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(71) **Demandeur/Applicant:**
MERZ PHARMA GMBH & CO. KGAA, DE
(72) **Inventeurs/Inventors:**
EISELE, KARL-HEINZ, DE;
MANDER, GERD, DE
(74) **Agent:** BORDEN LADNER GERVAIS LLP

(54) **Titre : GANGLIOSIDES POUR LA NORMALISATION ET L'AUGMENTATION DE LA SENSIBILITE DE CELLULES A DES
NEUROTOXINES BOTULINIQUES DANS DES SYSTEMES D'ESSAI IN VITRO**
(54) **Title: GANGLIOSIDES FOR STANDARDIZING AND INCREASING THE SENSITIVITY OF CELLS TO BOTULINUM
NEUROTOXINS IN IN VITRO TEST SYSTEMS**

(57) **Abrégé/Abstract:**

The present invention pertains to a method for standardizing the sensitivity of induced pluripotent stem cell (iPS)-derived neurons to a neurotoxin polypeptide, comprising the steps of: a) cultivating different batches of induced pluripotent stem cell-derived neurons in a cell culture medium comprising GT1b for at least 3 hours; b) contacting the different batches of induced pluripotent stem cell-derived neurons of step a) with a neurotoxin polypeptide; c) cultivating the different batches of induced pluripotent stem cell-derived neurons of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity, thereby standardizing the sensitivity of the induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide. The invention further relates to a method for the generation of induced pluripotent stem cell-derived neurons having a standardized sensitivity to a neurotoxin polypeptide, comprising the steps of: a) providing different batches of induced pluripotent stem cell-derived neurons; b) cultivating the different batches of induced pluripotent stem cell-derived neurons of step a) in a cell culture medium comprising GT1b for at least 3 hours, thereby standardizing the sensitivity of the induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide. In addition, encompassed by the present invention is a method for determining the biological activity of a neurotoxin polypeptide, comprising the steps of: a) cultivating induced pluripotent stem cell-derived neurons in a cell culture medium comprising GT1b for at least 3 hours; b) contacting the induced pluripotent stem cell-derived neurons of step a) with a neurotoxin polypeptide; c) cultivating the induced pluripotent stem cell-derived neurons of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity; and d) determining the biological activity of the neurotoxin polypeptide in said cells. Finally, the invention relates to the use of GT1b for a) standardizing the sensitivity of different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide; or b) reducing the variability of the sensitivity of different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide.



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(71) Applicant: MERZ PHARMA GMBH & CO. KGAA

[DE/DE]; Eckenheimer Landstrasse 100, 60318 Frankfurt am Main (DE).

(72) Inventors: EISELE, Karl-Heinz; Parlamentsplatz 2b,

60385 Frankfurt am Main (DE). MANDER, Gerd; Bertha-Bagge-Straße 46, 60438 Frankfurt (DE).

(74) Agent: DICK, Alexander; Herzog Fiesser & Partner Pat-

entanwälte PartG mbB, Dudenstrasse 46, 68167 Mannheim (DE).

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(57) Abstract: The present invention pertains to a method for standardizing the sensitivity of induced pluripotent stem cell (iPS)-derived neurons to a neurotoxin polypeptide, comprising the steps of: a) cultivating different batches of induced pluripotent stem cell-derived neurons in a cell culture medium comprising GT1b for at least 3 hours; b) contacting the different batches of induced pluripotent stem cell-derived neurons of step a) with a neurotoxin polypeptide; c) cultivating the different batches of induced pluripotent stem cell-derived neurons of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity, thereby standardizing the sensitivity of the induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide. The invention further relates to a method for the generation of induced pluripotent stem cell-derived neurons having a standardized sensitivity to a neurotoxin polypeptide, comprising the steps of: a) providing different batches of induced pluripotent stem cell-derived neurons; b) cultivating the different batches of induced pluripotent stem cell-derived neurons of step a) in a cell culture medium comprising GT1b for at least 3 hours, thereby standardizing the sensitivity of the induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide. In addition, encompassed by the present invention is a method for determining the biological activity of a neurotoxin polypeptide, comprising the steps of: a) cultivating induced pluripotent stem cell-derived neurons in a cell culture medium comprising GT1b for at least 3 hours; b) contacting the induced pluripotent stem cell-derived neurons of step a) with a neurotoxin polypeptide; c) cultivating the induced pluripotent stem cell-derived neurons of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity; and d) determining the biological activity of the neurotoxin polypeptide in said cells. Finally, the invention relates to the use of GT1b for a) standardizing the sensitivity of different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide; or b) reducing the variability of the sensitivity of different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide.

