal cells express the single chain polypeptide from a viral expression vector for at least 3 days prior to the contacting step. In some embodiments of any of the aspects described herein, the neuronal cells express the single chain polypeptide from a viral expression vector for at least 4 days prior to the contacting step. In some embodiments of any of the aspects described herein, the neuronal cells express the single chain polypeptide from a viral expression vector for at least 6 days prior to the contacting step. In some embodiments of any of the aspects described herein, the neuronal cells express the single chain polypeptide from a viral expression vector for 6 days prior to the contacting step. In some embodiments of any of the aspects described herein, the viral expression system is a lentivirus expression system. In some embodiments of any of the aspects described herein, the incubating step b) is from about 12 hours to about 72 hours. In some embodiments of any of the aspects described herein, the incubating step b) is about 48 hours. In some embodiments of any of the aspects described herein, the incubating step b) is 48 hours.

[0115] In some embodiments of any of the aspects described herein, the step of harvesting lysate can comprise addition of a lysis buffer. In some embodiments of any of the aspects described herein, the step of harvesting lysate can comprise centrifugation and/or sedimentation.

[0116] In some embodiments of any of the aspects described herein, a method can comprise the use of a combination of single chain polypeptides, each polypeptide having a different BoNT cleavage sites and/or combination of BoNT cleavage sites.

[0117] As used herein, "determining luciferase activity" refers to detecting, quantitatively or qualitatively, the level of light being generated by a luciferase (or a sample that may contain functional luciferase) in the presence of a luciferase substrate. Luminesence can be detected by any means known in the art, e.g., by luminescence microscopy, photometers, luminescence plate readers, photomultiplier detectors, and the like.

[0118] Luciferase substrates can include naturally-occurring luciferins as well as engineered substrates. Luciferins, for example, include firefly luciferin, *Cypridina* [also known as *Vargula*] luciferin [coelenterazine], bacterial luciferin, as well as synthetic analogs of these substrates. In some embodiments of any of the aspects described herein, the luciferin is coelenterazine and analogues thereof, which include molecules in U.S. Pat. No. 6,436,682, and for example, see Zhao et al, (2004), Mol Imaging, 3;43-54. Additional substrates are described in, e.g., U.S. Pat. Nos. 5,374,534; 5,098,828; 6,436,682; 5,004,565; 5,455,357; and 4,950,588.

[0119] In some embodiments of any of the aspects, the luciferase activity can be determined by addition of luciferase substrate to the single chain polypeptide and quantitative measurement of a luminescent signal produced. In some embodiments of any of the aspects described herein, the substrate for NANOLUC™ luciferase can be furimazine (2-furanylmethyl-deoxy-coelenterazine).

[0120] In some embodiments of any of the aspects described herein, the method can further comprise measuring the activity of a second luciferase comprised by the single chain polypeptide. In some embodiments of any of the aspects described herein, the activity of the second luciferase can be measured in the cell lysate. In some embodiments of any of the aspects described herein, the activity of the second luciferase can permit normalization of expression of the single chain polypeptide across samples, cells, cell populations, and/or wells. In some embodiments of any of the aspects described herein, the luciferase activity of the second luciferase polypeptide in the sample is determined and used as an indicator of total single chain polypeptide present in the harvested lysate.

[0121] In one aspect of any of the embodiments, described herein is a kit comprising: a) one or more single chain polypeptides as described herein, with each or a combination of the single chain polypeptides packaged into a separate container; one or more nucleic acids or nucleic acid vectors as described herein, with each or a combination of the nucleic acids or nucleic acid vectors packaged into a separate container; and/or one or more cells as described herein with each or a combination of the cells packaged into a separate container.

[0122] A kit is any manufacture (e.g., a package or container) comprising at least one reagent, e.g., a single chain polypeptide, nucleic acid encoding a single chain polypeptide, or cell comprising a nucleic acid encoding a single chain polypeptide as described herein, the manufacture being promoted, distributed, or sold as a unit for performing the methods or assays described herein.

[0123] The kits described herein include reagents and/or components that permit assaying the level of activity of a reporter, e.g., one or more types of luciferase. Such reagents comprise in addition to single chain polypeptides, for example, buffer solutions, substrates, or washing liquids etc. Furthermore, the kit can comprise an amount of a neurotoxin, e.g., BoNT, or luciferase which can be used for a calibration of the kit or as an internal control. Additionally, the kit may comprise an instruction leaflet and/or may provide information as to the relevance of the obtained results

[0124] For convenience, the meaning of some terms and phrases used in the specification, examples, and appended claims, are provided below. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. If there is an apparent discrepancy between the usage of a term in the art and its definition provided herein, the definition provided within the specification shall prevail.

[0125] For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here.

[0126] The terms "decrease", "reduced", "reduction", or "inhibit" are all used herein to mean a decrease by a statistically significant amount. In some embodiments of any of the aspects described herein, "reduce," "reduction" or "decrease" or "inhibit" typically means a decrease by at least 10% as compared to a reference level (e.g. the absence of a given treatment or agent) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% , or more.

[0127] The terms "increased", "increase", "enhance", or "activate" are all used herein to mean an increase by a statically significant amount. In some embodiments of any of the aspects described herein, the terms "increased", "increase", "enhance", or "activate" can mean an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. In the context of a marker, an "increase" is a statistically significant increase in such marker level.

[0128] As used herein, a "subject" means a human or animal. Usually the animal is a

vertebrate such as a primate, rodent, domestic animal or game animal. Primates include chimpanzees, cynomologous monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. In some embodiments of any of the aspects described herein, the subject is a mammal, e.g., a primate, e.g., a human. The terms, "individual," "patient" and "subject" are used interchangeably herein.

[0129] Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but is not limited to these examples. Mammals other than humans can be advantageously used as subjects that represent animal models of botulinum activity. A subject can be male or female.

[0130] As used herein, the term "binding activity" means that one molecule is contacting another molecule via at least one intermolecular or intramolecular force, including, without limitation, a covalent bond, an ionic bond, a metallic bond, a hydrogen bond, a hydrophobic interaction, a van der Waals interaction, and the like, or any combination thereof. "Bound" and "bind" are considered terms for binding.

[0131] In some embodiments of any of the aspects described herein, a single chain polypeptide can be isolated or purified. By "isolated" is meant a material that is free to varying degrees from components which normally accompany it as found in its native state. "Isolate" denotes a degree of separation from original source or surroundings, e.g. from a cell or cell lysate.

[0132] The term "purified" is used to refer to a substance such as a polypeptide that is "substantially pure", with respect to other components of a preparation (e.g., other polypeptides). It can refer to a polypeptide that is at least about 50%, 60%, 70%, or 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to other components. Recast, the terms "substantially pure" or "essentially purified", with regard to a polypeptide, refers to a preparation that contains fewer than about 20%, more preferably fewer than about 15%, 10%, 8%, 7%, most preferably fewer than about 5%, 4%, 3%, 2%, 1%, or less than 1%, of one or more other components (e.g., other polypeptides or cellular components).

