
5 **Methods for Enhancing the specific Uptake of Botulinum Neurotoxins into Cells**

[0001] The present invention provides a method for enhancing the specific uptake of a neurotoxin polypeptide into cells, the method comprising: incubating cells susceptible to
10 neurotoxin intoxication with a neurotoxin polypeptide for a time and under conditions which allow for the neurotoxin polypeptide to exert its biological activity, the incubation comprising at least one of the following steps: (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time and/or (iii) agitation of the cells during neurotoxin polypeptide exposition, thereby enhancing the specific uptake of the
15 neurotoxin polypeptide into said cells. In addition, the invention pertains to a method for directly determining the biological activity of a neurotoxin polypeptide in cells, comprising: a) incubating cells susceptible to neurotoxin intoxication with a neurotoxin polypeptide for a time and under conditions which allow for the neurotoxin polypeptide to exert its biological activity, the incubation comprising at least one of the following steps:
20 (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time and/or (iii) agitation of the cells during neurotoxin polypeptide exposition; b) fixing the cells and, optionally, permeabilizing the cells with a detergent; c) contacting the cells with at least a first capture antibody specifically binding to the non-cleaved and neurotoxin-cleaved substrate and with at least a second capture antibody specifically
25 binding to the cleavage site of the neurotoxin-cleaved substrate, under conditions which allow for binding of said capture antibodies to said substrates; d) contacting the cells with at least a first detection antibody specifically binding to the first capture antibody, under conditions which allow for binding of said first detection antibody to said first capture antibody, thus forming first detection complexes and with at least a second detection
30 antibody specifically binding to the second capture antibody, under conditions which allow for binding of said second detection antibody to said second capture antibody, thus forming second detection complexes; e) determining the amount of the first and second detection complexes of step d); and f) calculating the amount of substrate cleaved by said neurotoxin polypeptide in said cells by means of the second detection complexes, thereby determining
35 the biological activity of said neurotoxin polypeptide in said cells.

[0002] Clostridium botulinum and Clostridium tetani produce highly potent neurotoxins, i.e. Botulinum toxins (BoNTs) and Tetanus toxin (TeNT), respectively. These Clostridial neurotoxins (CNTs) specifically bind to neuronal cells and disrupt neurotransmitter release. Each toxin is synthesized as an inactive unprocessed approximately 150 kDa single-chain protein. The posttranslational processing involves formation of disulfide bridges, and limited proteolysis (nicking) by the bacterial protease(s). Active neurotoxin consists of two chains, an N-terminal light chain of approx. 50 kDa and a heavy chain of approx. 100 kDa linked by a disulfide bond. CNTs structurally and functionally consist of three domains, i.e. the catalytic light chain, the heavy chain encompassing the translocation domain (N-terminal half) and the receptor binding domain (C-terminal half); see, e.g., Krieglstein 1990, Eur. J. Biochem. 188, 39; Krieglstein 1991, Eur. J. Biochem. 202, 41; Krieglstein 1994, J. Protein Chem. 13, 49. The Botulinum neurotoxins are synthesized as molecular complexes comprising the 150 kDa neurotoxin protein and associated non-toxic proteins. The complex sizes differ based on the Clostridial strain and the distinct neurotoxin serotypes ranging from 300 kDa, over 500 kDa, and 900 kDa. The non-toxic proteins in these complexes stabilize the neurotoxin and protect it against degradation; see Silberstein 2004, Pain Practice 4, S19 – S26.

[0003] Clostridium botulinum secretes seven antigenically distinct serotypes designated A to G of the Botulinum neurotoxin (BoNT/A – BoNT/G). All serotypes together with the related Tetanus neurotoxin (TeNT) secreted by Clostridium tetani, are Zn²⁺-endoproteases that block synaptic exocytosis by cleaving SNARE proteins; see Couesnon, 2006, Microbiology, 152, 759. CNTs cause the flaccid muscular paralysis seen in botulism and tetanus; see Fischer 2007, PNAS 104, 10447. Recently, a new Botulinum toxin type, i.e. BoNT/H, has been identified; see Barash and Arnon, J. Infect. Dis. (2014), 209 (2): 183-191 and Dover, Barash, Hill, Xie and Arnon, J. Infect. Dis. (2014), 209(2): 192-202.

[0004] Despite its toxic effects, the Botulinum toxin complex has been used as a therapeutic agent in a large number of diseases. Botulinum toxin serotype A was approved for human use in the United States in 1989 for the treatment of strabism, blepharospasm, and other disorders. It is commercially available as Botulinum toxin A (BoNT/A) protein preparation, for example, under the trade name BOTOX (Allergan, Inc.) or under the trade name DYSPORT/RELOXIN (Ipsen, Ltd). An improved, complex-free Botulinum toxin A preparation is commercially available under the trade name XEOMIN (Merz Pharma GmbH & Co. KGaA). For therapeutic applications, the preparation is injected directly into the muscle to be treated. At physiological pH, the toxin is released from the protein complex and the desired pharmacological effect takes place. The effect of Botulinum toxin is only temporary, which is the reason why repeated administration of Botulinum toxin may be required to maintain a therapeutic affect.

[0005] The Clostridial neurotoxins weaken voluntary muscle strength and are effective therapy for strabism, focal dystonia, including cervical dystonia, and benign essential blepharospasm. They have been further shown to relief hemifacial spasm, and focal spasticity, and moreover, to be effective in a wide range of other indications, such as gastrointestinal disorders, hyperhidrosis, and cosmetic wrinkle correction; see Jost 2007, Drugs 67, 669.

[0006] During the manufacturing process of Clostridial neurotoxins, the qualitative and quantitative determination of said neurotoxins as well as the quality control of the biologically active neurotoxin polypeptides is of particular importance. In addition, governmental agencies accept only simple, reliable, and validated Botulinum toxin activity assays. At present the mouse LD₅₀ bioassay, a lethality test, remains the “gold standard” used by pharmaceutical manufacturers to analyze the potency of their preparations; see Arnon et al. (2001), JAMA 285, 1059-1070. However, in recent years, considerable effort has been undertaken to seek for alternative approaches to alleviate the need for animal testing and all the disadvantages, costs and ethical concerns associated with this type of animal-based assays. In addition, the regulatory agencies are engaging pharmaceutical companies to apply the three “Rs” principle to the potency testing of Botulinum neurotoxins: “Reduce, Refine, Replace”; see Straughan, Altern. Lab. Anim. (2006), 34, 305-313. As a consequence, cell-based test systems have been developed in order to provide reasonable alternatives to methods using live animals. Yet, only three cellular test systems are available for the determination of neurotoxin biological activity thus far which have been shown to be sufficiently sensitive to neurotoxin polypeptides. These cell-based test systems include the use of primary neurons isolated from rodent embryos which are differentiated *in vitro* (Pellett et al. (2011), Biochem. Biophys. Res. Commun. 404, 388-392), neuronal differentiated induced pluripotent stem cells (Whitemarsh et al. (2012), Toxicol. Sci. 126, 426-35), and a subclone of the SiMa cell line (WO 2010/105234 A1).

[0007] However, the isolation of primary neurons requires the killing of animals and is laborious and time consuming. Further, test systems using different primary neurons show large variances. Similarly, the generation of neuronal differentiated induced pluripotent stem cells is difficult and time consuming. In addition, storage of such cells is very problematic. Assays using tumor cell lines are frequently not sensitive enough to BoNT. Moreover, complex differentiation protocols are required for said tumor cell lines which result in large variances and/or high failure rates of assays using said cell lines.

[0008] In light of the above, further test systems for the determination of neurotoxin polypeptide activity are highly desirable.

[0009] Thus, the technical problem underlying the present invention may be seen as the provision of means and methods complying with the aforementioned needs. The technical problem is solved by the embodiments characterized in the claims and herein below.

5 Summary

[0009a] In one aspect, the present disclosure refers to an in vitro method for directly determining the biological activity of a neurotoxin polypeptide in cells, comprising:

- a) incubating cells susceptible to neurotoxin intoxication with a neurotoxin polypeptide for a time and under conditions which allow for the neurotoxin polypeptide to exert its biological activity, wherein the neurotoxin polypeptide is Botulinum neurotoxin BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, or BoNT/H, and wherein the incubation comprises (i) K⁺-mediated depolarization of the cells, wherein the K⁺-mediated depolarization of the cells is carried out at a final K⁺ concentration of 15 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM or 60 mM, for at least 30 minutes, and a neurotoxin polypeptide exposition time of at least 24 hours and less than 96 hours, or (ii) a neurotoxin polypeptide exposition time at least 24 hours and less than 96 hours and agitation of the cells during neurotoxin polypeptide exposition;
- b) fixing the cells and, optionally, permeabilizing the cells with a detergent;
- c) contacting the cells with at least a first capture antibody specifically binding to a non-cleaved and neurotoxin-cleaved substrate and with at least a second capture antibody specifically binding to the cleavage site of the neurotoxin-cleaved substrate, under conditions which allow for binding of said capture antibodies to said substrates;
- d) contacting the cells with at least a first detection antibody specifically binding to the first capture antibody, under conditions which allow for binding of said first detection antibody to said first capture antibody, thus forming first detection complexes and with at least a second detection antibody specifically binding to the second capture antibody, under conditions which allow for binding of said second detection antibody to said second capture antibody, thus forming second detection complexes;
- e) determining the amount of the first and second detection complexes of step d); and
- f) determining the amount of total neurotoxin substrate and the amount of substrate cleaved by said neurotoxin polypeptide at the same time, thereby determining the biological activity of said neurotoxin polypeptide in said cells.

Description

[0010] In a first aspect, the present invention pertains to a method for enhancing the specific uptake of a neurotoxin polypeptide into cells, the method comprising:

incubating cells susceptible to neurotoxin intoxication with a neurotoxin polypeptide for a time and under conditions which allow for the neurotoxin polypeptide to exert its biological activity, the incubation comprising at least one of the following steps: (i) K^+ - mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time and/or (iii) agitation of the cells during neurotoxin polypeptide exposition, thereby enhancing the specific uptake of the neurotoxin polypeptide into said cells. Preferably, this method is an in vitro method.