WO 2015/124618 A1

Gangliosides for standardizing and increasing the sensitivity of cells to Botulinum neurotoxins in *in vitro* test systems

5 [0001] The present invention pertains to a method for standardizing the sensitivity of induced pluripotent stem cell (iPS)-derived neurons to a neurotoxin polypeptide, comprising the steps of: a) cultivating different batches of induced pluripotent stem cell-derived neurons in a cell culture medium comprising GT1b for at least 3 hours;

10 b) contacting the different batches of induced pluripotent stem cell-derived neurons of step a) with a neurotoxin polypeptide; c) cultivating the different batches of induced pluripotent stem cell-derived neurons of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity, thereby standardizing the sensitivity of the induced pluripotent stem cell-derived neurons

15 to a neurotoxin polypeptide. The invention further relates to a method for the generation of induced pluripotent stem cell-derived neurons having a standardized sensitivity to a neurotoxin polypeptide, comprising the steps of: a) providing different batches of induced pluripotent stem cell-derived neurons; b) cultivating the different batches of induced pluripotent stem cell-derived neurons of step a) in a cell culture medium comprising GT1b

20 for at least 3 hours, thereby standardizing the sensitivity of the induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide. In addition, encompassed by the present invention is a method for determining the biological activity of a neurotoxin polypeptide, comprising the steps of: a) cultivating induced pluripotent stem cell-derived neurons in a cell culture medium comprising GT1b for at least 3 hours; b) contacting the induced

25 pluripotent stem cell-derived neurons of step a) with a neurotoxin polypeptide; c) cultivating the induced pluripotent stem cell-derived neurons of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity; and d) determining the biological activity of the neurotoxin polypeptide in said cells. Finally, the invention relates to the use of GT1b for

30 a) standardizing the sensitivity of different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide; or b) reducing the variability of the sensitivity of different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide.

[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). All serotypes together with the related Tetanus neurotoxin (TeNT) secreted by *Clostridium tetani*, are Zn^{2+} -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.

[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 Pharmaceuticals, LLC). 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 therapeutice ffect.

[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 robust, accurate, precise, reliable, and validated Botulinum toxin potency 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 clone derived from the SiMa cell line (WO 2010/105234 A1).

[0007] However, the isolation of primary neurons requires the killing of animals and is laborious, time consuming and validation of these assays appears to be a challenge. Further, test systems using different primary neurons show large variances. Similarly, the generation of neuronally 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.

5 [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.

[0010] In a first aspect, the present invention pertains to a method for the generation of
10 induced pluripotent stem cell (IPS)-derived neurons having a standardized sensitivity to a neurotoxin polypeptide, comprising the steps of:

- a) providing different batches of induced pluripotent stem cell-derived neurons;
- b) cultivating the different batches of induced pluripotent stem cell-derived
15 neurons of step a) in a cell culture medium comprising GT1b for at least 3 hours,

thereby standardizing the sensitivity of the induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide.

20 [0011] In this aspect, different batches of induced pluripotent stem cell-derived neurons are provided, in a first step. The batches can differ, e.g., in the number of passages and/or the number of freeze/thaw cycles and/or in other properties mentioned elsewhere herein. Subsequently, the different batches of induced pluripotent stem cell-derived neurons are cultivated in an appropriate cell culture medium comprising GT1b for at least 3 hours, at
25 least 4 hours, at least 5 hours, at least 6 hours, at least 12 hours, at least 24 hours (1 day), at least 36 hours, at least 48 hours (2 days), at least 72 hours (3 days), at least 4 days, at least 5 days or even longer. Preferably, said cultivation is for a few hours, e.g., for 3 hours, 4 hours, 5 hours, 6 hours or 12 hours. As an appropriate cell culture medium, for example, Neurobasal[®] medium comprising B27 supplement, iCell[®] neuron medium (Cellular
30 Dynamics international; CDI) or other cell culture media provided by manufacturer's or providers of induced pluripotent stem cell-derived neurons can be used. It has been found by the present inventors, that, thereby, the variability of the sensitivity of the different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide can be reduced significantly, in comparison to control batches of induced pluripotent stem cell-
35 derived neurons without GT1b treatment, as set forth in more detail below.

[0012] In another aspect, the above-indicated method of the invention further comprises

- c) contacting the different batches of induced pluripotent stem cell-derived neurons of step b) with a neurotoxin polypeptide; and
- d) cultivating the different batches of induced pluripotent stem cell-derived neurons of step c) for at least 24 hours, in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity.