[0133] As used herein, "contacting" refers to any suitable means for delivering, or exposing, a sample or agent to a single chain polypeptide as described herein (or a cell comprising said polypeptide). Exemplary delivery methods include, but are not limited to, direct delivery to a sample or to a cell culture medium, perfusion, injection, or other delivery method well known to one skilled in the art.

[0134] The term "sample" or "test sample" as used herein denotes a sample taken or isolated from any source, e.g., biological, environmental, industrial, or otherwise. The term also

includes a mixture of the above-mentioned samples. The term "test sample" also includes untreated or pretreated (or pre-processed) samples. The test sample can be obtained by removing a sample from the source, but can also be accomplished by using a previously isolated sample (e.g. isolated at a prior timepoint and isolated by the same or another person).

[0135] In some embodiments of any of the aspects described herein, the test sample can be an untreated test sample. As used herein, the phrase "untreated test sample" refers to a test sample that has not had any prior sample pre-treatment except for dilution and/or suspension in a solution. Exemplary methods for treating a test sample include, but are not limited to, centrifugation, filtration, sonication, homogenization, heating, freezing and thawing, and combinations thereof. In some embodiments of any of the aspects described herein, the test sample can be a frozen test sample, e.g., a frozen environmental sample, frozen industrial sample, or frozen tissue. The frozen sample can be thawed before employing methods, assays and systems described herein. After thawing, a frozen sample can be centrifuged before being subjected to methods, assays and systems described herein. In some embodiments of any of the aspects described herein, the test sample is a clarified test sample prepared, for example, by centrifugation and collection of a supernatant. In some embodiments of any of the aspects described herein, a test sample can be a pre-processed test sample, for example, supernatant or filtrate resulting from a treatment selected from the group consisting of centrifugation, filtration, thawing, purification, and any combinations thereof. In some embodiments of any of the aspects described herein, the test sample can be treated with a chemical and/or biological reagent. Chemical and/or biological reagents can be employed to protect and/or maintain the stability of the sample, including biomolecules (e.g., protein) therein, during processing. The skilled artisan is well aware of methods and processes appropriate for pre-processing of biological samples required for determination of the level of an expression product as described herein.

[0136] As used herein, the term "neuronal cell" or "neuron" refers to cells found in the nervous system that are specialized to receive, process, and transmit information as nerve signals. Neurons can include a central cell body or soma, and two types of projections: dendrites, by which, in general, the majority of neuronal signals are conveyed to the cell body; and axons, by which, in general, the majority of neuronal signals are conveyed from the cell body to effector cells, such as target neurons or muscle. Neurons can convey information from tissues and organs into the central nervous system (afferent or sensory neurons) and transmit signals from the central nervous systems to effector cells (efferent or motor neurons). Other neurons, designated interneurons, connect neurons within the nervous system.

[0137] As used herein, the term "specific" refers to a particular interaction (e.g. binding and/or cleavage) between two molecules wherein a first entity interacts with a second, target entity with greater specificity and affinity than it interacts with a third entity which is a nontarget. In some embodiments of any of the aspects described herein, specific can refer to an interaction of the first entity with the second target entity which is at least 10 times, at least 50 times, at least 100 times, at least 500 times, at least 1000 times or greater than the interaction with the third nontarget entity. A reagent specific for a given target is one that exhibits, e.g. specific

binding for that target under the conditions of the assay being utilized or specific cleavage of a target sequence under the conditions of the assay being utilized.

[0138] As used herein, the terms "protein" and "polypeptide" are used interchangeably herein to designate a series of amino acid residues, connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein", and "polypeptide" refer to a polymer of amino acids, including modified amino acids (e.g., phosphorylated, glycated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. "Protein" and "polypeptide" are often used in reference to relatively large polypeptides, whereas the term "peptide" is often used in reference to small polypeptides, but usage of these terms in the art overlaps. The terms "protein" and "polypeptide" are used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary polypeptides or proteins include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

[0139] In the various embodiments described herein, it is further contemplated that variants (naturally occurring or otherwise), alleles, homologs, conservatively modified variants, and/or conservative substitution variants of any of the particular polypeptides described are encompassed. As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid and retains the desired activity of the polypeptide. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles consistent with the disclosure.

[0140] A given amino acid can be replaced by a residue having similar physiochemical characteristics, e.g., substituting one aliphatic residue for another (such as Ile, Val, Leu, or Ala for one another), or substitution of one polar residue for another (such as between Lys and Arg; Glu and Asp; or Gln and Asn). Other such conservative substitutions, e.g., substitutions of entire regions having similar hydrophobicity characteristics, are well known. Polypeptides comprising conservative amino acid substitutions can be tested in any one of the assays described herein to confirm that a desired activity, e.g. polypeptide binding activity and specificity of a native or reference polypeptide is retained.

[0141] Amino acids can be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)): (1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M); (2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q); (3) acidic: Asp (D), Glu (E); (4) basic: Lys (K), Arg (R), His (H). Alternatively, naturally occurring residues can be divided into groups based on common side-chain properties: (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; (6) aromatic: Trp,

Tyr, Phe. Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Particular conservative substitutions include, for example; Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

[0142] In some embodiments of any of the aspects described herein, the polypeptide described herein (or a nucleic acid encoding such a polypeptide) can be a functional fragment of one of the amino acid sequences described herein. As used herein, a "functional fragment" is a fragment or segment of a peptide which retains at least 50% of the wildtype reference polypeptide's activity according to the assays described below herein. A functional fragment can comprise conservative substitutions of the sequences disclosed herein.

[0143] In some embodiments of any of the aspects described herein, the polypeptide described herein can be a variant of a sequence described herein. In some embodiments of any of the aspects described herein, the variant is a conservatively modified variant. Conservative substitution variants can be obtained by mutations of native nucleotide sequences, for example. A "variant," as referred to herein, is a polypeptide substantially homologous to a native or reference polypeptide, but which has an amino acid sequence different from that of the native or reference polypeptide because of one or a plurality of deletions, insertions or substitutions. Variant polypeptide-encoding DNA sequences encompass sequences that comprise one or more additions, deletions, or substitutions of nucleotides when compared to a native or reference DNA sequence, but that encode a variant protein or fragment thereof that retains activity. A wide variety of PCR-based site-specific mutagenesis approaches are known in the art and can be applied by the ordinarily skilled artisan.

[0144] A variant amino acid or DNA sequence can be at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more, identical to a native or reference sequence. The degree of homology (percent identity) between a native and a mutant sequence can be determined, for example, by comparing the two sequences using freely available computer programs commonly employed for this purpose on the world wide web (e.g. BLASTp or BLASTn with default settings).

[0145] Alterations of the native amino acid sequence can be accomplished by any of a number of techniques known to one of skill in the art. Mutations can be introduced, for example, at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion. Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered nucleotide sequence having particular codons altered according to the substitution, deletion, or insertion required. Techniques for making such alterations are very well established and include, for example, those disclosed by

Walder et al. (Gene 42:133, 1986); Bauer et al. (Gene 37:73, 1985); Craik (BioTechniques, January 1985, 12-19); Smith et al. (Genetic Engineering: Principles and Methods, Plenum Press, 1981); and U.S. Pat. Nos. 4,518,584 and 4,737,462. Any cysteine residue not involved in maintaining the proper conformation of the polypeptide also can be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) can be added to the polypeptide to improve its stability or facilitate oligomerization.