[0011] Clostridial neurotoxin polypeptides act within the synaptic terminal to block neurotransmitter release. The neurotoxin enters the neuron by binding to neuronal membrane receptors, being taken up into an endosome-like compartment, and penetrating the endosome membrane via a pH-dependent translocation process. Once within the synaptic cytoplasm, the Clostridial neurotoxins cleave their corresponding SNARE protein substrates, required for synaptic vesicle fusion.

[0012] More specifically, Clostridial neurotoxins are characterized in that they specifically inhibit the secretion of neurotransmitters from pre-synaptic nerve endings. The selectivity for peripheral neurons is mediated by the recognition of different receptors, such as SV2 and GT1b. For example, the specific uptake of BoNT/A into pre-synaptic nerve terminals is a tightly controlled multistep process, involving a combination of high and low affinity receptors. Binding to the ganglioside GT1b mediates an initial binding step and via this concentrates BoNT/A on the cell surface. Once anchored in the membrane, lateral movements within the plasma membrane facilitate intermolecular interactions of BoNT/A with additional (protein) receptors, such as SV2 or FGFR3. The receptor for BoNT/A is the ganglioside GT1b with a binding pocket within the C-terminal portion of the receptor binding domain. According to the APR receptor model, an array of presynaptic receptors (APRs), clustered in microdomains at the presynaptic membrane, are responsible for specific uptake of neurotoxins, including BoNT/A. It is the binding to ganglioside GT1b that mediates the initial binding step and concentrates BoNT/A on the cell surface. Once anchored in the membrane, lateral movements within the plasma membrane facilitate intermolecular interaction of BoNT/A with protein receptors, including the three isoforms of Synaptic Vesicle (SV) glycoprotein 2, SV2A (ENSG00000159164), B (ENSG00000185518) and C (ENSG00000122012) that are exposed on the outer plasma

membrane after fusion of synaptic vesicles to the presynaptic membrane. BoNT/A specifically recognizes the fourth luminal domain (LD4) of SV2. The specific sequence in the BoNT/A binding domain that interacts with SV2 has not yet been identified. Glycosylated SV2A, B, and C have also been identified as receptors for BoNT/F and glycosylated SV2A and B have been identified as receptors for BoNT/E. BoNT/D was reported to enter neurons via two ganglioside binding sites, one site at a position previously identified in BoNT/A, B, E, F, and G, and the other site resembling the second ganglioside-binding pocket of TeNT. Recently, BoNT/D has also been shown to use SV2 (all three isoforms) to enter hippocampal neurons, but BoNT/D bound SV2 via a mechanism distinct from BoNT/A and BoNT/E. SV2A and SV2B have also been reported to mediate binding and entry of TeNT into central neurons; see Jacky et al., PLoS Pathog. 2013 May; 9(5): e1003369 and references cited therein.

[0013] The physiological effect of the neurotoxins is based on the cleavage of a protein of the SNARE complex subsequent to the binding of the receptor and the translocation of the neurotoxin's light chain. The determination of the biological activity of Clostridial neurotoxins is an important aspect in the characterization of said neurotoxin proteins and is required, *inter alia*, by regulatory authorities for the clearance of Clostridial neurotoxin - containing products. A reliable test for the measurement of the biological activity of Clostridial neurotoxins is, therefore, basis for research, development and marketing of products containing Clostridial neurotoxins. Furthermore, cell-based test systems shall replace the thus far predominant animal tests for ethical reasons. For establishing such cell-based test systems, a sufficient high sensitivity of neuronal cells or cell lines towards Clostridial neurotoxins is essential.

[0014] It has advantageously been found by the present inventors that the specific uptake of Clostridial neurotoxin polypeptides into cells susceptible to Clostridial neurotoxin intoxication can be increased by at least one of the following steps: (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time or (iii) agitation of the cells during neurotoxin polypeptide exposition. At the same time, the unspecific uptake of the neurotoxin polypeptide or degradation products thereof can be reduced, by these measures. In particular, the unspecific cellular uptake of the mentioned neurotoxin or degradation products thereof is decreased, by a reduction of the time period in which the cells are incubated with the neurotoxin. The K^+ -mediated depolarization of the cells stimulates the specific uptake of the neurotoxin. Further, agitation of the cells upon neurotoxin intoxication influences the biophase concentration adjacent to the neuronal membrane and is thereby able to reduce the unspecific uptake as well as to increase the specific uptake of the neurotoxin, into the cells. The corresponding data is shown in the following examples. Further, Figure 1 shows a comparison of three cell based

assay examples where the stressed samples show a comparable kinetic in decay to the referenced assay, the mouse LD₅₀ bioassay.

5 [0015] Accordingly, in one aspect of the method of the invention, the unspecific cellular uptake of the neurotoxin polypeptide or degradation products thereof is reduced.

[0016] The present invention also comprises selectivity for impaired molecules, e.g. by degradation as an effect of stability storage of Botulinum neurotoxin. As the release of neurotransmitter is induced by K⁺-mediated depolarization, the following increase in re-
10 uptake of vesicles comprises specific uptake by binding to receptors like GT1b and / or members of the SV2 protein family. An impaired molecule may exhibit a weaker or no binding to a receptor. The same holds true for physical influences like shaking. A less stable binding of the impaired molecule will even be reduced by shaking.

15 [0017] In order to enhance the specific uptake of a neurotoxin polypeptide into cells which are susceptible to neurotoxin intoxication are incubated with a neurotoxin polypeptide for a time period and under conditions which allow for the neurotoxin polypeptide to exert its biological activity. Such time periods and cell culture conditions are known in the art. In the methods of the invention, the cell culture conditions comprise at least one of the
20 following steps: (i) K⁺-mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time or (iii) agitation of the cells during neurotoxin polypeptide exposition. The method of the invention can also encompass at least two of the mentioned steps, for example, steps (i) and (ii), steps (i) and (iii), steps (ii) and (iii), or all three of the steps (i) to (iii). It is preferred that the cell culture conditions comprise each of the
25 following steps, *i.e.* (i) K⁺-mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time and (iii) agitation of the cells during neurotoxin polypeptide exposition.

[0018] Usually, the cell culture medium used in neurotoxin activity assays in the art
30 contains about 5 mM K⁺. Neurons usually establish an ion gradient by actively transporting K⁺ ions from the medium into the cell and Na⁺ ions from the cytosol through the plasma membrane to the outside of the cell. The result is a membrane potential which is strictly regulated by the cell and which is the basis of several different cellular processes including communication between cells. A sudden change of the specific ion permeability of the
35 plasma membrane or rapid alteration of the ionic composition at either side of the membrane leads to a change of the membrane potential called depolarization. The K⁺-mediated depolarization of the cells can be carried out at an increased K⁺ concentration in the cell culture medium, for example, an additional K⁺ concentration of about 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, or 55 mM. The final concentration of K⁺ in the methods of

the invention can be about 15 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM or 60 mM K^+ . The time period for the K^+ -mediated depolarization is at least for about 30 minutes, 1 hour, 1 and a half hours, 2 hours, 2 and a half hours, 3 hours or even longer.

5 [0019] The K^+ -mediated depolarization of the cells and/or the neurotoxin polypeptide exposition can be performed in the presence of the ganglioside GT1b. GT1b can be used in a concentration of about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 μM , preferably between about 15 μM and about 50 μM , more preferably about 20 μM .

10 [0020] The neurotoxin polypeptide exposition time used in the art is usually between about 4 hours and about 96 hours, frequently about 72 hours. The reduced neurotoxin polypeptide exposition time is intoxication of the cells with the neurotoxin polypeptide for at least 24 hours and less than 96 hours, preferably for at least 48 hours, at least 60 hours, and 72 hours at maximum.

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[0021] The agitation of the cells during neurotoxin polypeptide exposition can be carried out by using means and methods known in the art, e.g., a magnetic stirrer, rotating spinner flasks or shaking of the cells by utilizing shakers. Agitation of the cells is performed at an appropriate cell culture medium flow rate which avoids the detachment of the cells from
20 the tissue flask. Suitable mean flow rates of the medium are, for example, between about 25 cm / min and about 300 cm / min.

[0022] The aforementioned K^+ -mediated depolarization of the cells can be also carried out in the presence of GT1b and neurotoxin polypeptide, followed by a neurotoxin polypeptide
25 exposition for an additional time period, such as about 24 hours, 36 hours, 48 hours, 60 hours or 72 hours, under agitation.

[0023] It is also envisaged that an incubation time without neurotoxin polypeptide precedes the neurotoxin polypeptide exposition. For instance, the incubation time without
30 neurotoxin polypeptide can be, about 6, 12, 18, 24, 30, 36, 42, 48, 54, or 60 hours, preferably between about 16 and about 48 hours.

[0024] Further preferred embodiments of the methods of the invention can be derived from the following examples.