[0013] After cultivating the different batches of induced pluripotent stem cell-derived neurons in a cell culture medium comprising GT1b for at least 3 hours, the different batches of induced pluripotent stem cell-derived neurons can first be contacted and then intoxicated with a neurotoxin polypeptide for at least 24 hours (1 day), at least 36 hours, at least 48 hours (2 days), at least 60 hours, at least 72 hours (3 days), at least 4 days, at least 5 days, at least 6 days, at least 7 days (1 week), at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 5 weeks, at least 6 weeks or even longer, in a next step. Preferably, intoxication is for at least 72 hours or longer. The neurotoxin polypeptide can be, for example, BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/H or TeNT, or a subtype thereof, as defined in more detail elsewhere herein. The different batches of induced pluripotent stem cell-derived neurons are cultivated for the above-indicated time period in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity. Appropriate cell culture conditions which allow the neurotoxin polypeptide to exert its biological activity and the biological activity of a neurotoxin polypeptide is as defined elsewhere herein. By this treatment, the variability of the sensitivity of the different batches of induced pluripotent stem cell-derived neurons to said neurotoxin polypeptide can be reduced further, in comparison to control batches of intoxicated induced pluripotent stem cell-derived neurons without GT1b treatment.

[0014] In a further aspect, the present invention relates to a method for standardizing the sensitivity of induced pluripotent stem cell (iPS)-derived neurons to a neurotoxin polypeptide, comprising the steps of:

- a) cultivating different batches of induced pluripotent stem cell-derived neurons in a cell culture medium comprising GT1b for at least 3 hours;
- b) contacting the different batches of induced pluripotent stem cell-derived neurons of step a) with a neurotoxin polypeptide;
- c) cultivating the different batches of induced pluripotent stem cell-derived neurons of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity;

thereby standardizing the sensitivity of the induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide.

[0015] In a further aspect, the aforementioned methods of the invention can comprise one or more additional steps. For example, said additional steps can encompass steps for determining the biological activity of a neurotoxin polypeptide as defined herein. To this end, the induced pluripotent stem cell (iPS)-derived neurons which have been cultivated in the presence of GT1b as described herein are brought in contact with a neurotoxin polypeptide as defined herein. The term “contacting” as used in accordance with the methods of the invention refers to bringing the aforementioned cells and the neurotoxin polypeptide which may be comprised, e.g., in a sample, in physical proximity as to allow physical and/or chemical and/or biological interaction. Suitable conditions which allow for specific interaction are well known to the skilled worker. Said conditions will depend on the cells and neurotoxins to be applied in the methods of the present invention and can be adapted by the skilled artisan without further ado. Moreover, a time being sufficient to allow interaction can also be determined by the skilled worker without further ado. For example, a specific amount of an isolated or recombinant neurotoxin polypeptide or a variant thereof as defined herein or a sample comprising a neurotoxin polypeptide can be added to the GT1b-treated induced pluripotent stem cell (iPS)-derived neurons. Thereafter, the cells are incubated with the neurotoxin polypeptide for at least 24 hours under conditions which allow for the neurotoxin polypeptide to exert its biological activity, again in the presence of GT1b. “Conditions which allow for the neurotoxin polypeptide to exert its biological activity” as used herein are known in the art. Subsequently, the exertion of the biological activity is stopped, for example by the addition of a lysis buffer to the cells, and the biological activity of the neurotoxin polypeptide is determined, for instance, by a Western blot assay specifically detecting the cleaved neurotoxin substrate or a specific ELISA technique. For instance, 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.

[0016] 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 two different receptors, SV2 and GT1b. The physiological effect of the neurotoxins is based on the cleavage of a protein of the so-called 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 BoNT is an important aspect in the characterization of said neurotoxin proteins and is

required, *inter alia*, by regulatory authorities for the commercial release of BoNT-containing products. A reliable test for the measurement of the biological activity of BoNT is, therefore, basis for research, development and marketing of products containing BoNT. 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 Botulinum neurotoxins is essential.

[0017] To determine the biological activity of Botulinum toxins in pharmaceutical products, the neuronal cells or cell lines shall have the following properties: First, the cells should be of human, neuronal origin in order to resemble the target as close as possible, i.e. the human patient. Second, the cell system shall be robust towards excipients in the final product and, preferably, also towards impurities in intermediate stages of the production process (process controls). Third, the cell-based test system shall exhibit a dynamic measuring range which allows for the accurate determination of the biological activity of BoNT in a vial (for example, 50 LD₅₀U BoNT/A). Considering technical factors such as the solubility of excipients, volumes of cell culture media etc., a BoNT concentration of less than 1 pM has to be determined accurately.

[0018] One of the available cell-based test systems having sufficiently high sensitivity to BoNT uses neuronal differentiated induced pluripotent stem cells. The present inventors have evaluated a test system using commercially available human induced pluripotent stem cell-derived neurons (Cellular Dynamics International, Inc., Madison). Said human induced pluripotent stem cell-derived neurons had been obtained as cryopreserved cells and were thawed and cultivated for 4 days according to the manufacturer's manual. Said cells are finally differentiated to neuronal cells characterized in that they do not proliferate any more and exhibit a terminally differentiated, neuronal phenotype which cannot be altered any more. After having formed said phenotype, the cells were incubated with neurotoxin polypeptide for 72 hours. Thereafter, the neurotoxin substrate cleavage product was quantified by Immuno-Western blot analysis of the cell lysates or ELISA methods, as exemplified for the neurotoxin polypeptide BoNT/A, and its substrate SNAP-25. As a result of the evaluation of said test, high sensitivity, reproducibility and intermediate precision of said test system could be confirmed, as long as the test had been carried out by using the same cell batch of the mentioned provider. However, when using different cell batches of said provider, unexplainable high variability with respect to the sensitivity of said cells towards neurotoxin polypeptide was found although the characterization of said cell batches by the provider with regard to cell number, viability, phenotype etc. did not give any clue as regards the mentioned variability. Specifically, the sensitivity (EC₅₀) of