[0146] As used herein, the term "nucleic acid" or "nucleic acid sequence" refers to any molecule, preferably a polymeric molecule, incorporating units of ribonucleic acid, deoxyribonucleic acid or an analog thereof. The nucleic acid can be either single-stranded or double-stranded. A single-stranded nucleic acid can be one nucleic acid strand of a denatured double- stranded DNA. Alternatively, it can be a single-stranded nucleic acid not derived from any double-stranded DNA. In one aspect, the nucleic acid can be DNA. In another aspect, the nucleic acid can be RNA. Suitable DNA can include, for example, genomic DNA or cDNA. Suitable RNA can include, for example, mRNA.

[0147] In some embodiments of any of the aspects described herein, a polypeptide, nucleic acid, or cell as described herein can be engineered. As used herein, "engineered" refers to the aspect of having been manipulated by the hand of man. For example, a polypeptide is considered to be "engineered" when at least one aspect of the polypeptide, e.g., its sequence, has been manipulated by the hand of man to differ from the aspect as it exists in nature. As is common practice and is understood by those in the art, progeny of an engineered cell are typically still referred to as "engineered" even though the actual manipulation was performed on a prior entity.

[0148] The term "vector", as used herein, refers to a nucleic acid construct designed for delivery to a host cell or for transfer between different host cells. As used herein, a vector can be viral or non-viral. The term "vector" encompasses any genetic element that is capable of replication when associated with the proper control elements and that can transfer gene sequences to cells. A vector can include, but is not limited to, a cloning vector, an expression vector, a plasmid, phage, transposon, cosmid, chromosome, virus, virion, etc.

[0149] As used herein, the term "expression vector" refers to a vector that directs expression of an RNA or polypeptide from sequences linked to transcriptional regulatory sequences on the vector. The sequences expressed will often, but not necessarily, be heterologous to the cell. An expression vector may comprise additional elements, for example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in human cells for expression and in a prokaryotic host for cloning and amplification. The term "expression" refers to the cellular processes involved in producing RNA and proteins and as appropriate, secreting proteins, including where applicable, but not limited to, for example, transcription, transcript processing, translation and protein folding, modification and processing. "Expression products" include RNA transcribed from a gene, and polypeptides obtained by translation of mRNA transcribed from a gene. The term "gene" means the nucleic

acid sequence which is transcribed (DNA) to RNA in vitro or in vivo when operably linked to appropriate regulatory sequences. The gene may or may not include regions preceding and following the coding region, e.g. 5' untranslated (5'UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons).

[0150] As used herein, the term "viral vector" refers to a nucleic acid vector construct that includes at least one element of viral origin and has the capacity to be packaged into a viral vector particle. The viral vector can contain the nucleic acid encoding a polypeptide as described herein in place of non-essential viral genes. The vector and/or particle may be utilized for the purpose of transferring any nucleic acids into cells either *in vitro* or *in vivo.* Numerous forms of viral vectors are known in the art.

[0151] By "recombinant vector" is meant a vector that includes a heterologous nucleic acid sequence, or "transgene" that is capable of expression *in vivo.* It should be understood that the vectors described herein can, In some embodiments of any of the aspects described herein, be combined with other suitable compositions and therapies. In some embodiments of any of the aspects described herein, the vector is episomal. The use of a suitable episomal vector provides a means of maintaining the nucleotide of interest in a subject or cell in high copy number extra chromosomal DNA thereby eliminating potential effects of chromosomal integration.

[0152] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) or greater difference.

[0153] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean ±1%.

[0154] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the method or composition, yet open to the inclusion of unspecified elements, whether essential or not.

[0155] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0156] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment.

[0157] As used herein, the term "corresponding to" refers to an amino acid or nucleotide at the enumerated position in a first polypeptide or nucleic acid, or an amino acid or nucleotide that is

equivalent to an enumerated amino acid or nucleotide in a second polypeptide or nucleic acid. Equivalent enumerated amino acids or nucleotides can be determined by alignment of candidate sequences using degree of homology programs known in the art, e.g., BLAST.

[0158] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[0159] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims. Definitions of common terms in immunology and molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 19th Edition, published by Merck Sharp & Dohme Corp., 2011 (ISBN 978-0-911910-19-3); Robert S. Porter et al. (eds.), The Encyclopedia of Molecular Cell Biology and Molecular Medicine, published by Blackwell Science Ltd., 1999-2012 (ISBN 9783527600908); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006; Janeway's Immunobiology, Kenneth Murphy, Allan Mowat, Casey Weaver (eds.), Taylor & Francis Limited, 2014 (ISBN 0815345305, 9780815345305); Lewin's Genes XI, published by Jones & Bartlett Publishers, 2014 (ISBN-1449659055); Michael Richard Green and Joseph Sambrook, Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2012) (ISBN 1936113414); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (2012) (ISBN 044460149X); Laboratory Methods in Enzymology: DNA, Jon Lorsch (ed.) Elsevier, 2013 (ISBN 0124199542); Current Protocols in Molecular Biology (CPMB), Frederick M. Ausubel (ed.), John Wiley and Sons, 2014 (ISBN 047150338X, 9780471503385), Current Protocols in Protein Science (CPPS), John E. Coligan (ed.), John Wiley and Sons, Inc., 2005; and Current Protocols in Immunology (CPI) (John E. Coligan, ADA M Kruisbeek, David H Margulies, Ethan M Shevach, Warren Strobe, (eds.) John Wiley and Sons, Inc., 2003 (ISBN 0471142735, 9780471142737).

[0160] Other terms are defined herein within the description of the various aspects of the invention.

[0161] All patents and other publications; including literature references, issued patents, published patent applications, and co-pending patent applications; cited throughout this

application are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[0162] The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. Moreover, due to biological functional equivalency considerations, some changes can be made in protein structure without affecting the biological or chemical action in kind or amount. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

[0163] Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

[0164] The technology described herein is further illustrated by the following examples which in no way should be construed as being further limiting.

EXAMPLES

[0165] EXAMPLE 1: Novel *in vitro* and cell-based assays for measuring the activity of botulinum neurotoxins

[0166] Botulinum neurotoxins are a family of seven bacterial toxins (BoNT/A-G). They are one of the six Category A potential bioterrorism agents. These toxins have also been widely used to treat a growing list of medical conditions, with a market over two billion dollars. Detection and characterization of BoNTs have relied on the classic mouse lethal dose (LD50) assay. There is a pressing need, from both biodefense purposes and for medical product development to replace the animal assay with more convenient, sensitive, and accurate *in vitro* and cell-based

assays.

[0167] Currently available in vitro assays for detecting and characterizing toxin activity have been developed, with various sensitivity. There are two major commercialized assays, both of which are based on fluorescence detection of the cleavage of the substrates by the enzymatic domain of toxins. The first of these assays is SNAPtide™ (List Biologies), involving the use of synthetic peptides containing the native cleavage site for BoNTs and labeled with two fluorescent dyes. The fluorescence signals are normally quenched between two dyes, and the signals rise once the peptide is cleaved by BoNTs. The second assay is BoTest™ (BioSentinel LLC). A cyan fluorescent protein (CFP) is linked through a peptide sequence to a yellow florescent protein (YFP). Cleavage of the linker by BoNTs abolishes the fluorescence resonance energy transfer (FRET) between CFP and YFP that can be detected.