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[0025] The term “neurotoxin polypeptide” or briefly “neurotoxin” or “toxin” as referred to herein denotes Clostridial neurotoxins, *i.e.* Clostridium botulinum and Clostridium tetani neurotoxins, in particular Botulinum neurotoxins (BoNTs) and Tetanus neurotoxin

(TeNT). More specifically, said term encompasses BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/H (Barash and Arnon, J. Infect. Dis. (2014), 209 (2): 183-191) and Tetanus neurotoxin (TeNT), as well as subtypes thereof. For example, the subtypes of BoNT/A include BoNT/A1, BoNT/A2, BoNT/A3, BoNT/A4, and BoNT/A5. The BoNT/B subtypes encompass, for instance, BoNT/B1, BoNT/B2, BoNT/B3, BoNT/B4, BoNT/B5, BoNT/B6, BoNT/B7 and BoNT/B8. The BoNT/C subtypes comprise, e.g., BoNT/C1-1 and BoNT/C1-2. Encompassed is also the BoNT/D-C subtype. The BoNT/E subtypes include, e.g., BoNT/E1, BoNT/E2, BoNT/E3, BoNT/E4, BoNT/E5, BoNT/E6, BoNT/E7, BoNT/E8 and BoNT/E9. Further, the BoNT/F subtypes comprise, for instance, BoNT/F1, BoNT/F2, BoNT/F3, BoNT/F4, BoNT/F5, BoNT/F6, and BoNT/F7. Further subtypes are described, e.g., in Hill et al. (J Bacteriol. 2007 Feb; 189(3): 818-32. Epub 2006 Nov 17.), the disclosure content of which is incorporated herewith by reference. The neurotoxin polypeptide and, in particular, its light chain and heavy chain are derivable from one of the antigenically different serotypes of Botulinum neurotoxins or subtypes indicated above. In an aspect, said light and heavy chain of the neurotoxin polypeptide are the light and heavy chain of a neurotoxin selected from the group consisting of: BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/H or TeNT. In another aspect, the polynucleotide encoding said neurotoxin polypeptides comprises a nucleic acid sequence as shown in SEQ ID NO: 1 (BoNT/A), SEQ ID NO: 3 (BoNT/B), SEQ ID NO: 5 (BoNT/C1), SEQ ID NO: 7 (BoNT/D), SEQ ID NO: 9 (BoNT/E), SEQ ID NO: 11 (BoNT/F), SEQ ID NO: 13 (BoNT/G) or SEQ ID NO: 15 (TeNT). Moreover, encompassed is, in an aspect, a polynucleotide comprising a nucleic acid sequence encoding an amino acid sequence as shown in any one of SEQ ID NO: 2 (BoNT/A), SEQ ID NO: 4 (BoNT/B), SEQ ID NO: 6 (BoNT/C1), SEQ ID NO: 8 (BoNT/D), SEQ ID NO: 10 (BoNT/E), SEQ ID NO: 12 (BoNT/F), SEQ ID NO: 14 (BoNT/G) or SEQ ID NO: 16 (TeNT). Further encompassed is in an aspect of the methods of the present invention, a neurotoxin polypeptide comprising or consisting of an amino acid sequence selected from the group consisting of: SEQ ID NO: 2 (BoNT/A), SEQ ID NO: 4 (BoNT/B), SEQ ID NO: 6 (BoNT/C1), SEQ ID NO: 8 (BoNT/D), SEQ ID NO: 10 (BoNT/E), SEQ ID NO: 12 (BoNT/F), SEQ ID NO: 14 (BoNT/G) and SEQ ID NO: 16 (TeNT). Also encompassed is the corresponding nucleic and amino acid sequence of the recently described BoNT/H as shown, e.g., in Dover, N. et al., (2014), J Infect Dis 209(2): 192-202.

[0026] In another aspect, the said polynucleotide is a variant of the aforementioned polynucleotides or polypeptides. The variant can be a naturally occurring neurotoxin polynucleotide or polypeptide, such as the aforementioned Clostridial neurotoxin isoforms or subtypes. For example, it is recognized by those of skill in the art that within each serotype of Botulinum neurotoxin there can be naturally occurring Botulinum neurotoxin

variants that differ somewhat in their amino acid sequence, and also in the nucleic acids encoding these proteins.

[0027] The variant can also be a non-naturally occurring neurotoxin polypeptide. As used
5 herein, the term "non-naturally occurring variant" of a Clostridial neurotoxin means a Clostridial neurotoxin produced with the aid of human manipulation, including, without limitation, Clostridial neurotoxin produced by genetic engineering or recombinant methods, e.g., using random mutagenesis or rational design, enzymatically modified variants of Clostridial neurotoxins that are modified by the activity of enzymes, such as
10 endo- or exoproteolytic enzymes, or Clostridial neurotoxins produced by chemical synthesis. "Genetic manipulation" refers to methods known in the art for modifying the native Clostridial neurotoxin of any serotype/subtype by means of modifying the gene encoding for the Clostridial neurotoxin or respective nucleic acids like DNA or mRNA. Recombinant methods for genetic engineering of a polynucleotide encoding a neurotoxin
15 polypeptide or a neurotoxin polypeptide are well described in the art; see, e.g. Sambrook, J. & Russell, D. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

[0028] Moreover, a naturally or non-naturally occurring variant polynucleotide as referred
20 to herein shall in another aspect comprise a nucleic acid sequence variant being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to the nucleic acid sequence as shown in any one of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13 or 15, or the nucleic acid sequence of BoNT/H as shown in in Dover, N. et al., (2014), *J Infect*
25 *Dis* 209(2): 192-202, or a nucleic acid sequence variant which encodes an amino acid sequence being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to the amino acid sequence as shown in any one of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, or 16, or the amino acid sequence of BoNT/H as shown in in Dover, N. et
30 al., (2014), *J Infect Dis* 209(2): 192-202. The term "identical" as used herein refers to sequence identity characterized by determining the number of identical amino acids between two nucleic acid sequences or two amino acid sequences wherein the sequences are aligned so that the highest order match is obtained. It can be calculated using published techniques or methods codified in computer programs such as, for example, BLASTP, BLASTN or FASTA (Altschul 1990, *J Mol Biol* 215, 403). The percent identity values are,
35 in one aspect, calculated over the entire amino acid sequence, e.g. the light chain or heavy chain of the neurotoxin polypeptide or both. A series of programs based on a variety of algorithms is available to the skilled worker for comparing different sequences. In this context, the algorithms of Needleman and Wunsch or Smith and Waterman give

particularly reliable results. To carry out the sequence alignments, the program PileUp (Higgins 1989, CABIOS 5, 151) or the programs Gap and BestFit (Needleman 1970, J Mol Biol 48; 443; Smith 1981, Adv Appl Math 2, 482), which are part of the GCG software packet (Genetics Computer Group 1991, 575 Science Drive, Madison, Wisconsin, USA 53711), may be used. The sequence identity values recited above in percent (%) are to be determined, in another aspect of the invention, using the program GAP over the entire sequence region with the following settings: Gap Weight: 50, Length Weight: 3, Average Match: 10.000 and Average Mismatch: 0.000, which, unless otherwise specified, shall always be used as standard settings for sequence alignments. In an aspect, each of the aforementioned variant polynucleotides encodes a polypeptide retaining one or more and, in another aspect, all of the herein defined biological properties of the respective neurotoxin polypeptide, i.e. the BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/H or Tetanus neurotoxin (TeNT). Those of skill in the art will appreciate that full biological activity is maintained only after proteolytic activation, even though it is conceivable that the unprocessed precursor can exert some biological functions or be partially active. *In vivo* assays for assessing the biological activity of a Clostridial neurotoxin include the mouse LD50 assay and the *ex vivo* mouse hemidiaphragm assay, as described by Pearce et al. (Pearce 1994, Toxicol. Appl. Pharmacol. 128: 69-77) and Dressler et al. (Dressler 2005, Mov. Disord. 20:1617-1619, Keller 2006, Neuroscience 139: 629-637). The biological activity is commonly expressed in Mouse Units (MU). As used herein, 1 MU is the amount of neurotoxic component, which kills 50% of a specified mouse population after intraperitoneal injection, i.e. the mouse i.p. LD50. In a further aspect, the variant polynucleotides can encode neurotoxins having improved or altered biological properties, e.g., they may comprise cleavage sites which are improved for enzyme recognition, and/or may be improved for receptor binding, internalization, translocation across the endosomal membrane into the cytosol or endoproteolytic cleavage of the corresponding substrate of the SNARE protein family.

[0029] Non-limiting examples of non-naturally occurring Clostridial neurotoxin variants include, e.g., conservative Clostridial neurotoxin variants. As used herein, the term "conservative Clostridial neurotoxin variant" means a Clostridial neurotoxin that has at least one amino acid substituted by another amino acid or an amino acid analog that has at least one property similar to that of the original amino acid from the reference Clostridial neurotoxin sequence as set forth elsewhere herein, e.g. the amino acid sequence shown in SEQ ID NO. 2, 4, 6, 8, 10, 12, 14 or 16 or the amino acid sequence of BoNT/H as shown in Dover, N. et al., (2014), J Infect Dis 209(2): 192-202. The variant may have one, two, three, four, five or even more conservative amino acid substitutions compared to the reference sequence. The variant shall have comparable or even improved properties of the reference Clostridial neurotoxin sequence. Examples of properties include, without

limitation, similar size, topography, charge, hydrophobicity, hydrophilicity, lipophilicity, covalent-bonding capacity, hydrogen-bonding capacity, a physicochemical property, of the like, or any combination thereof. Preferably, the property is a biological property as defined elsewhere herein, i.e. (a) receptor binding, (b) internalization, (c) translocation across the endosomal membrane into the cytosol, and/or (d) endoproteolytic cleavage of proteins involved in synaptic vesicle membrane fusion. A conservative Clostridial neurotoxin variant can function in substantially the same manner as the reference Clostridial neurotoxin on which the conservative Clostridial neurotoxin variant is based, and can be substituted for the reference Clostridial neurotoxin in any aspect of the present invention. The Clostridial neurotoxin described herein will typically contain naturally occurring amino acid residues, but in some cases non-naturally occurring amino acid residues may also be present. Therefore, so-called "peptide mimetics" and "peptide analogues", which may include non-amino acid chemical structures that mimic the structure of a particular amino acid or peptide, may also be used within the context of the invention. Such mimetics or analogues are characterized generally as exhibiting similar physical characteristics such as size, charge or hydrophobicity, and the appropriate spatial orientation that is found in their natural peptide counterparts. A specific example of a peptide mimetic compound is a compound in which the amide bond between one or more of the amino acids is replaced by, for example, a carbon-carbon bond or other non-amide bond, as is well known in the art; see, for example Sawyer, in *Peptide Based Drug Design*, pp. 378-422, ACS, Washington D.C. 1995.