different cell batches of the human induced pluripotent stem cell-derived neurons of the provider varied in a range from 1.7 to more than 10 U/ml.

[0019] It has surprisingly been found by the present inventors that the external application
5 of gangliosides such as GT1b resulted in a drastic reduction of the variability of the sensitivity between different cell batches of the human induced pluripotent stem cell-derived neurons. This finding is unusual for the following reasons: Firstly, cells exhibiting a neuronal phenotype produce endogenously sufficient GT1b themselves. This has been found, for example, for primary neurons. Moreover, even different preparations of primary
10 neuron cell cultures did not show such variability in the sensitivity towards neurotoxin polypeptides, in the inventors' experience. In addition, such effects could not be observed in neuroblastoma cell line-based tests in which, for example, SiMa cells have been used, neither for different passage numbers nor when testing different cryopreserved batches. Secondly, the provider's manual by Cellular Dynamics International did not contain any
15 information with respect to such variability of the sensitivity of different cell batches of the human induced pluripotent stem cell-derived neurons towards neurotoxin polypeptides. When using the methods of the present invention, said variability could advantageously be reduced by the present inventors from about 30 % to about 15 % (standard deviation) by cultivating and neurotoxin incubation in the presence of 30 μ M GT1b which has been
20 added to the cell culture medium. Accordingly, the methods of the present invention provide for a sensitive, accurate and reproducible cell-based test system in order to determine the biological activity of neurotoxins. Said methods can be used as an alternative to conventional animal-based test systems. Further, the comparatively simple methods of the invention for standardizing the sensitivity of human induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide result in an improved sensitivity of said cells:
25 Whereas an EC50 of about 5 U/ml corresponding to 167 fM has been found for cells without addition of GT1b, an EC50 of about 0.75 U/ml corresponding to 25 fM has been found for cells to which GT1b has been added to the cell culture medium, corresponding to a ~7-fold increase of sensitivity. Accordingly, it has been found by the present inventors
30 that the sensitivity of human induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide can be increased by the addition of GT1b, in comparison to human induced pluripotent stem cell-derived neurons cultivated in the absence of GT1b. Specifically, the sensitivity of each single batch of human induced pluripotent stem cell-derived neurons could be improved by the incubation with said ganglioside. Interestingly, the sensitivity of
35 parental SiMa cells to a neurotoxin polypeptide could also be enhanced by the addition of GT1b. In this case, it was possible to increase the sensitivity of said neuroblastoma cells by a factor of 10, in comparison to SiMa cells not treated with GT1b. These results were not a trivial task or self-evident finding because other neuroblastoma cells such as Neuro2a did

not exhibit a comparable increase in sensitivity to the neurotoxin polypeptide upon incubation with GT1b, or only a slight increase, such as SH-SY5Y (DSMZ and ECACC), PC12, or NG108-15 cells, as demonstrated in the following examples.

5 [0020] Accordingly, in another aspect, the present invention pertains to a method for the generation of induced pluripotent stem cell (IPS)-derived neurons or SiMa cells having an increased sensitivity to a neurotoxin polypeptide, comprising the steps of:

- a) providing induced pluripotent stem cell-derived neurons or SiMa cells;
- b) cultivating the induced pluripotent stem cell-derived neurons or SiMa cells

10 of step a) in a cell culture medium comprising GT1b for at least 3 hours, thereby increasing the sensitivity of the induced pluripotent stem cell-derived neurons or SiMa cells to said neurotoxin polypeptide. In a further aspect, SH-SY5Y cells, PC12 cells, or NG108-15 cells having an increased sensitivity to a neurotoxin polypeptide can be produced, by this method.

15 [0021] In still another aspect, the above-indicated method of the invention further comprises

- c) contacting the induced pluripotent stem cell-derived neurons or SiMa cells of step b) with a neurotoxin polypeptide; and
- 20 d) cultivating the induced pluripotent stem cell-derived neurons or SiMa cells for at least 24 hours, in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity.

Alternatively, SH-SY5Y cells, PC12 cells, or NG108-15 cells can be used in this aspect, of the method of the invention, as indicated above.