[0168] Both assays provide sensitive and convenient ways to detect toxins and characterize toxin activities and are now widely used in laboratories. A significant drawback with these assays is that these fluorescent assays are prone to the interference from serum albumin proteins that present at high levels in the pharmaceutical products of toxins and clinical samples. A current solution is to purify toxins out of these samples with specific toxin antibodies prior to these fluorescence assays, but it adds significant cost to the assay and requires special agents (toxin antibodies) that are not readily available.

[0169] Numerous mice are currently utilized every year to quantify the toxin activity by pharmaceutical companies. There is a pressing need to develop a cell based assay to replace the mouse bio-assay, in order to reduce the use of animals in production of BoNTs. Cell based assays offer the ability to examine all aspects of toxin activity, including receptor binding, membrane translocation, and enzymatic activities. These aspects are not captured by any *in vitro* assays. Currently, there is only one cell based assay that is well established by a major pharmaceutical company (Allergan). This assay is an ELISA-type assay based on using a monoclonal antibody that recognizes the SNAP-25 protein cleaved by BoNT/A. Briefly, cells are exposed to BoNT/A and cleaved SNAP-25 is pulled down and immobilized by a special antibody that only recognizes the cleaved SNAP-25, but not the full length protein. Immobilized SNAP-25 is then detected by a second SNAP-25 antibody. This assay offers good sensitivity and can potentially replace the mouse assay. It is approved by the FDA for quantifying toxin products. The major drawback is that it relies on a specialized antibody generated by Allergan.

[0170] Alternative assays that are under development include using CFP-SNAP-25-YFP as substrates in cells (BioSentinel LLC). Cleavage of this fusion protein in cells abolishes the FRET signals between CFP and YFP. The major drawback is that this assay also requires complicated equipment such as a florescence microscope.

[0171] Described herein is a new assay developed to detect and measure the activity of BoNTs *in vitro* and *in vivo.* The assay is based on the development of a split form of luciferase. NANOLUC™ luciferase is a new generation of luciferase with superb protein stability and

greatly increased luminescent signals (>100-fold higher than the classic firefly luciferase). This luciferase can be split into two pieces. When these two pieces are close to each other, they can reconstitute into a full active form that generates luminescent signals. Luminescent signals are abolished when these two pieces are separated from each other. In an illustrative embodiment depicted in FIG. 1, a linker region derived from the toxin substrates (SNAP-25 for BoNT/A, E, and C, synaptobrevin for BoNT/B, D, F, G) is inserted between two fragments of NANOLUC™ luciferase. Cleavage of this linker region by BoNTs separates the two fragments of NANOLUC™ luciferase and abolishes its activity, resulting in a reduction in luminescent signals. To demonstrate the feasibility of this approach, the following toxin sensors were designed and tested.

[0172] *In vitro* toxin sensors: Three different in vitro prototype toxin sensors were created and tested (FIG. 2A-FIG. 2B, FIG. 3A-FIG. 3B). The first sensor contains a SNAP-25 fragment that can be cleaved by BoNT/A, E, and C between the two split NANOLUC™ fragments (N-nano and C-nano, respectively, FIG. 2A-FIG. 2B). The second sensor contains an additional fragment of SV2C that serves as a toxin receptor, which can increase the cleavage efficiency by enhancing the interactions between toxins and sensor. The third version contains a synaptobrevin fragment that can be cleaved by BoNT/B, D, F, and G (FIG. 3A-FIG. 3B).

[0173] These sensor proteins were purified as recombinant proteins. The two versions of sensors containing SNAP-25 were incubated with a gradient of concentrations of BoNT/A for either 4 or 24 hours. The remaining luciferase activities were measured and plotted as shown in FIG. 2A. As a control, it was found that BoNT/B did not affect the luciferase activity of these two sensors, demonstrating the specificity of these two sensors for BoNT/A. As shown in FIG. 2B, the sensor that contains SV2C fragment has an EC50 at 9.7 pM after four hours' incubation. This is approximately 5-fold more sensitive than the sensor without SV2C, indicating that including the fragment of toxin receptors may result in a more sensitive toxin sensor.

[0174] The third sensor protein was incubated with a gradient of BoNT/B. The remaining luciferase activities were measured and plotted as shown in FIG. 3A. The EC50, listed in FIG. 3B, is approximately 75.7 pM after 4 hours incubation. Incubating toxins with the sensor for 24 hours significantly improved the EC50 to 3.9 pM.

[0175] *Advantage compared to available in vitro sensors:* These luciferase based toxin sensors provides similar sensitivity as fluorescence based sensors. The major advantage of these split luciferase based sensors is that they are not affected by the presence of serum albumin proteins.

[0176] *Cell based assay*: An illustrative embodiment of a sensor for cell based assays is illustrated in FIG. 4. It contains a full length SNAP-25 between split NANOLUC™ luciferase fragments. In addition, it also contains a firefly luciferase between the N-nano and SNAP-25, which provides an internal control for expression levels of the sensor proteins in cells. This

sensor protein is expressed in neurons via lentivirus mediated infection. Neurons were then exposed to various concentrations of BoNT/A. Cell lysates were harvested 48 hours later and the luciferase activities of both firefly luciferase and NANOLUC™ luciferase were measured. The levels of NANOLUC™ luciferase are normalized using firefly luciferase signals, and then normalized to the control neurons that were not exposed to any toxins (FIG. 4). Incubating neurons with toxins reduced the signals of NANOLUC™ luciferase (FIG. 4), with an EC50 at 2.9 pM.

[0177] *Advantage compared to available cell based assays:* The assay described herein provides sensitivity similar to the ELISA based assay established by Allergan. The major advantage of the present assay is that it does not require any special antibody. It is also easier, cheaper and faster than the ELISA assay.

[0178] It is specifically contemplated herein that toxin sensors and cell based assays as described herein offer faster, cheaper, and easier ways for pharmaceutical companies to measure and quantify the activity of BoNT/A and BoNT/B, potentially replacing the mouse bio-assay.

[0179] *In vitro and in vivo constructs for BoNTs detection.* For BoNT/A detection, the construct was cloned into pET28a vector with NocI/NotI and the inserts were Nnano-SV2C(529-566)-G4S-SNAP25(141-206)-Cnano and Nnano-SNAP25(141-206)-Cnano ("G4S" disclosed as SEQ ID NO: 6). Split NANOLUC™ luciferase sequence is available from Promega. Overlap PCR was used to obtain full inserts and then inserts were ligated into a cut vector. For BoNT/B detection, the construct was Nnano-VAMP(35-96)-Cnano.

[0180] For BoNT/A in vivo detection, the constructs were designed either using split firefly or split NANOLUC, then the renilla and firefly as internal controls were separately applied. The internal control is located just in front of SNAP25 (Full length). The vector was based on pcDNA3.1 and syn-lox lentiviral vectors.