[0030] The neurotoxin polypeptide variant as used herein further encompasses chemically modified neurotoxin polypeptides. "Chemical modification" as used herein refers generally to methods known in the art for modifying the native or recombinant Clostridial neurotoxin of any serotype or subtype by means of chemical reactions or the like; it refers especially to substitutions, deletions, insertions, additions or posttranslational modifications of amino acids of the Clostridial neurotoxin. A chemically modified neurotoxin polypeptide may be one that is modified by pyruvation, phosphorylation, sulfatation, lipidation, pegylation, glycosylation and/or the chemical addition of an amino acid or a polypeptide comprising, e.g., between about two and about 500 amino acids. For example, by incorporating hyaluronic acid or polyvinylpyrrolidone or polyethyleneglycol or mixtures thereof into the neurotoxin polypeptide, the Clostridial neurotoxin, or the toxin which is derived from Clostridial toxin by chemical modification or by genetic manipulation, can be stabilized.

[0031] Another non-naturally occurring variant of the Clostridial neurotoxin which can be used in the methods of the invention is a hybrid Clostridial neurotoxin. In one aspect, the hybrid Clostridial neurotoxin comprises a combination of a Clostridial neurotoxin heavy

chain and light chain, wherein the light chain and heavy chain are not of the same serotype or subtype.

[0032] Methods for making such chemically, enzymatically or genetically modified variants of Clostridial neurotoxins, and methods for identifying whether such variants maintain the biological properties referred to herein, such as (a) receptor binding, (b) internalization, (c) translocation across the endosomal membrane into the cytosol, and/or (d) endoproteolytic cleavage of proteins involved in synaptic vesicle membrane fusion, are well known to anyone of ordinary skill in the art; see, e.g., Sambrook, loc. cit.

[0033] The term "biological activity of a neurotoxin polypeptide" as used herein means the biological properties characteristic for a neurotoxin polypeptide, namely, a) receptor binding, (b) internalization, (c) translocation across the endosomal membrane into the cytosol, and/or (d) endoproteolytic cleavage of proteins involved in synaptic vesicle membrane fusion. It is envisaged that the neurotoxin polypeptide as used herein exhibits at least one of the properties a) to d) mentioned above, preferably endoproteolytic cleavage of proteins involved in synaptic vesicle membrane fusion, or two or three or all four biological properties listed in a) to d). Assays for determining the biological activity of neurotoxin polypeptides are well known in the art and also described elsewhere herein; see, e.g., Pellett et al. (2011), *Biochem. Biophys. Res. Commun.* 404, 388-392; Whitemarsh et al. (2012), *Toxicol. Sci.* 126, 426-35.

[0034] SNAP-25 is a known substrate of and cleaved by BoNT/A, BoNT/C1 and BoNT/E. VAMP/Synaptobrevin is a substrate of and cleaved by BoNT/B, BoNT/D, BoNT/F, BoNT/G and TeNT, whereas Syntaxin is a substrate of and cleaved by BoNT/C1. The mentioned substrates and the corresponding nucleic acid and amino acid sequences are well known in the art; see, e.g., WO 2014/207109.

[0035] As used herein, the term "cell" refers to any eukaryotic cell susceptible to neurotoxin intoxication by a neurotoxin such as, e.g., BoNT/A, or any eukaryotic cell that can uptake a neurotoxin. Aspects of the present disclosure comprise, in part, a cell from an established cell line. The term "cell" encompasses cells from a variety of organisms, such as, e.g., murine, rat, porcine, bovine, equine, rhesus, primate and human cells; from a variety of cell types such as, e.g., neuronal and non-neuronal; and can be isolated from or part of a heterogeneous cell population, tissue or organism. It is to be understood that human embryonic cells are excluded from the scope of the methods of the invention. As used herein, the term "established cell line" is synonymous with "immortal cell line," or "transformed cell line" and refers to a cell culture of cells selected for indefinite propagation from a cell population derived from an organism, tissue, or organ source. By

definition, an established cell line excludes a cell culture of primary cells. As used herein, the term "primary cells" are cells harvested directly from fresh tissues or organs and do not have the potential to propagate indefinitely. For example, primary neuronal cells can be used in the methods of the invention. An established cell line can comprise a heterogeneous population of cells or a uniform population of cells. An established cell line derived from a single cell is referred to as a clonal cell line. An established cell line can be one whose cells endogenously express all component necessary for the cells to undergo the overall cellular mechanism whereby a neurotoxin, such as BoNT/A, proteolytically cleaves a substrate, such as SNAP-25, and encompasses the binding of a neurotoxin to a neurotoxin receptor, such as BoNT/A, to a BoNT/A receptor, the internalization of the neurotoxin/receptor complex, the translocation of the neurotoxin light chain from an intracellular vesicle into the cytoplasm and the proteolytic cleavage of a neurotoxin substrate. Alternatively, an established cell line can be one whose cells have had introduced from an exogenous source at least one component necessary for the cells to undergo the overall cellular mechanism whereby a neurotoxin, such as BoNT/A, proteolytically cleaves a substrate, such as SNAP-25, and encompasses the binding of a neurotoxin to a receptor, such as BoNT/A to a BoNT/A receptor, the internalization of the neurotoxin/receptor complex, the translocation of the neurotoxin light chain from an intracellular vesicle into the cytoplasm and the proteolytic cleavage of a neurotoxin substrate. Also referred to as a genetically-engineered cell line, cells from such an established cell line may, e.g., express an exogenous FGFR2, an exogenous FGFR3, an exogenous SV2, an exogenous neurotoxin substrate such as SNAP-25, or any combination thereof.

[0036] "Cell culture" as used herein refers in the broadest sense to the removal of cells from an animal or human and their subsequent growth in a favourable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established. Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate, *i.e.* reach confluence. At this stage, the cells have to be sub-cultured, *i.e.* passaged by transferring them to a new vessel with fresh growth medium to provide more room for continued growth. Normal cells usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as senescence; these cell lines are known as finite. However, some cell lines become immortal through a process called transformation, which can occur spontaneously or can be chemically or virally induced. When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line. Culture conditions vary widely for each cell type, but the artificial

environment in which the cells are cultured invariably consists of a suitable vessel containing the following: a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, gases (O₂, CO₂), a regulated physico-chemical environment (pH, osmotic pressure, temperature) etc. Most cells are anchorage-dependent and must be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture), while others can be grown floating in the culture medium (suspension culture).

[0037] The term “cell(s) susceptible to neurotoxin intoxication” as denoted herein means a cell that can undergo the overall cellular mechanisms whereby a neurotoxin polypeptide (e.g., BoNT/A) cleaves a neurotoxin substrate (e.g., the BoNT/A substrate SNAP-25) and encompasses the binding of the neurotoxin polypeptide to its corresponding receptor (e.g., binding of BoNT/A to the BoNT/A receptor), the internalization of the neurotoxin/receptor complex, the translocation of the neurotoxin light chain from an intracellular vesicle into the cytoplasm and the proteolytic cleavage of the neurotoxin substrate. Accordingly, a “cell susceptible to neurotoxin intoxication” as used herein means a neurotoxin sensitive cell. The mentioned term comprises a cell or a cell line, for example, an isolated, primary cell or a cell line thereof or a cell of an established cell line or an established cell line, for example, tumor cells or tumor cell lines which are capable of differentiating to neuronal cells, such as neuroblastoma cells or neuroblastoma cell lines as defined elsewhere herein. For example, said neuroblastoma cell line can be a SiMa cell line which is commercially available from DSMZ (ACC 164). Specific clones of the cell line SiMa are furthermore disclosed in WO 2010/105234. Other neuroblastoma cell lines which can be used in the method of the invention can be obtained from ATCC or DSMZ, under the following ATCC or DSMZ numbers: Cell line N1E-115 under CRL-2263, cell line Neuro2a under CCL-131, cell line SH-SY5Y under CRL-2266, cell line PC12 under CRL-1721, cell line MHH-NB-11 under ACC 157 (DSMZ) and cell line SK-N-BE(2) under CRL-2271. Other tumor cells which are susceptible to neurotoxin intoxication are P-19 cells (murine embryonal carcinoma cell line) (DSMZ no. ACC 316). In some aspects, e.g. for activity assays, it can be necessary to differentiate said cells into neuronal cells. Such differentiation methods are well described in the literature. Further encompassed by cells susceptible to neurotoxin intoxication are induced pluripotent stem cell (iPS)-derived neurons, preferably human induced pluripotent stem cell (iPS)-derived neurons; see, e.g., Whitemarsh et al. (2012), loc. cit. Such human iPS-derived neurons are also commercially available, for instance, from Cellular Dynamics. Methods of generating iPS cells are described, for example, in Yu et al. (Science 2009 May 8; 324(5928): 797-801. Epub 2009), WO 2011/056971 and WO 2011/025852. In some aspects, iPS are differentiated into neurons using suitable methods, e.g., those described in WO 2012/135621 and U.S. Patent Applications US 2010/0279403 and US 2010/0216181.

[0038] The terms “differentiation”, “differentiating” or “differentiated” as used herein denote the process by which an unspecialized or a relatively less specialized cell becomes relatively more specialized. In the context of cell ontogeny, the adjective “differentiated” is a relative term. Hence, a “differentiated cell” is a cell that has progressed further down a certain developmental pathway than the cell it is being compared with. A differentiated cell may, for example, be a terminally differentiated cell, i.e., a fully specialized cell that takes up specialized functions in various tissues and organs of an organism, and which may but need not be post-mitotic. For instance, iCell[®] neurons are terminally differentiated from human iPS cells and exhibit neuronal characteristics and functions. In another example, a differentiated cell may also be a progenitor cell within a differentiation lineage, which can further proliferate and/or differentiate. Similarly, a cell is “relatively more specialized” if it has progressed further down a certain developmental pathway than the cell it is being compared with, wherein the latter is therefore considered “unspecialized” or “relatively less specialized”. A relatively more specialized cell may differ from the unspecialized or relatively less specialized cell in one or more demonstrable phenotypic characteristics, such as, for example, the presence, absence or level of expression of particular cellular components or products, e.g., RNA, proteins, specific cellular markers or other substances, activity of certain biochemical pathways, morphological appearance, proliferation capacity and/or kinetics, differentiation potential and/or response to differentiation signals, etc., wherein such characteristics signify the progression of the relatively more specialized cell further along the said developmental pathway. Cell culture conditions for differentiating cells into neuronal cells are well known in the art as evident from the literature cited herein.