25 [0022] In a further aspect, the present invention relates to a method for determining the biological activity of a neurotoxin polypeptide, comprising the steps of:

- a) cultivating induced pluripotent stem cell-derived neurons or SiMa cells, in a cell culture medium comprising GT1b for at least 3 hours;
- 30 b) contacting the induced pluripotent stem cell-derived neurons or SiMa cells of step a) with a neurotoxin polypeptide;
- c) cultivating the induced pluripotent stem cell-derived neurons or SiMa cells of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity;
- 35 and
- d) determining the biological activity of the neurotoxin polypeptide in said cells.

SH-SY5Y cells, PC12 cells, or NG108-15 cells can alternatively be used for determining the biological activity of a neurotoxin polypeptide, in other aspects of this method of the invention.

- 5 [0023] Preferably, single batches of said induced pluripotent stem cell-derived neurons, SiMa cells, SH-SY5Y cells, PC12 cells, or NG108-15 cells are used in the methods of the invention for generating induced pluripotent stem cell-derived neurons, SiMa cells, SH-SY5Y cells, PC12 cells, or NG108-15 cells, having an increased sensitivity to a neurotoxin polypeptide, or in the methods of the invention for increasing the sensitivity of the
- 10 mentioned cells of the invention. It is preferred that the SiMa cells are parental SiMa cells (DSMZ no. ACC164). Preferably, the concentration of GT1b is between 10 and 50 μ M, more preferably 30 μ M. Cultivating the induced pluripotent stem cell-derived neurons, SiMa cells, SH-SY5Y cells, PC12 cells, or NG108-15 cells in a cell culture medium comprising GT1b is preferably for at least 12 hours, at least 24 hours, at least 36 hours, at
- 15 least 48 hours, at least 60 hours, at least 72 hours or at least 96 hours, or even longer. Intoxication with the neurotoxin polypeptide is preferably carried out for at least 36 hours, 48 hours, 60 hours, 72 hours, 96 hours or even longer. Preferably, the neurotoxin polypeptide is BoNT/A. The increase of the sensitivity to a neurotoxin polypeptide of GT1b-treated induced pluripotent stem cell-derived neurons is preferably at least 2-fold, at
- 20 least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, or at least 6.7-fold, in comparison to induced pluripotent stem cell-derived neurons not treated with GT1b. Further, the increase of the sensitivity to a neurotoxin polypeptide of GT1b-treated SiMa cells is preferably at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at
- 25 least 7-fold, at least 8-fold, at least 9-fold, or at least 10-fold, in comparison to SiMa not treated with GT1b. The increase of the sensitivity to a neurotoxin polypeptide of GT1b-treated SH-SY5Y cells is preferably at least 1.2-fold, at least 1.4-fold, at least 1.6-fold, at least 1.8-fold, or at least 2-fold, in comparison to SH-SY5Y cells not treated with GT1b. The increase of the sensitivity to a neurotoxin polypeptide of GT1b-treated PC12 cells is
- 30 preferably at least 1.1-fold, at least 1.2-fold, at least 1.3-fold, or at least 1.4-fold, in comparison to PC12 cells not treated with GT1b. Moreover, the increase of the sensitivity to a neurotoxin polypeptide of GT1b-treated NG108-15 cells is preferably at least 1.1-fold, at least 1.2-fold, at least 1.3-fold, at least 1.4-fold, at least 1.5-fold, or at least 1.6-fold, in comparison to NG108-15 cells not treated with GT1b.
- 35 [0024] The methods of the present invention allow for high dilutions of neurotoxin containing samples to be analyzed. Further, the methods of the invention are robust towards excipients and impurities in the samples to be analyzed which allows for high dilutions of said samples. Such high dilutions of samples are important with respect to

excipients and impurities within the samples in order to apply said potentially disturbing substances in a concentration as low as possible.

[0025] “Induced pluripotent stem cell (iPS)-derived neuron(s)” as used herein means in a broad sense, a cell which is susceptible to a neurotoxin polypeptide exhibiting 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. Accordingly, an “induced pluripotent stem cell (iPS)-derived neuron” as referred to herein is susceptible to neurotoxin intoxication. More specifically, “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 to its corresponding receptor (e.g. binding of BoNT/A to 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. Assays for determining the biological activity of a neurotoxin polypeptide are well known in the art and also described elsewhere herein (see, e.g., Pellett et al., Withemars et al., loc. cit.) 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. 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, about 0.1 pM, about 50 fM, about 40 fM, about 30 fM, about 20 fM, about 10 fM, about 5 fM, about 4 fM, about 3 fM, about 2 fM, or about 1 fM of neurotoxin polypeptide, or even 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. 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, about 0.1 pM, about 50 fM or less, about 40 fM or less, about 30 fM or less, about 20 fM or less, about 10 fM or less, about 5 fM or less, about 4 fM or less, about 3 fM or less, about 2 fM or less, or even about 1 fM or less of neurotoxin polypeptide. For example, an extremely low EC50 value of about 3 fM has been found by the present inventors for induced pluripotent stem cell (iPS)-derived neurons to which GT1b has externally been added to the cell culture medium in the methods of the present invention. 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 an 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. *In vivo* assays for assessing the biological activity of neurotoxins include, for example, the already mentioned mouse LD₅₀ assay and the *ex vivo* mouse hemidiaphragm assay as described by Pearce et al. and Dressier et al.; see Pearce 1994, Toxicol. Appl. Pharmacol. 128: 69-77 and Dressier 2005, Mov. Disord. 20:1617-1619. As known to those skilled in the art, the biological activity of neurotoxins is commonly expressed in Mouse LD₅₀ Units (MU). One MU is the amount of neurotoxic component, which kills 50% of a specified mouse population after intraperitoneal injection.