Cloning of in vitro assay constructs

Sensor 1: NNano-SV2C-p25-CNano
NcoI-Nnano(1-159)-GSSGGGGSGGGGSSG-SacI-SV2C(529-566)-GGGGS-P25(141-206)-EcoRI-GSSGGGGSGGGGSSG-Cnano (160-170)-NotI-His6- stop ("GSSGGGGSGGGGSSG" disclosed as SEQ ID NO: 4, "GGGGS" disclosed as SEQ ID NO: 6, "GSSGGGGSGGGGSSG" disclosed as SEQ ID NO: 5 and "His6" disclosed as SEQ ID NO: 12)

Sensor 2: NNano-p25-CNano
NcoI-Nnano-(1-159)-GSSGGGGSGGGGSSG-SacI-p25(141-206)-EcoRI-GSSGGGGSGGGGSSG-Cnano(160-170)-NotI-His6-stop("GSSGGGGSGGGGSSG" disclosed as SEQ ID NO: 4, "GSSGGGGSGGGGSSG" disclosed as SEQ ID NO: 5 and "His6" disclosed as SEQ ID NO: 12)

Sensor 3: NNano-hVAMP1-CNano
NcoI - Nnano-(1-159)-GSSGGGGSGGGGSSG-SacI -human V AMP 1 (35-96)-EcoRI-GSSGGGGSGGGSSG-Cnano(160-170)-NotI-His6-stop("GSSGGGGSGGGGSSG" disclosed as SEQ ID NO: 4, "GSSGGGGSGGGSSG" disclosed as SEQ ID NO: 5 and "His6" disclosed as SEQ ID NO: 12)

Cloning of in vivo assay construct
BamHI-Nnano-(1-159)-GSSGGGGSGGGSSG-SacI-Firefly (Full length)-GGGGS-p25(FullLength)-EcoRI- GSSGGGGSGGGSSG-Cnano(160-170)-NotI (Stop codon) ("GGGGS" disclosed as SEQ ID NO: 6 and "GSSGGGGSGGGSSG" disclosed as SEQ ID NO: 5)

[0181] *IMAC purification of sensor proteins for in vitro assay.* Different constructs of sensor proteins were purified using Immobilized Metal Affinity Columns (IMAC). The His6 tag (SEQ ID NO: 12) was cloned into pET28a vector at C terminal. BL21 containing correct plasmid of in vitro construct was inoculated over night and cultivated at 37°C until O.D. around 0.6-0.8, then induced by 0.25 mM of IPTG (final concentration) for over night at 20 °C. The cells are harvested by centrifuging at 4500g, 10 minutes. After that, cells pellets were dissolve in 50 mM HEPES, 150 mM NaCl, pH7.4 buffer and followed by sonication for 3 minutes. The supernatant of cell lysate was saved and mixed with Ni2+ beads for 1 h with rotation in 4°C. Later, the mixture was loaded into column and the resin was washed with 10 fold of bed column of 20 mM Imidizole in 50 mM HEPES, 150 mM NaCl, pH7.4 buffer. Finally, the protein was eluted in 4 fold of bed column 500 mM Imidizole in 50 mM HEPES, 150 mM NaCl, pH7.4 buffer. The purified sensor protein was dialyzed into 50 mM HEPES, pH 7.1 buffer to perform the assay immediately.

[0182] *In vitro assay of toxin detection.* Sensor protein concentration is estimated by SDS-PAGE with standard BSA as reference. 30 nM of sensor protein was prepared in 50 mM HEPES, 20 µM $ZnCl_2$, 2 mM DTT, 1 mg/ml BSA, pH 7.1 buffer. The botulinum toxin A was diluted and added into sensor protein solution with a concentration series from 1 nM to 1 fM with dilution factor 10. After 4 h and 24 h incubation at 37°C, Nano-Glo™ susbrate (Promega) was added into each sample with equal volume. The luminescent signal was measured in plate reader. The assay was performed in duplicate. Negative controls were also designed and performed. BoNT/A was mixed with Nnano-VAMP-Cnano sensor protein or BoNT/B was mixed with Nnano-SV2C-p25-Cnano sensor protein.

[0183] *In vivo assay (cell based assay) of toxin detection.* Virus of dual luciferase construct (firefly as internal control and split NANOLUC™ for cleavage detection) was added into 7-days neuron cells and then botulinum toxin A of a series concentration from 300 pM to 1 pM was added into 13-day neuron cells with triplicate. After 48h challenge with toxin, neuron cells were lysed by adding 200 µl passive lysis buffer (Promega) and incubated at room temperature for 20 minutes. 50 µl of cell lysate was mixed with 50 µl firefly luciferase substrate (ONE-Glo (TM) EX reagent) and light emissions measured. Subsequently, 50 µl NANOLUC™ luciferase

substrate (NanoDLR™ Stop & Glo (R) reagent) was added and light emissions measured.

SEQ ID NO:	Description	Sequence
23	Human SNAP25	MAEDADMRNELEEMQRRADQLADESLESTRRMLQL VEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMK EAEKNLTDLGKFCGLCVCPCNKLKSSDAYKKAWGNN QDGVVASQPARVVDEREQMAISGGFIRRVTNDARENE
	UniProtKB - P60880 (SNP25_HUMAN)	MDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIM EKADSNKTRIDEANQRATKMLGSG
24	Human VAMP-1 UniProtKB - P23763 (VAMP1_HUMAN):	MSAPAQPPAEGTEGTAPGGGPPGPPPNMTSNRRLQQT QAQVEEVVDIIRVNVDKVLERDQKLSELDDRADALQ AGASQFESSAAKLKRKYWWKNCKMMIMLGAICAIIV VVIVIYFFT
25	Human VAMP-2 UniProtKB - P63027 (VAMP2 HUMAN)	MSATAATAPPAAPAGEGGPPAPPPNLTSNRRLQQTQA QVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAG ASQFETSAAKLKRKYWWKNLKMMIILGVICAIILIIIV YFST
26	Human VAMP-3 UniProtKB - Q15836 (VAMP3_HUMAN):	MSTGPTAATGSNRRLQQTQNQVDEVVDIMRVNVDKV LERDQKLSELDDRADALQAGASQFETSAAKLKRKYW WKNCKMWAIGITVLVIFIIIIVWVVSS
27	Human Syntaxin 1A UniProtKB - Q16623 (STX1A_HUMAN)	MKDRTQELRTAKDSDDDDDVAVTVDRDRFMDEFFEQ VEEIRGFIDKIAENVEEVKRKHSAILASPNPDEKTKEEL EELMSDIKKTANKVRSKLKSIEQSIEQEEGLNRSSADL RIRKTQHSTLSRKFVEVMSEYNATQSDYRERCKGRIQ RQLEITGRTTTSEELEDMLESGNPAIFASGIIMDSSISKQ ALSEIETRHSEIIKLENSIRELHDMFMDMAMLVESQGE MIDRIEYNVEHAVDYVERAVSDTKKAVKYQSKARRK KIMIIICCVILGIVIASTVGGIFA
28	Human Syntaxin 1B	mkdrtqelrs akdsddeeev vhvdrdhfmd effeqveeir gcieklsedv eqvkkqhsai laapnpdekt kqeledltad ikktankvrs klkaieqsie qeeglnrssa dlrirktqhs tlsrkfvevm teynatqsky rdrckdriqr qleitgrttt neeledmles gklaiftddi kmdsqmtkqa lneietrhne iikletsire lhdmfvdmam lvesqgemid rieynvehsv dyveravsdt kkavkyqska rrkkimiiic cvvlgvvlas siggtlgl