[0039] Furthermore, cell culture conditions for incubating cells susceptible to neurotoxin intoxication with a neurotoxin polypeptide for a time and under conditions which allow for the neurotoxin polypeptide to exert its biological activity are well described in the art and can also be derived from the publications recited herein.

[0040] The term “specific uptake of a neurotoxin polypeptide” as used herein means a process in which the neurotoxin polypeptide enters the neuron by binding to its specific neuronal membrane receptor(s), for example, SV2, gangliosides, GD1a, Synaptotagmin II for BoNT/B, or Synaptotagmin I for BoNT/D/-C, being taken up into an endosome-like compartment and penetrating the endosome membrane via a pH-dependent translocation process. Accordingly, the mentioned term encompasses a) receptor binding of the neurotoxin polypeptide, (b) internalization of the neurotoxin polypeptide, and (c) translocation of the neurotoxin polypeptide across the endosomal membrane into the cytosol. As appreciated by those skilled in the art, the neurotoxin-sensitive cell is

preferably able to first uptake a neurotoxin and then undergoes the overall cellular mechanisms listed above. The expression “non-specific or unspecific uptake of a neurotoxin polypeptide” as denoted herein means a process in which the neurotoxin polypeptide or a degradation product thereof enters the neuron by binding to non-specific neuronal membrane receptor(s) or by entering the cell via unspecific mechanisms, e.g. by an accidental co-transport in the event of pinocytosis. A neurotoxin-sensitive cell as used herein can uptake, e.g., about 100 nanomolar (nM), about 10 nM, about 1 nM, about 500 picomolar (pM), about 400 pM, about 300 pM, about 200 pM, about 100 pM, about 90 pM, about 80 pM, about 70 pM, about 60 pM, about 50 pM, about 40 pM, about 30 pM, about 20 pM, about 10 pM, about 9 pM, about 8 pM, about 7 pM, about 6 pM, about 5 pM, about 4 pM, about 3 pM, about 2 pM, about 1 pM, about 0.5 pM, or about 0.1 pM of neurotoxin polypeptide or less than one of the indicated values. EC50 values above 100 pM have been reported in the literature. By definition, a cell susceptible to neurotoxin intoxication must express, or be engineered to express, at least one neurotoxin receptor and at least one neurotoxin substrate. Receptors and substrates for neurotoxins are described in the art and mentioned elsewhere herein. Accordingly, said cell is preferably susceptible to a biologically active or mature neurotoxin polypeptide as defined herein. Preferably, the neurotoxin-sensitive cell as used herein is susceptible to neurotoxin intoxication by, e.g., about 1 nM or less, 500 pM or less, about 400 pM or less, about 300 pM or less, about 200 pM or less, about 100 pM or less, about 90 pM or less, about 80 pM or less, about 70 pM or less, about 60 pM or less, about 50 pM or less, about 40 pM or less, about 30 pM or less, about 20 pM or less, about 10 pM or less, about 9 pM or less, about 8 pM or less, about 7 pM or less, about 6 pM or less, about 5 pM or less, about 4 pM or less, about 3 pM or less, about 2 pM or less, about 1 pM or less, about 0.9 pM or less, about 0.8 pM or less, about 0.7 pM or less, about 0.6 pM or less, about 0.5 pM or less, about 0.4 pM or less, about 0.3 pM or less, about 0.2 pM or less, or even about 0.1 pM or less. 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 a 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 exposure duration. Methods for the identification of cells or cell lines susceptible to neurotoxin intoxication and/or having neurotoxin uptake capacity, i.e. neurotoxin-sensitive cells as defined herein, are known in the art; see, e.g. US 2012/0122128 A1. The biological activity of the neurotoxin polypeptides, in an aspect, results from all of the aforementioned biological properties. Only a few cell-based assays with sufficient high sensitivity towards neurotoxins which can be used for the

determination of the biological activity of a neurotoxin have been described in the prior art so far, as indicated elsewhere herein.

[0041] The term “enhancing” as utilized herein means that the specific uptake of a neurotoxin polypeptide into the cell is improved or increased, in comparison to Clostridial neurotoxin intoxication not using (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time and (iii) agitation of the cells during neurotoxin polypeptide exposition. The specific uptake of a neurotoxin polypeptide into the cell is preferably increased at least 1.5-fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold or even at least 10-fold, in comparison to methods using cell culture conditions that do not comprise (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time and (iii) agitation of the cells during neurotoxin polypeptide exposition. Generally, the methods described in the art use a final concentration of K^+ of about 5 mM, a neurotoxin polypeptide exposition time of about 72 hours and no agitation of the cells. It has found by the present inventors that the above measures (i), (ii) and/or (iii) not only improve the specific uptake of the neurotoxin into the cells but also result in a reduced unspecific uptake of the mentioned neurotoxin or degradation products into the cells. The nonspecific uptake of a neurotoxin polypeptide into the cell is preferably reduced at least 1.5-fold, at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold or even at least 10-fold, in comparison to methods using cell culture conditions that do not comprise (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time and/or (iii) agitation of the cells during neurotoxin polypeptide exposition. Means and methods for measuring the specific or unspecific uptake of a neurotoxin polypeptide into the cell are well described in the literature and herein and further shown in the following examples. For example, the effect of BoNT/A, BoNT/C and BoNT/E neurotoxin exposure on SNAP-25 proteolysis in neuronal cell cultures can be used as an indicator of neurotoxin translocation. The same holds true for BoNT/C neurotoxin exposure on Syntaxin proteolysis in neuronal cells and BoNT/E, BoNT/D and BoNT/G neurotoxin exposure on VAMP proteolysis.

[0042] The aforementioned method of the invention is followed by a method for determining the biological activity of the neurotoxin polypeptide in the cells, in a further aspect of the method of the invention.

[0043] As demonstrated in the following examples, a cell-based potency assay could advantageously be improved by increasing the specific uptake of Clostridial neurotoxin polypeptides into the cells, by using K^+ -mediated depolarization of the cells, a reduced neurotoxin polypeptide exposition time and/or agitation of the cells during neurotoxin

polypeptide exposition. At the same time, a decreased unspecific uptake of the mentioned neurotoxin or degradation products into the cells could be observed.

[0044] A number of assays for determining the biological activity of neurotoxin polypeptides has been described in the art, such as light chain assays (ELISA), Endopep-MS, FRET, HPLC-UPLC, DARET or cell-based assays using, for instance, SH-SY5Y or Neuro2a cells, embryonic chicken neurons, primary neurons from spinal cord or dorsal root ganglia (Pellett et al. (2011), Biochem. Biophys. Res. Commun. 404, 388-392), embryonic stem cell-derived neurons (Whitemarsh et al. (2012), Toxicol. Sci. 126, 426-35) relying on Western blot read-out, or differentiated human neuroblastoma SiMa cells; see, e.g. Fernández-Salas, E. et al., (2012). PLoS One 7 (11).

[0045] In a second aspect, the invention provides for a method for directly determining the biological activity of a neurotoxin polypeptide in cells, comprising:

- a) incubating cells susceptible to neurotoxin intoxication with a neurotoxin polypeptide for a time and under conditions which allow for the neurotoxin polypeptide to exert its biological activity, the incubation comprising at least one of the following steps: (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time and/or (iii) agitation of the cells during neurotoxin polypeptide exposition;
- b) fixing the cells and, optionally, permeabilizing the cells with a detergent;
- c) contacting the cells with at least a first capture antibody specifically binding to the non-cleaved and neurotoxin-cleaved substrate and with at least a second capture antibody specifically binding to the cleavage site of the neurotoxin-cleaved substrate, under conditions which allow for binding of said capture antibodies to said substrates;
- d) contacting the cells with at least a first detection antibody specifically binding to the first capture antibody, under conditions which allow for binding of said first detection antibody to said first capture antibody, thus forming first detection complexes and with at least a second detection antibody specifically binding to the second capture antibody, under conditions which allow for binding of said second detection antibody to said second capture antibody, thus forming second detection complexes;
- e) determining the amount of the first and second detection complexes of step d); and
- f) calculating the amount of substrate cleaved by said neurotoxin polypeptide in said cells by means of the second detection complexes, thereby determining the biological activity of said neurotoxin polypeptide in said cells.

[0046] Preferably, this method is an in vitro method. This method of the invention allows for the direct determination of the biological activity of a neurotoxin polypeptide in cells. This means that no lysis of the cells and no isolation or concentration of the cleaved neurotoxin substrate from cell lysates is necessary any longer, as in the methods described in the art. For example, in the Western blot analysis-based assay of the art, the neurotoxin substrate is concentrated by the separation and concentration of the components of the respective sample in the SDS polyacrylamide gel. In the ECL sandwich ELISA described in the art, the concentration of the neurotoxin substrate is carried out by using antibodies which bind specifically to the cleaved neurotoxin substrate on a microtiter plate to which the cell lysate is added. The cleaved neurotoxin substrate is isolated from the lysate by binding of the mentioned antibody which results in a concentration of said cleaved Clostridial neurotoxin substrate.