[0026] More specifically, “induced pluripotent stem cell (iPS)-derived neurons” as used herein are described in the literature; see, for example, Whitemarsh et al., loc. cit; WO 2012/135621; US 2010/0279403 and US 2010/0216181. In particular, human induced pluripotent stem cells (hiPSC) hold great promise for providing various differentiated cell models for *in vitro* toxigenicity testing. hiPSC-derived neurons were differentiated and cryopreserved, e.g., by Cellular Dynamics International (Madison, WI) and consist of an

almost pure pan-neuronal population of predominantly gamma aminoisobutyric acidergic and glutamatergic neurons. Said hiPSC-derived neurons are known as iCell[®] neurons. Western blot and quantitative PCR data showed that these neurons express all the necessary receptors and substrates for BoNT intoxication, according to the provider.

5 BoNT/A intoxication studies demonstrated that the hiPSC-derived neurons reproducibly and quantitatively detect biologically active BoNT/A with high sensitivity. Additionally, the quantitative detection of BoNT serotypes B, C, E, and BoNT/A complex was demonstrated, and BoNT/A specificity was confirmed through antibody protection studies. A direct comparison of BoNT detection using primary rat spinal cord cells and hiPSC-

10 derived neurons showed equal or increased sensitivity, a steeper dose-response curve and a more complete SNARE protein target cleavage for hiPSC-derived neurons; see Whitemarsh et al., loc. cit. These data suggested that neurons derived from hiPSCs provide an ideal and highly sensitive platform for BoNT potency determination, neutralizing antibody detection and for mechanistic studies. In an aspect of the methods of the

15 invention, the induced pluripotent stem cell-derived neurons are mammalian (such as rodent, cynomolgus, macaque or chimpanzee) induced pluripotent stem cell (iPS)-derived neurons, preferably human induced pluripotent stem cell (iPS)-derived neurons. Preferably, said induced pluripotent stem cell-derived neurons are iCell[®] neurons (Cellular Dynamics International, Inc. (CDI)). According to the provider, iCell[®] neurons are derived from

20 human induced pluripotent stem (iPS) cells and provide a unique *in vitro* system for preclinical drug discovery, neurotoxicity testing and disease research. Moreover, iCell[®] neurons offer high quality and highly pure human neuronal cells that possess typical phenotypic characteristics and functionality of mature neurons. Historically, *in vitro* models have played an important role in the drug discovery process including use during

25 early stage disease modeling and candidate in the identification as well as pharmacokinetic and safety testing. Because of the complexity of the human brain, scientists currently use simplified models such as primary cells isolated from rodent tissues and transformed cell lines. Issues of biological relevance, reproducibility, and scalability can arise and the reliance on inferior models may result in drug-induced neurotoxicity not being observed

30 until late-stage clinical trials or after marketplace introduction in the field of neurotoxins. iCell[®] neurons overcome these limitations providing a robust, well characterized highly reproducible *in vitro* model for preclinical neurotoxin safety testing. iCell[®] neurons are terminally differentiated from human iPS cells and exhibit neuronal characteristics and functions. iCell[®] neurons are highly pure, providing biologically relevant and reproducible

35 results. iCell[®] neurons remain viable and pure in culture for weeks, enabling assessment of both acute and subchronic responses. Further, iCell[®] neurons are shipped cryopreserved with cell culture media specifically formulated for optimal cell performance. They are simple to thaw and use, according to the provider's manual. However, different batches of

iCell[®] neurons have been found by the present inventors to differ drastically with respect to the sensitivity of said batches to neurotoxin polypeptides. As a result, strong divergences in the measured values of the biological activity of neurotoxins have been obtained for different batches. In order to reduce the variability of the sensitivity of the different batches
5 of iPS-derived neurons to a neurotoxin polypeptide, the external addition of GT1b to the cell culture medium can advantageously be used in accordance with the methods of the invention.

[0027] As used herein, the singular forms “a”, “an” and “the” include both singular and
10 plural reference unless the context clearly dictates otherwise. By way of example, “a cell” refers to one or more than one cell.