REFERENCES CITED IN THE DESCRIPTION

Cited references

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Patent documents cited in the description

- WO2005038029A2 [0004]
- US6890745B1 [0006]
- WO9532738A [0033]
- WO9633273A [0033]
- WO9807864A [0033]
- WO9917806A [0033]
- US8557970B [0040]
- US6436682B [0118] [0118]
- US5374534A [0118]
- US5098828A [0118]
- US5004565A [0118]
- US5455357A [0118]
- US4950588A [0118]
- US4518584A [0145]
- US4737462A [0145]

Non-patent literature cited in the description

- **WAUD et al.**Engineering the C-terminus of firefly luciferase as an indicator of covalent modification of proteins.Biochim Biophys Acta, 1996, [0005]
- **LENG J et al.**Cleavalite™: A Novel Bioluminescent Caspase Activity AssayAbstracts of the annual meeting of the society for neuroscience, 2001, [0006]
- **HILL et al.**Journal of Bacteriology, 2007, vol. 189, 3818-832 [0033]

- **YANN HUMEAU et al.**How Botulinum and Tetanus Neurotoxins Block Neurotransmitter ReleaseBiochimie., 2000, vol. 82, 5427-446 [0036]
- **KATHRYN TURTON et al.**Botulinum and Tetanus Neurotoxins: Structure, Function and Therapeutic UtilityTrends Biochem. Sci., 2002, vol. 27, 11552-558 [0036]
- **GIOVANNA LALLI et al.**The Journey of Tetanus and Botulinum Neurotoxins in NeuronsTrends Microbiol., 2003, vol. 11, 9431-437 [0036]
- **HEISE, K. et al.**Dual luciferase assay for secreted luciferase based on Gaussia and NANOLUCAssay Drug Dev. Technol., 2013, [0039]
- **TAKENAKA, Y. et al.**Computational analysis and functional expression of ancestral copepod luciferaseGene, 2013, [0039]
- **BINZ, TH. et al.**Clostridial neurotoxins: mechanism of SNARE cleavage and outlook on potential substrate specificity reengineering.Toxins, 2010, vol. 2.4, 665-682 [0052]
- **CHEN, X. et al.**Fusion protein linkers: proterty, design and functionalityAdv. Drug Deliv. Rev., 2013, [0082]
- INSECT CELL CULTURE ENGINEERINGMarcel Dekker19930000 [0105]
- INSECT CELL CULTURES: FUNDAMENTAL AND APPLIED ASPECTSKluwer Academic Publishers19960000 [0105]
- **MAUREEN A. HARRISONIAN F. RAE**GENERAL TECHNIQLTES OF CELL CULTURECambridge University Press19970000 [0105]
- CELL AND TISSUE CULTURE: LABORATORY PROCEDURESJohn Wiley and Sons19980000 [0105]
- **R. IAN FRESHNEY**CLTLTURE OF ANIMAL CELLS: A MANUAL OF BASIC TECHNIQUEWiley-Liss20000000 [0105]
- ANIMAL CELL CULTURE: A PRACTICAL APPROACHOxford University Press20000000 [0105]
- MOLECULAR CLONING A LABORATORY MANUAL, 2001, [0105]
- **JOHN M. DAVIS**BASIC CELL CULTURE: A PRACTICAL APPROACHOxford Press20020000 [0105]
- CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, 2004, [0105]
- **ZHAO et al.**Mol Imaging, 2004, vol. 3, 43-54 [0118]
- **A. L. LEHNINGER**BiochemistryWorth Publishers1975000073-75 [0141]
- **WALDER et al.**Gene, 1986, vol. 42, 133- [0145]
- **BAUER et al.**Gene, 1985, vol. 37, 73- [0145]
- **CRAIK**BioTechniques, 1985, 12-14 [0145]
- **SMITH et al.**Genetic Engineering: Principles and MethodsPlenum Press19810000 [0145]
- The Merck Manual of Diagnosis and TherapyMerck Sharp & Dohme Corp.20110000 [0159]
- **BLACKWELL SCIENCE LTD. et al.**The Encyclopedia of Molecular Cell Biology and Molecular Medicine19990000 [0159]
- Molecular Biology and Biotechnology: a Comprehensive Desk ReferenceVCH Publishers, Inc.19950000 [0159]
- **WERNER LUTTMANN**ImmunologyElsevier20060000 [0159]
- Janeway's ImmunobiologyTaylor & Francis Limited20140000 [0159]

- Lewin's Genes XIJones & Bartlett Publishers20140000 [0159]
- **MICHAEL RICHARD GREENJOSEPH SAMBROOK**Molecular Cloning: A Laboratory ManualCold Spring Harbor Laboratory Press20120000 [0159]
- **DAVIS et al.**Basic Methods in Molecular BiologyElsevier Science Publishing, Inc.20120000 [0159]
- Methods in Enzymology: DNAElsevier [0159]
- Current Protocols in Molecular Biology (CPMB), Frederick M. AusubelJohn Wiley and Sons20140000 [0159]
- Current Protocols in Protein Science (CPPS)John Wiley and Sons, Inc.20050000 [0159]
- **JOHN E. COLIGAN**Current Protocols in Immunology (CPI)John Wiley and Sons, Inc.20030000 [0159]

Patentkrav

1. Enkeltkædet polypeptid omfattende, fra N-terminal til C-terminal:

a. et N-terminalt fragment af et reporterprotein;

b. en linker omfattende: (i) et *C. botulinum* neurotoksin (BoNT) spaltningssted og (ii) et bindingsfragment af en BoNT-receptor; og

c. et C-terminalt fragment af reporterproteinet;

hvor reporterproteinet er et luciferaseprotein, og hvor de N-terminale og C-terminale fragmenter tilsammen omfatter en funktionel luciferaseproteinsekvens, når fragmenterne er bundet sammen ved hjælp af linkeren, og ikke når linkeren er blevet spaltet ved spaltningsstedet.

2. Enkeltkædet polypeptid ifølge krav 1, hvor:

i) det N-terminale fragment omfatter en sekvens med mindst 90% sekvensidentitet med aminosyrerne 1-159 af luciferasen af SEQ ID NO: 1 (N-nano$_{1-159}$), og det C-terminale fragment omfatter en sekvens med mindst 90% sekvensidentitet med aminosyrerne 160-170 af luciferasen af SEQ ID NO: 1 (C-nano$_{160-170}$); eller

ii) det N-terminale fragment omfatter en sekvens med mindre end 10 aminosyrerestsubstitutioner, - deletioner eller - additioner i forhold til aminosyrerne 1-159 af luciferasen af SEQ ID NO: 1 (N-nano$_{1-159}$), og det C-terminale fragment omfatter en sekvens med mindre end 3 aminosyrerestsubstitutioner, - deletioner eller - additioner i forhold til aminosyrerne 160-170 af luciferasen af SEQ ID NO: 1 (C-nano$_{160-170}$).