[0047] In contrast, the cleaved neurotoxin substrate, for example SNAP-25, can be directly detected in the cell, in this method of the invention. To this end, cells which are susceptible to neurotoxin intoxication as defined in more detail elsewhere herein are incubated with a neurotoxin polypeptide for a time and under conditions which allow for the neurotoxin polypeptide to exert its biological activity. The incubation comprises K^+ -mediated depolarization of the cells, a reduced neurotoxin polypeptide exposition time and/or agitation of the cells during neurotoxin polypeptide exposition, as set forth elsewhere herein. These measures enhance the specific cellular uptake of the neurotoxin while at the same time reducing the unspecific uptake of the neurotoxin or degradation products into the cells. In a next step, the cells are fixed, for example, by addition of a fixation agent such as methanol, ethanol, acetone, formaldehyde or mixtures of the mentioned fixation agents. Optionally, the cells can be permeabilized by using at least one detergent such as Triton X-100, Tween 20, Saponin, Digitonin or n-Octyl- β -glucopyranoside. The detergent can be comprised in an appropriate buffer such as PBS. Thereafter, the cells are contacted with at least a first capture antibody which specifically binds to the non-cleaved and neurotoxin-cleaved substrate and with at least a second capture antibody specifically binding to the cleavage site of the neurotoxin-cleaved substrate, under conditions which allow for binding of said capture antibodies to said substrates. Herein, the first capture antibody is able to determine the total content or amount of neurotoxin substrate in the cells, by binding specifically to an appropriate epitope present in both the non-cleaved and neurotoxin-cleaved neurotoxin substrate. The second capture antibody recognizes and binds specifically to an epitope present only in the cleaved neurotoxin substrate, for example, by binding specifically to the neurotoxin-cleaved site in the neurotoxin substrate. Alternatively, the cells can be contacted with a mixture of said first and second capture antibodies, i.e. the cells are contacted with at least a first capture antibody and at least a

second capture antibody simultaneously, under the mentioned conditions. In the next step, the cells are contacted with at least a first detection antibody specifically binding to the first capture antibody under conditions which allow for binding of said first detection antibody to said first capture antibody, thus forming first detection complexes. In a subsequent step, the cells are contacted with at least a second detection antibody specifically binding to the second capture antibody, under conditions which allow for binding of said second detection antibody to said second capture antibody, thus forming second detection complexes. Alternatively, the cells can be contacted with a mixture of said first and second detection antibodies, i.e. the cells are contacted with at least a first detection antibody and at least a second detection antibody simultaneously, under the mentioned conditions. Alternatively, after permeabilization of the cells, they can be contacted with a mixture of said first and second capture antibodies and said first and second detection antibodies simultaneously, under the mentioned conditions. In the next step, the amounts of the first and second detection complexes are determined. Finally, the amount of substrate cleaved by said neurotoxin polypeptide in said cells is calculated by means of the second detection complexes. Thereby, the biological activity of said neurotoxin polypeptide is determined directly in the cells.

[0048] In the following, this method of the invention is described in more detail. For cell culture, the cells susceptible to neurotoxin intoxication as defined herein, such as neuronal cells, SiMa cells or iPS-derived neurons, are first seeded on 96-well microtiter plates. SiMa cells are differentiated to a neuronal phenotype, for example, according to the procedures disclosed in WO 2010/105234, and iPS-derived neurons are differentiated to a neuronal phenotype, e.g. , according to assays described in WO 2012/135621. Then, the cells are incubated with a neurotoxin polypeptide, such as BoNT/A for a time and under conditions which allow for the neurotoxin polypeptide to exert its biological activity. The incubation comprises K^+ -mediated depolarization of the cells, a reduced neurotoxin polypeptide exposition time and/or agitation of the cells during neurotoxin polypeptide exposition, as described elsewhere herein.

[0049] In the subsequent step, the cells are fixed on the microtiter plate, prior to the ELISA assay. For fixing the cells, for example ice-cold methanol (-20 °C) can be added to the cells for 20 minutes at -20 °C.

[0050] For performing the ELISA assay, the cells are first washed. As a wash buffer, e.g., 0.1 % Triton X-100 in 10 mM PBS buffer (pH 7.4) can be used. Thereafter, endogenous proteases are quenched by a quenching buffer such as 0.6% H_2O_2 in 10 mM PBS (pH 7.4), followed by another wash step. In the following step, free binding sites on the microtiter plate are blocked by an appropriate blocking buffer such as, for instance, 2 % BSA in

10 mM PBS buffer (pH 7.4) and 0.05 % Triton X-100. Then, the cells are permeabilized, by using an appropriate detergent. As a permeabilization buffer, e.g., 0.5% Triton X-100 in 10 mM PBS buffer can be utilized. Permeabilization allows the diffusion of the antibodies through the pores formed in the cells. Thereafter, the cells are washed by washing buffer as
5 mentioned above.

[0051] In the next step, the permeabilized cells are incubated, e.g., with a mixture of two different antibodies. The mixture comprises a first capture antibody specifically binding to the non-cleaved and neurotoxin-cleaved substrate and a second capture antibody
10 specifically binding to the cleavage site of the neurotoxin-cleaved substrate. Said first and second capture antibodies can also be applied subsequently. For example, the first capture antibody can specifically bind to both non-cleaved and neurotoxin-cleaved SNAP-25, thereby allowing for the quantification of the total amount or content of SNAP-25 in the cells. Further, this first capture antibody can be used for the normalization of the amount of
15 cleaved SNAP-25 in the cells, upon evaluation. The second capture antibody specifically binds to the cleavage site of the neurotoxin-cleaved substrate and therefore allows the determination and detection of the cleaved neurotoxin substrate, such as BoNT/A-cleaved SNAP-25.

[0052] The following detection of the total neurotoxin substrate and the neurotoxin-cleaved neurotoxin substrate in the method of the invention can be carried out directly on the microtiter plate or cell culture dish, i.e. within the cells. Advantageously, it is, therefore, not necessary to prepare cell extracts and to isolate and/or concentrate the neurotoxin substrate from the cell lysate in the method of the invention, as in the methods
25 described in the art. Thereafter, the cells are washed in order to remove excess antibody not bound to the respective antigen. In the subsequent step, the permeabilized cells are contacted with at least a first detection antibody and at least a second detection antibody. Said antibodies can be applied as a mixture, i.e. simultaneously, or subsequently. The first detection antibody specifically binds to the first capture antibody. Thereby, first detection
30 complexes are being formed. The first detection antibody can be directed against the species from which the first capture antibody is derived from. For example, in case the rabbit polyclonal anti-SNAP-25 antibody S9684 (Sigma) is used as a first capture antibody specifically binding to the non-cleaved and BoNT/A-cleaved substrate SNAP-25, an anti-rabbit alkaline phosphatase-conjugated antibody can be used as a first detection antibody.
35 The second detection antibody specifically binds to the second capture antibody. Thereby, second detection complexes are being formed. The second detection antibody can be directed against the species from which the second capture antibody is derived from. For instance, in case the mouse monoclonal antibody (mAb) 20-2-5 described WO 2014/207109 in is used as a second capture antibody specifically binding to the

BoNT/A-cleaved SNAP-25, an anti-mouse horseradish peroxidase (HRP)-conjugated antibody can be used as a second detection antibody. It is evident to those skilled in the art that the first detection antibody and the second detection antibody are conjugated with different enzymes in order to allow for the specific detection of the respective first and second capture antibody as used in the method of the invention. For instance, the HRP-based detection as described elsewhere herein can be used for the BoNT/A-cleaved SNAP-25 and the alkaline phosphatase-based detection for the total (BoNT/A-cleaved and non-cleaved) SNAP-25. Thereafter, the cells are washed again. In a subsequent step, a fluorogenic HRP substrate is added to the cells. As a HRP substrate, e.g., Amplex UltraRed (Invitrogen) can be used which is excited at 540 nm and which emits at 600 nm. Incubation with the HRP substrate is carried out for a time sufficient for sufficient conversion of substrate by the horseradish peroxidase. Subsequent to the incubation with the HRP substrate, for example, the AP substrate DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate; excitation 360 nm; emission 450 nm) can be added to the HRP substrate and the cells are incubated with a mixture of said two substrates. Incubation with said AP substrate is carried out for a time which allows for sufficient conversion of substrate by the alkaline phosphatase. As known in the art, a substrate has to be converted in an amount which is sufficient so that the measured signal is at least as high as the mean value of the blank plus three standard deviations of the mean, according to the definition of limit of detection. The limit of detection can be determined as described in the literature; see, e.g., Armbruster and Pry, Clinical Biochem. Rev. 2008, 29 (Supplement 1): S49-S52. Because the pH optimum of the alkaline phosphatase is in the alkaline region, the corresponding substrate buffer is strongly alkaline. If the alkaline phosphatase substrate is added to the HRP substrate, the reaction of the horseradish peroxidase is stopped by the alkaline pH and the alkaline phosphatase converts DiFMUP. Converted HRP substrate is not influenced by the alkaline pH. Finally, the fluorescence of the two substrates is measured as follows:

Amplex UltraRed: Excitation 540 nm; emission 600 nm

DiFMUP: Excitation 360 nm; emission 450 nm

[0053] As appreciated by those skilled in the art, only those fluorogenic substrates are appropriate for detection of the first and second capture antibody in the method of the invention which exhibit different excitation/emission wave lengths of the used substrates. Only in this case, they allow for the specific detection of each antigen, i.e. the total neurotoxin substrate (such as non-cleaved and neurotoxin-cleaved SNAP-25) and the cleaved neurotoxin substrate (such as neurotoxin-cleaved SNAP-25). Thereby, it is possible to quantify the total content of neurotoxin substrate and the content of cleaved neurotoxin substrate in every well or cell culture dish at the same time. In light of this, it is

advantageously possible to automatize the method of the invention. As set forth elsewhere herein it is envisaged that the fluorogenic substrates chosen for the method of the invention exhibit a sufficient shift between the excitation/emission spectra in order to allow for the specific detection of the respective substrate. This requirement is fulfilled, for example, for the HRP substrate Amplex and its derivatives and for the AP substrate DiFMUP. Whereas, in an optimal case, there is no overlap between the excitation/emission spectra of the used fluorogenic substrates, it has been experienced that an overlap of up to 30% in the peak area of the excitation spectra of the used fluorogenic substrates is tolerable. Further details as regards this method of the invention are described, e.g., in WO 2014/207109.