[0028] 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
15 percent, 6 percent, 5 percent, 4 percent, 3 percent, 2 percent or 1 percent of the value of the stated item, number, percentage, or term. Preferred is a range of plus or minus 10 percent.

[0029] The terms “comprising”, “comprises” and “comprised of” as used herein are synonyms with “including”, “includes” or “containing”, “contains”, and are inclusive or
20 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, 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
25 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 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).

[0030] In case numerical ranges are used herein such as “GT1b in a concentration from 10
30 to 50 μ M the range includes not only 10 and 50 μ M, but also any numerical value in between 10 and 50 μ M, for example, 15 μ M, 20 μ M, 25 μ M, 30 μ M, 35 μ M, 40 μ M and 45 μ M GT1b.

[0031] The term “*in vitro*” as used herein denotes outside, or external to, the animal or
35 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

body and maintained or propagated outside the body, e.g., in a culture vessel. The term “*in vivo*” as used herein denotes inside, or internal to, the animal or human body.

[0032] 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.

[0033] The term “neurotoxin polypeptide” as used herein denotes Clostridium botulinum and Clostridium tetani neurotoxins (or Clostridial neurotoxins), i.e. Botulinum toxins (BoNTs) and Tetanus toxin (TeNT). 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. More specifically, said term encompasses BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/H and Tetanus neurotoxin (TeNT), or 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 and BoNT/B7. 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, and BoNT/E8. Further, the BoNT/F subtypes comprise, for instance, BoNT/F1, BoNT/F2, BoNT/F3, BoNT/F4, BoNT/F5, BoNT/F6, and BoNT/F7. The

neurotoxin polypeptide and, in particular, its light chain and heavy chain are derivable from one of the antigenically different serotypes of Botulinum neurotoxins 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 means and 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). The corresponding sequences of BoNT/H are shown in the publication by Dover et al., J. Infect. Dis. (2014), 209 (2): 192-202. Said BoNT/H sequences are also encompassed, in specific aspects of the means and methods of the invention.

[0034] In another aspect, the said polynucleotide is a variant of the aforementioned polynucleotides comprising one or more nucleotide substitutions, deletions and/or additions which in still another aspect may result in a polypeptide having one or more amino acid substitutions, deletions and/or additions. Moreover, a variant polynucleotide 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 (preferably complete) 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 of BoNT/H, 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 (preferably complete) 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. 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, in one aspect, calculated over the entire amino acid sequence. 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. The variant of a Clostridial neurotoxin as referred to herein includes, e.g. 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 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 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. 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. In an aspect, each of the aforementioned variant polynucleotides encodes a polypeptide retaining one or more and, in another aspect, all of the 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. "Biological properties" as used herein refers to (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. More specifically, the overall cellular mechanisms whereby a neurotoxin (e.g. BoNT/A) cleaves a neurotoxin substrate (e.g. SNAP-25) encompasses the binding of the neurotoxin to its corresponding receptor (e.g. binding of BoNT/A to 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. *In vitro* and *in vivo* assays for determining the biological activity of a neurotoxin polypeptide are well known in the art. *In vivo* assays for assessing biological activity 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 or may be improved for receptor binding or any other property specified above. In some aspects, the neurotoxin polypeptide can be included in a sample. The sample can be, for example, a clinical sample, a biological sample, a food sample, a pharmaceutical or toxicological sample, an antibody sample or the like.

[0035] Accordingly, the term "determining the biological activity of a neurotoxin polypeptide" as used herein means measuring the biological activity of a neurotoxin protein, 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.

[0036] The term "amount" as used herein encompasses the absolute amount of, e.g., a neurotoxin polypeptide or a neurotoxin substrate polypeptide, the relative amount or the

concentration of the said polypeptide as well as any value or parameter which correlates thereto or can be derived there from.

[0037] The term “determining the amount” of, e.g., a neurotoxin polypeptide or a neurotoxin substrate polypeptide relates to measuring the absolute amount, relative amount or concentration of, e.g., the neurotoxin polypeptide or neurotoxin substrate polypeptide in a quantitative or semi-quantitative manner. Suitable measures for detection are well known to those skilled in the art. It will be understood that the determination of the amount of neurotoxin polypeptides or neurotoxin substrate polypeptides, in an aspect, also requires calibration of the method by applying standard solutions with predefined amounts of neurotoxin polypeptides or neurotoxin substrate polypeptides. How to carry out such a calibration is well known to those skilled in the art.

[0038] In an aspect of the methods of the invention, the induced pluripotent stem cell (iPS)-derived neurons are cultivated in a cell culture medium comprising GT1b. The ganglioside GT1b binds to neurotoxin polypeptide and potentially mediates the selectivity of neurotoxins for neurons. Accordingly, GT1b can be used for standardizing the sensitivity of induced pluripotent stem cell (iPS)-derived neurons to a neurotoxin polypeptide. It has been shown by the present inventors that the external addition of GT1b to the iPS-derived neurons reduces drastically the variability of the sensitivity of different batches of said iPS-derived neurons to a neurotoxin polypeptide, in comparison to control batches of iPS-derived neurons treated without GT1b. Preferably, said GT1b is present in a concentration of about 10 to about 50 μM , i.e. in a concentration of about 10 μM , about 15 μM , about 20 μM , about 25 μM , about 30 μM , about 35 μM , about 40 μM , about 45 μM , or about 50 μM , more preferably in a concentration of about 30 μM .