3. Enkeltkædet polypeptid ifølge et hvilket som helst af de foregående krav, hvor det N-terminale fragment har sekvensen af aminosyrerne 1-159 af luciferasen af SEQ ID NO: 1 (N-nano$_{1-159}$), og det C-terminale fragment har sekvensen af aminosyrerne 160-170 af luciferasen af SEQ ID NO: 1 (C-nano$_{160-170}$).

4. Enkeltkædet polypeptid ifølge et hvilket som helst af de foregående krav, hvor BoNT-spaltningsstedet genkendes af BoNT af serotype A, E, C, B, D, F eller G.

5. Enkeltkædet polypeptid ifølge et hvilket som helst af de foregående krav, hvor:

i) BoNT-spaltningsstedet er fra et SNARE-protein;

ii) BoNT-spaltningsstedet er fra et SNARE-protein, og SNARE-proteinet er SNAP-25, synaptobrevin (VAMP) eller syntaksin;

iii) BoNT-spaltningsstedet er aminosyrerne 141-206 af human SNAP-25b;

iv) BoNT-spaltningsstedet er aminosyrerne 35-96 af human VAMP1; eller

v) BoNT-spaltningsstedet er aminosyrerne 1-206 af human SNAP-25b.

6. Enkeltkædet polypeptid ifølge et hvilket som helst af de foregående krav, hvor bindingsfragmentet af en BoNT-receptor er placeret mellem det N-terminale fragment og BoNT-spaltningsstedet.

7. Enkelt polypeptidkæde ifølge krav 6, hvor bindingsfragmentet af en BoNT-receptor er et fragment af en menneske-, muse-, rotte- eller primat-BoNT-receptor.

8. Enkeltkædet polypeptid ifølge krav 7, hvor BoNT-receptoren:

i) er SV2C;

ii) omfatter aminosyrerne 529-566 af human SV2C;

iii) er SYTI;

iv) omfatter aminosyrerne 32-52 af SYTI;

v) er SYT2; eller

vi) omfatter aminosyrerne 40-60 af SYT2.

9. Enkeltkædet polypeptid ifølge et hvilket som helst af de foregående krav, hvor:

i) linkeren yderligere omfatter et intakt andet luciferase-reporterpolypeptid placeret mellem det N-terminale fragment og BoNT-spaltningsstedet; eller

ii) linkeren yderligere omfatter et intakt andet luciferase-reporterpolypeptid placeret mellem det N-terminale fragment og BoNT-spaltningsstedet, og hvor det andet luciferase-reporterpolypeptid er ildflue luciferase (f.eks. *Photinus pyralis*), bakteriel luciferase (f.eks. *Vibrio fischeri, Vibrio harveyi*), nældecelledyr luciferase (*Renilla reniformis*), dinoflagellat luciferase, *Gaussia* luciferase eller copepod luciferase.

10. Enkeltkædet polypeptid ifølge et hvilket som helst af de foregående krav, yderligere omfattende et polyhistidin-affinitetsmærke.

11. Enkeltkædet polypeptid ifølge et hvilket som helst af de foregående krav, hvor linkeren omfatter en eller flere spacere placeret mellem BoNT-spaltningsstedet og det N-terminale fragment, en eller flere spacere placeret mellem BoNT-spaltningsstedet og det C-terminale fragment eller en kombination deraf.

12. Enkeltkædet polypeptid ifølge krav 6, hvor linkeren yderligere omfatter en eller flere spacere placeret mellem bindingsfragmentet af en BoNT-receptor og BoNT-spaltningsstedet.

13. Enkeltkædet polypeptid ifølge krav 9, hvor linkeren yderligere omfatter en eller flere spacere placeret mellem det andet luciferase-reporterpolypeptid og BoNT-spaltningsstedet.

14. Nukleinsyre omfattende en nukleotidsekvens, der koder for det enkeltkædede polypeptid ifølge et hvilket som helst af de foregående krav.

15. Nukleinsyrevektor omfattende nukleinsyren ifølge krav 14.

16. Celle omfattende nukleinsyren ifølge krav 14.

17. *In vitro* fremgangsmåde til at bestemme styrken af *C. botulinum* neurotoksin (BoNT), omfattende:

a) at bringe BoNT i kontakt med et enkeltkædet polypeptid under betingelser passende for BoNT-aktivitet, hvor det enkeltkædede polypeptid omfatter:

i) et N-terminalt fragment af et reporterprotein;

ii) en linker omfattende: (i) et *C. botulinum* neurotoksin (BoNT) spaltningssted, (ii) et bindingsfragment af en BoNT-receptor; og

iii) et C-terminalt fragment af reporterproteinet;

hvor reporterproteinet er et luciferaseprotein, og hvor de N-terminale og C-terminale fragmenter tilsammen omfatter en funktionel luciferaseproteinsekvens, når fragmenterne er bundet sammen ved hjælp af linkeren, og ikke når linkeren er blevet spaltet ved spaltningsstedet; og

b) bestemmelse af luciferaseaktivitet af polypeptidet sammenlignet med en reference, hvorved styrken bestemmes.

18. Fremgangsmåde til at detektere *C. botulinum* neurotoksin (BoNT) aktivitet i en prøve, omfattende:

a) at bringe prøven i kontakt med et enkeltkædet polypeptid under betingelser passende for BoNT-aktivitet, hvor det enkeltkædede polypeptid omfatter:

i) et N-terminalt fragment af et reporterprotein;

ii) en linker omfattende: (i) et *C. botulinum* neurotoksin (BoNT) spaltningssted og (ii) et bindingsfragment af en BoNT-receptor; og

iii) et C-terminalt fragment af reporterproteinet;

hvor reporterproteinet er et luciferaseprotein, og hvor de N-terminale og C-terminale fragmenter tilsammen omfatter en funktionel luciferaseproteinsekvens, når fragmenterne er bundet sammen ved hjælp af linkeren, og ikke når linkeren er blevet spaltet ved spaltningsstedet; og

b) bestemmelse af luciferaseaktivitet af polypeptidet sammenlignet med luciferaseaktivitet af polypeptidet i fravær af prøven, hvor et fald i luciferaseaktivitet indikerer BoNT-aktivitet i prøven.

19. Fremgangsmåde ifølge krav 18, hvor det enkeltkædede polypeptid udtrykkes af en neuronal celle, og fremgangsmåden yderligere omfatter, efter kontakttrinnet:

inkubering af de neuronale celler i en periode på fra ca. 12 timer til ca. 60 timer og høst af lysat fra de neuronale celler.

20. Kit omfattende:

a) et eller flere enkeltkædede polypeptider beskrevet i et hvilket som helst af kravene 1-13, med hver eller en kombination af de enkeltkædede polypeptider pakket i en separat beholder;

b) en eller flere nukleinsyrer eller nukleinsyrevektorer omfattende en nukleotidsekvens, der koder for det ene eller flere enkeltkædede polypeptider fra a), med hver eller en kombination af nukleinsyrerne eller nukleinsyrevektorerne pakket i en separat beholder; og/eller

c) en eller flere celler, der udtrykker et eller flere enkeltkædede polypeptider fra a), med hver eller en kombination af cellerne pakket i en separat beholder.