[0054] As further acknowledged by those skilled in the art, the method of the present invention allows for the direct detection and quantification of neurotoxin substrate cleaved by the neurotoxin polypeptide in the cells, thereby determining the biological activity of said neurotoxin polypeptide in said cells. Advantageously, the method of the invention does not require the preparation of cell lysates or extracts and the isolation or concentration of the cleaved neurotoxin substrate from the cell lysates/extracts, which is necessary for the methods known in the art. As a consequence of this, sample material can be saved. Further, the sample preparation and the number of samples can be reduced by the method of the invention since the amount of total neurotoxin substrate and the amount of cleaved neurotoxin substrate in the sample can be determined at the same time. In the assays described in the art, the samples have to be subdivided in order to detect both antigens, i.e. total neurotoxin substrate and cleaved neurotoxin substrate, separately from each other. The method of the invention renders the subdivision of the sample unnecessary. Thereby, inhomogeneities resulting from the subdivision of samples can be avoided and sample material can be saved. Furthermore, antigens can be degraded in the assays described in the art which can falsify the detection of the cleaved neurotoxin substrate. This is because in the assays described in the art, the cells are incubated with detergent-containing lysis buffers which, however, are not able to inactivate the neurotoxin polypeptide or other endogenous proteases resulting in degradation of the neurotoxin substrate upon longer storage of the samples. Stronger lysis buffers cannot be used in the ECL sandwich ELISA described in the prior art due to the required use of the cell lysate in said assay. This is because the aggregation of the above-mentioned antigens can result in unspecific adsorption of the antigens to the plastic surface of the cell culture dishes or microtiter plates which in turn disturbs the detection of the antigens by appropriate antibodies. Since the antibodies for the detection of the antigens get into contact with the lysate, too, the antibodies can also aggregate. In this case, no reliable and accurate detection of the antigen is possible anymore. The present inventors have experienced such degradation reactions by using Western blot assays for the detection of the biological activity of neurotoxin activity described in the art. Upon longer storage of lysates at -20 °C, in comparison to fresh lysate

samples the detection signal of total SNAP-25 has been found to be strongly reduced and the ratio of cleaved neurotoxin substrate SNAP-25 to un-cleaved neurotoxin substrate SNAP-25 had shifted due to degradation processes during the freezing. It has been found by the present inventors that the degradation of the neurotoxin substrate and/or the
5 instability of the samples can be avoided by directly fixing the cells on the cell culture dish because both the neurotoxin substrate and the neurotoxin or other endogenous proteases are inactivated immediately by aggregation on the cell culture dish. This can be achieved by using, for example, fixing of the cells by methanol or other fixatives or fixation agents known in the art, such as ethanol, acetone, formaldehyde or mixtures thereof or other
10 fixation agents described herein. The analysis of the stability of, e.g., parental SiMa cells (human neuroblastoma cells; DSMZ no.: ACC 164) and iPS-derived neurons (Whitemarsh et al. (2012), Toxicol. Sci. 126, 426-35) using this fixation method did not reveal any differences between fresh and cell culture dishes stored seven days in the refrigerator.

[0055] Suitable antibodies specifically binding to the non-cleaved and neurotoxin-cleaved substrate which can be used as first capture antibody in the method of the invention encompass, e.g., the rabbit polyclonal anti-SNAP-25 antibody S9684 (Sigma), the rabbit polyclonal anti-SNAP25 antibody PA5-19708 (Pierce Antibodies), the rabbit polyclonal anti-SNAP25 antibody PA5-19701 (Pierce Antibodies), or the rabbit monoclonal anti-
20 SNAP25 antibody ab108990 (Abcam).

[0056] Appropriate antibodies specifically binding to the cleavage site of the neurotoxin-cleaved substrate that can be utilized as second capture antibody in the method of the invention include, for example, the mouse monoclonal antibody clone 20-2-5 (WO
25 2014/207109), the mouse monoclonal antibody described in EP 14199282.6, the mouse monoclonal antibody MC-6053 (clone 4F3-2C1, R&D Systems), MAB4733 (Abnova), orb26633 (Biorbyt), or GWB-T00279 (Genway).

[0057] Suitable detection antibodies that can be used as first and second detection
30 antibodies are known in the art. For example, the first detection antibody can be an alkaline phosphatase (AP)-conjugated antibody, a horseradish-peroxidase (HRP)-conjugated antibody or an antibody conjugated to a fluorescence dye. As a second detection antibody, e.g., an alkaline phosphatase (AP)-conjugated antibody, a horseradish-peroxidase (HRP)-conjugated antibody, a glucose oxidase-conjugated antibody, a tyrosinase-conjugated
35 antibody or a β -Galactosidase antibody can be used. Preferably, the first and second detection antibodies differ from each other, when used in the method of the invention.

[0058] As used herein, the singular forms “a”, “an” and “the” include both singular and plural reference unless the context clearly dictates otherwise. By way of example, “a cell” refers to one or more than one cell.

5 [0059] As used herein, the term “about” when qualifying a value of a stated item, number, percentage, or term refers to a range of plus or minus 10 percent, 9 percent, 8 percent, 7 percent, 6 percent, 5 percent, 4 percent, 3 percent, 2 percent, 1 percent or 0 percent of the value of the stated item, number, percentage, or term. Preferred is a range of plus or minus 10 percent.

10 [0060] The terms “comprising”, “comprises” and “comprised of” as used herein are synonyms with “including”, “includes” or “containing”, “contains”, and are inclusive or open-ended and do not exclude additional, non-recited members, elements or method steps. Evidently, the term “comprising” encompasses the term “consisting of”. More specifically,
15 the term “comprise” as used herein means that the claim encompasses all the listed elements or method steps, but may also include additional, unnamed elements or method steps. For example, a method comprising steps a), b) and c) encompasses, in its narrowest sense, a method which consists of steps a), b) and c). The phrase “consisting of” means that the composition (or device, or method) has the recited elements (or steps) and no more. In
20 contrast, the term “comprises” can encompass also a method including further steps, e.g., steps d) and e), in addition to steps a), b) and c).

[0061] In case numerical ranges are used herein such as “in a concentration between 1 and 5 micromolar”, the range includes not only 1 and 5 micromolar, but also any numerical
25 value in between 1 and 5 micromolar, for example, 2, 3 and 4 micromolar.

[0062] The term “*in vitro*” as used herein denotes outside, or external to, the animal or human body. The term “*in vitro*” as used herein should be understood to include “*ex vivo*”. The term “*ex vivo*” typically refers to tissues or cells removed from an animal or human
30 body and maintained or propagated outside the body, e.g., in a culture vessel. It is preferred, that the methods of the invention are *in vitro* methods. The term “*in vivo*” as used herein denotes inside, or internal to, the animal or human body.

[0063] In a further aspect of the methods of the invention, the K⁺-mediated depolarization
35 of the cells is carried out at an additional K⁺ concentration of about 20 mM to about 55 mM, for at least 2 hours.

[0064] Usually, the cell culture medium used in the art contains 5 mM K^+ . Accordingly, the final concentration of K^+ in the methods of the invention is preferably between about 25 mM and about 60 mM K^+ .

5 [0065] In a still further aspect of the methods of the invention, the K^+ -mediated depolarization of the cells and/or the neurotoxin polypeptide exposition is carried out in the presence of GT1b.

[0066] In another aspect of the methods of the invention, GT1b is used in a concentration
10 between 15 and 50 μ M, preferably 20 μ M.

[0067] In a further aspect of the methods of the invention, the reduced neurotoxin polypeptide exposition time is exposition of the cells to the neurotoxin polypeptide for at least 24 hours and less than 96 hours, preferably for at least 48 hours and 72 hours at
15 maximum.

[0068] In a further aspect of the methods of the invention, agitation of the cells during neurotoxin polypeptide exposition is achieved with a magnetic stirrer or rotating spinner flasks or shaking of the cells. Agitation of the cells is carried out at an appropriate cell
20 culture medium flow rate, preferably a mean (or average) medium flow rate of about 25 cm/min to about 300 cm/min, more preferably of about 25 cm/min to about 150 cm/min.

[0069] In a further aspect of the methods of the invention, the K^+ -mediated depolarization of the cells is carried out at an additional K^+ concentration of at least about 20 mM to about
25 55 mM, for at least 2 hours in the presence of 20 μ M GT1b and neurotoxin polypeptide, followed by neurotoxin polypeptide exposition for additional 70 hours under agitation.

[0070] In a further aspect of the methods of the invention, an incubation time without neurotoxin polypeptide precedes the neurotoxin polypeptide exposition.
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[0071] In a further aspect of the methods of the invention, the incubation time without neurotoxin polypeptide is between 16 and 48 hours.

[0072] In a further aspect of the methods of the invention, the method is a fluorescence
35 method.

[0073] In a further aspect of the methods of the invention, the neurotoxin polypeptide is BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/F, BoNT/G, BoNT/H or TeNT, or subtypes thereof as defined elsewhere herein.

[0074] In a further aspect of the methods of the invention, the substrate is VAMP/Synaptobrevin, SNAP-25 or Syntaxin.

[0075] In a further aspect of the methods of the invention, the cells are neuronal cells or neuronal differentiated cells selected from the group consisting of: primary neuronal cells, tumor cells which are capable of differentiating to neuronal cells such as neuroblastoma cells, P19 cells or induced pluripotent stem cell (IPS)-derived neurons.

[0076] Further preferred embodiments of the methods of the invention can be derived from the following examples.

The Figure shows:

Figure 1: Kinetics in loss of activity of stressed drug product samples. Drug product samples containing BoNT/A were stored at 70°C for up to four weeks. After 0, 1, 2 and 4 weeks samples were drawn and subjected to analysis in the mouse LD₅₀ bioassay as well as in the cell based assay (CBA) employing different protocols. On the x-axis the storage time in weeks is given whereas on the y-axis the relative potency is given. The potency at the start point was set to 100% and the consecutive test time points are expressed relative to the start point. The values for the LD₅₀ bioassay are depicted as diamonds. The cell based assays protocol employing K⁺-depolarization is depicted in squares, the protocol employing an 8 hour toxin incubation time followed by a 64 hour toxin-free incubation time is depicted in triangles and the protocol employing shaking during the 72 hour incubation time is depicted in circles. A CBA protocol which was not modified is depicted in line symbols. In sum, Figure 1 shows a comparison of three cell based assay examples where the stressed samples show a comparable kinetic in decay to the referenced assay, the mouse LD₅₀ bioassay.

The invention will now be illustrated by the following examples which shall, however, not be construed as limiting the scope of the present invention.