DRAWINGS

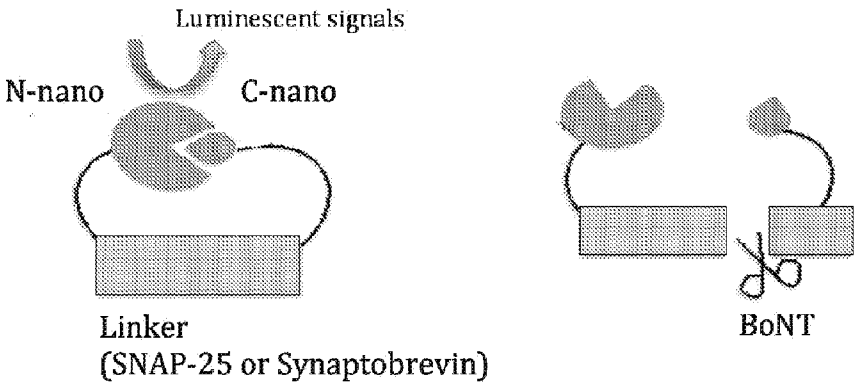

FIG. 1

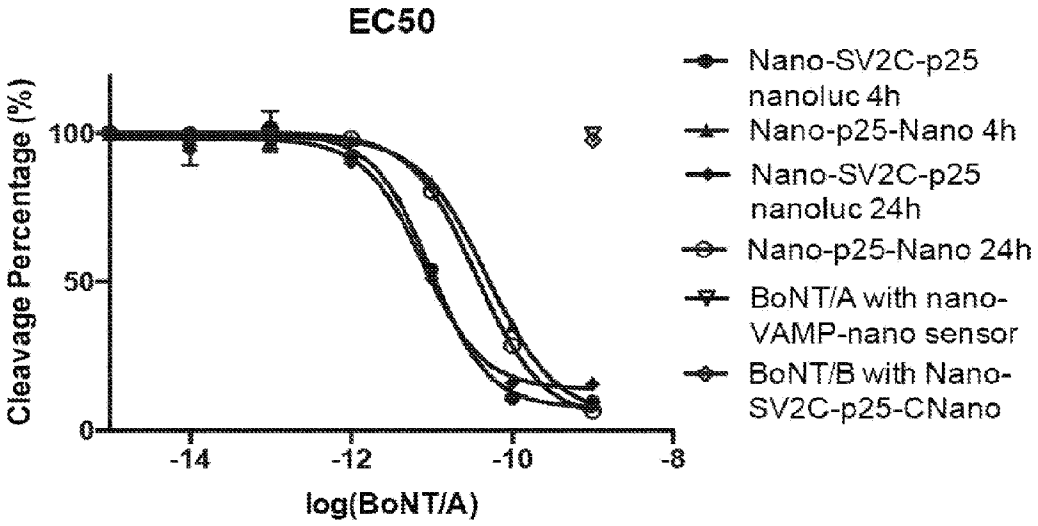

FIG. 2A

	4h	24h
NNano-SV2C-p25-CNano	9.73 pM	7.86 pM
NNano-p25-CNano	48.26 pM	35.87 pM

FIG. 2B

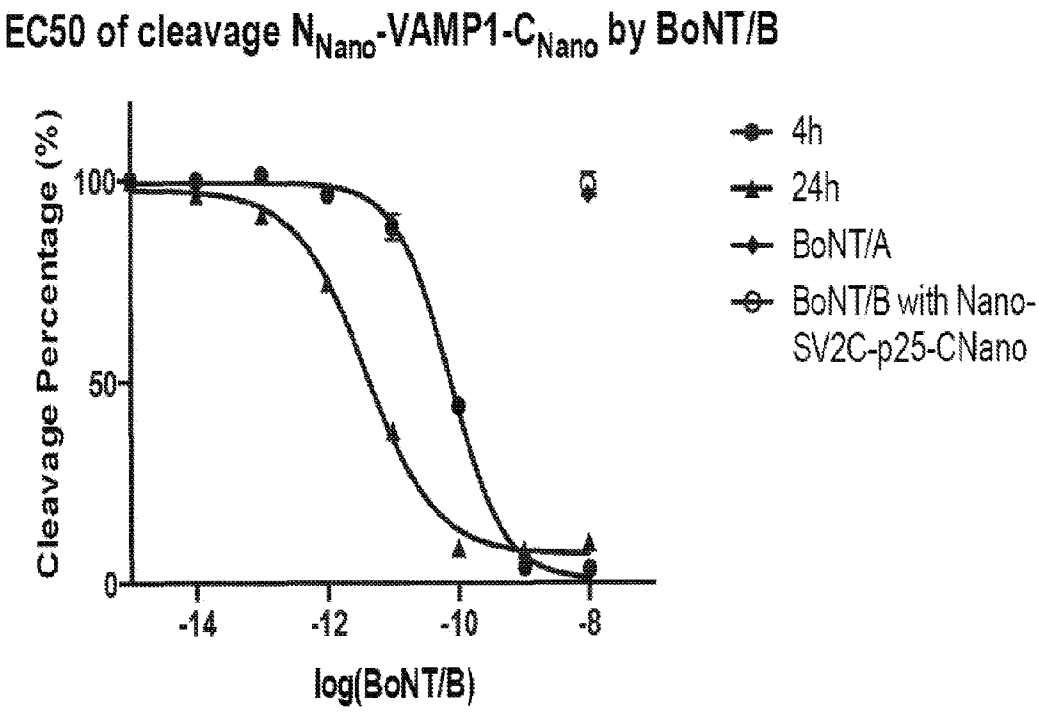

FIG. 3A

	4h	24h
N Nano-VAMP1-C Nano	75.7 pM	3.9 pM

FIG. 3B

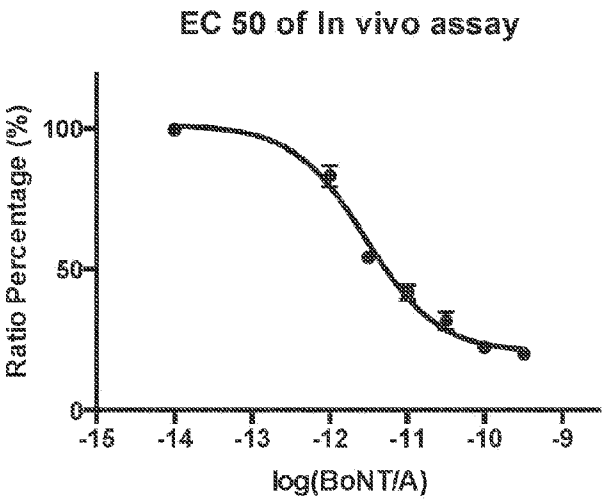
EC 50 of In vivo assay
Ratio Percentage (%)
100
50
0
-15
-14
-13
-12
-11
-10
-9
log(BoNT/A)

FIG. 4

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.