Examples:

Example 1: Double-Fluorescence-cell based-BoNT/A activity ELISA

Fixation of cells

1. Remove the media/toxin solution. Add 100 µl/well ice-cold methanol (-20 °C) and incubate for 20 min at -20 °C.

Note: Perform all subsequent steps at room temperature.

After cell fixation:

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1. Remove the methanol solution and add 100 μ l/well PBS buffer. For longer storage (> 1 day) one should add 300 μ l/well PBS buffer and seal the plates with parafilm. The plates should be stored in the refrigerator.

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2. Remove the PBS buffer and wash the cells 3 times with 200 μ l/well of PBS buffer. Each step should be performed for 1 minute with gentle shaking.

3. Remove the PBS buffer and add 100 μ l/well of quenching buffer and incubate for 20 minutes with gentle shaking.

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4. Remove the quenching buffer and wash the cells once with 300 μ l/well of PBS buffer for 3 minutes under gentle shaking.

5. Remove the PBS buffer, and add 200 μ l/well of blocking buffer and incubate for 1 hour with gentle shaking.

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6. Remove the blocking buffer and add 100 μ l of the primary antibody mixture (antibody dilution in blocking buffer) to each well. Incubate overnight (16-18 h) with gentle shaking. The cells are simultaneously incubated with two primary antibodies: a mouse antibody specific for the BoNT/A-cleaved SNAP-25 and a polyclonal rabbit antibody that recognizes SNAP-25 (antibody for determining the total amount of SNAP 25 for normalization).

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7. Remove the primary antibody mixture and wash the cells 4 times with 200 μ l of PBS buffer. Each step should be performed for 3 minutes with gentle shaking.

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8. Remove the PBS buffer, and add 100 μ l of the secondary antibody mixture: HRP-conjugated anti-mouse and AP-conjugated anti-rabbit secondary antibodies (antibody dilution in blocking buffer) to each well and incubate for 2.5 to 3 hours with gentle shaking.

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9. Remove the secondary antibody mixture and wash the cells 5 times with 200 μ l/well of PBS buffer, followed by 1 washing step with 300 μ l/well of HEPES buffer. Each wash step should be performed for 3 minutes with gentle shaking.

10. Remove the HEPES buffer from the plate and add 75 µl of a fluorogenic substrate for horseradish-peroxidase (HRP substrate) to each well. Incubate for 50 minutes with gentle shaking. Protect the plates from direct light.

- 5 11. Add 75 µl of a fluorogenic substrate for alkaline phosphatase (AP substrate) to each well and incubate for an additional 50 minutes at with gentle shaking. Protect the plates from direct light.

12. Read the plates using a fluorescence plate reader:

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excitation at 540 nm; emission at 600 nm.

excitation at 360 nm; emission at 450 nm.

13. Calculation

- 15 For normalization, the RFU value for cleaved SNAP-25 (fluorescence at 600 nm) is normalized to RFU of total SNAP-25 (450 nm) in each well. For better illustration of RFUs in a diagram all values are multiplied with a factor 1000 using the following equation:

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$$\frac{\text{RFU (600 nm)}}{\text{RFU (450 nm)}} \times 1000$$

Subsequently the resulting RFU values are averaged for each standard or sample.

- 25 Reagent Preparation

PBS buffer (10 mM):

Phosphate buffered saline (Sigma, # P5368) (pH 7.4)

- 30 Quenching buffer:

0.6 % H₂O₂ in 10 mM PBS buffer (pH 7.4)

Blocking buffer:

2 % BSA in 10 mM PBS buffer (pH 7.4) + 0.05 % Triton X-100

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HEPES buffer:

50 mM HEPES (pH 7.4)

HRP substrate:

50 mM HEPES (pH 7.4)

0.007% H₂O₂

150 pM Amplex UltraRed

5 AP substrate:

25 mM Diethanolamine (pH 9.8)

2 mM MgCl₂

100 µl M DiFMUP

10 **Example 2: Enhancement of specific uptake of Clostridial neurotoxin polypeptides into cells**

a) iCell® neurons were thawed and plated according to the Cellular Dynamics International (CDI) user manual on 96 well plates from 4 different cell batches. 24 hours
15 (h) after plating the medium was replaced by fresh maintenance medium as described in the user manual.

After further 72 h incubation time, the medium was removed and replaced by fresh medium containing BoNT/A in varying concentrations and K⁺-ions in a total concentration
20 of 30 mM (, *i.e.* 25 mM additional K⁺ compared to the medium as such). After 2 hours the high-K⁺-medium was removed and fresh medium containing BoNT/A in varying concentrations was added to the cells.

Another 70 h later, the medium was aspirated, the cells were fixed and an ELISA readout
25 was performed as described in Example 1. The results of this protocol are given in Figure 1, squares.

b) iCell® neurons were thawed and plated according to the Cellular Dynamics International (CDI) user manual on 96 well plates from 4 different cell batches. 24 hours
30 (h) after plating the medium was replaced by fresh maintenance medium as described in the user manual.

After further 72 h incubation time, the medium was removed and replaced by fresh medium containing BoNT/A in varying concentrations. After 8 hours the BoNT/A-
35 containing-medium was removed and fresh medium without BoNT/A was added to the cells.

Another 64 h later, the medium was aspirated, the cells were fixed and an ELISA readout was performed as described in Example 1. The results of this protocol are given in Figure 1, triangles.

- 5 c) iCell® neurons were thawed and plated according to the Cellular Dynamics International (CDI) user manual on 96 well plates from 4 different cell batches. 24 hours (h) after plating the medium was replaced by fresh maintenance medium as described in the user manual.
- 10 After further 72 h incubation time, the medium was removed and replaced by fresh medium containing BoNT/A in varying concentrations. The cells were put on a plate shaker in the incubator and were shaken at an average flow rate of 300 cm/min during toxin exposition time.
- 15 Another 72 h later, the medium was aspirated, the cells were fixed and an ELISA readout was performed as described in Example 1. The results of this protocol are given in Figure 1, circles.

- d) iCell® neurons were thawed and plated according to the Cellular Dynamics
20 International (CDI) user manual on 96 well plates from 4 different cell batches. 24 hours (h) after plating the medium was replaced by fresh maintenance medium as described in the user manual.

- After further 72 h incubation time, the medium was removed and replaced by fresh
25 medium containing BoNT/A in varying concentrations.

- Another 72 h later, the medium was aspirated, the cells were fixed and an ELISA readout was performed as described in Example 1. The results of this protocol are given in Figure 1.

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Conclusion:

- In sum, K^+ -mediated depolarization of the cells, a reduced neurotoxin polypeptide exposition time or agitation of the cells during neurotoxin polypeptide exposition facilitate
35 comparable stability indicating kinetics of the cell based assay of Example 1 when compared to the mouse LD₅₀ bioassay.

Claims

1. An in vitro method for directly determining the biological activity of a neurotoxin polypeptide in cells, comprising:
 - a) incubating cells susceptible to neurotoxin intoxication with a neurotoxin polypeptide for a time and under conditions which allow for the neurotoxin polypeptide to exert its biological activity, wherein the neurotoxin polypeptide is Botulinum neurotoxin BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, or BoNT/H, and wherein the incubation comprises (i) K^+ -mediated depolarization of the cells, wherein the K^+ -mediated depolarization of the cells is carried out at a final K^+ concentration of 15 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM or 60 mM, for at least 30 minutes, and a neurotoxin polypeptide exposition time of at least 24 hours and less than 96 hours, or (ii) a neurotoxin polypeptide exposition time of at least 24 hours and less than 96 hours and agitation of the cells during neurotoxin polypeptide exposition;
 - b) fixing the cells and, optionally, permeabilizing the cells with a detergent;
 - c) contacting the cells with at least a first capture antibody specifically binding to a non-cleaved and neurotoxin-cleaved substrate and with at least a second capture antibody specifically binding to the cleavage site of the neurotoxin-cleaved substrate, under conditions which allow for binding of said capture antibodies to said substrates;
 - d) contacting the cells with at least a first detection antibody specifically binding to the first capture antibody, under conditions which allow for binding of said first detection antibody to said first capture antibody, thus forming first detection complexes and with at least a second detection antibody specifically binding to the second capture antibody, under conditions which allow for binding of said second detection antibody to said second capture antibody, thus forming second detection complexes;
 - e) determining the amount of the first and second detection complexes of step d); and
 - f) determining the amount of total neurotoxin substrate and the amount of substrate cleaved by said neurotoxin polypeptide at the same time, thereby determining the biological activity of said neurotoxin polypeptide in said cells.
2. The method of claim 1, wherein the neurotoxin polypeptide exposition is carried out in the presence of a ganglioside GT1b.

3. The method of claim 2, wherein GT1b is used in a concentration between 15 and 50 μ M, preferably 20 μ M.
- 5 4. The method of any of claims 1 to 3, wherein the neurotoxin polypeptide exposition time is exposition of the cells to the neurotoxin polypeptide for at least 48 hours and 72 hours at maximum.
- 10 5. The method of any of claims 1 to 4, wherein agitation of the cells during neurotoxin polypeptide exposition is carried out with a magnetic stirrer, rotating spinner flasks or shaking of the cells by a shaker, preferably at a mean medium flow rate of 25 cm/min to 300 cm/min.
- 15 6. The method of any of claims 1 to 5, wherein an incubation time without neurotoxin polypeptide precedes the neurotoxin polypeptide exposition.
7. The method of claim 6, wherein the incubation time without neurotoxin polypeptide is between 16 and 48 hours.
- 20 8. The method of any of claims 1 to 7, wherein the method is a fluorescence method.
9. The method of any of claims 1 to 8, wherein the substrate is VAMP/Synaptobrevin, SNAP-25 or Syntaxin.
- 25 10. The method of any of claims 1 to 9, wherein the cells are neuronal cells or neuronal differentiated cells selected from the group consisting of: primary neuronal cells, tumor cells which are capable of differentiating to neuronal cells such as neuroblastoma cells, P19 cells or induced pluripotent stem cell (IPS)-derived neurons.
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