[0039] In a further aspect, the present invention relates to a method for determining the biological activity of a neurotoxin polypeptide, comprising the steps of:

- a) cultivating induced pluripotent stem cell-derived neurons in a cell culture medium comprising GT1b for at least 3 hours;
- b) contacting the induced pluripotent stem cell-derived neurons of step a) with a neurotoxin polypeptide;
- c) cultivating the induced pluripotent stem cell-derived neurons of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity; and
- d) determining the biological activity of the neurotoxin polypeptide in said cells.

[0040] In a specific aspect of this method of the invention, different batches of induced pluripotent stem cell-derived neurons are used, as defined elsewhere herein.

[0041] In one aspect of the methods of the invention, “standardizing of the sensitivity” (of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide) is a reduction in the variability of the sensitivity of the different batches of induced pluripotent stem cell (iPS)-derived neurons to a neurotoxin polypeptide, in comparison to control batches of induced pluripotent stem cell (iPS)-derived neurons treated under the same conditions, however, without GT1b.

[0042] In another aspect, the reduction in the variability of the sensitivity of the different batches of induced pluripotent stem cell (iPS)-derived neurons to a neurotoxin polypeptide is an at least 1.1-fold, at least 1.2-fold, at least 1.3-fold, at least 1.4-fold, at least 1.5-fold, at least 1.6-fold, at least 1.7-fold, at least 1.8-fold, at least 1.9-fold, at least 2-fold, at least 2.1-fold, at least 2.2-fold, at least 2.3-fold, at least 2.4-fold, at least 2.5-fold, or even at least 3-fold reduction, in comparison to control batches of induced pluripotent stem cell (iPS)-derived neurons treated under the same conditions, however, without GT1b.

[0043] In other aspects of the methods of the invention, the induced pluripotent stem cell (iPS)-derived neurons are human induced pluripotent stem cell (iPS)-derived neurons. Preferably, said induced pluripotent stem cell (iPS)-derived neurons are iCell[®] neurons (Cellular Dynamics International; loc. cit.).

[0044] In one aspect of the methods of the invention, the different batches of induced pluripotent stem cell (iPS)-derived neurons differ in the number of passages, the number of freeze/thaw cycles, the cultivation conditions, the storage time, the growth time, the differentiation conditions, or combinations thereof.

[0045] In another aspect of the methods of the invention, the cell culture medium comprises Neurobasal medium, B27 Supplement (2%), and Glutamin or Glutamax (1%). Optionally the cell culture medium can comprise antibiotics (1%), N2 supplement (1%) and/or Serum Albumin (0.2%).

[0046] In further aspects of the methods of the invention, GT1b is added in a concentration of 1 to 300 μ M, preferably 30 μ M.

[0047] In another aspect of the methods of the invention, the neurotoxin polypeptide is BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/G, BoNT/F, BoNT/H or TeNT, or a subtype thereof.

5 [0048] In a still further aspect of the methods of the invention, the biological activity of the neurotoxin polypeptide is determined by quantification of the neurotoxin-cleaved substrate by Immuno-Western blot analysis, SDS-PAGE immunoblot analysis or ELISA (see, e.g., Pellet et al. (2010), J. Pharmacol. Toxicol. Methods 61, 304-310).

10 [0049] In a further aspect, the invention relates to the use of GT1b for

- a) standardizing the sensitivity of different batches of induced pluripotent stem cell (iPS)-derived neurons to a neurotoxin polypeptide; or
- b) reducing the variability of the sensitivity of different batches of induced pluripotent stem cell (IPS)-derived neurons to a Neurotoxin polypeptide.

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[0050] Specific aspects of the methods and uses of the present invention are shown in the following Examples.

20 [0051] The Figures show:

Figure 1: SiMa cells were cultivated and intoxicated as described in Example 2 and the ratio of cleaved to uncleaved SNAP-25 was determined by Western Blot analysis. On the X-axis the concentration of the Botulinum Neurotoxin type is given, whereas on the Y-axis

25 the relative amount of cleaved SNAP-25, i.e. the ratio of cleaved to uncleaved SNAP-25 is plotted. The circles symbolize SiMa cells cultivated without GT1b, the squares symbolize SiMa cells cultivated with 30 μ M GT1b. The cultivation with GT1b led to an increase in sensitivity of about 10-fold.

30 Figure 2: SH-SY5Y cells were cultivated and intoxicated as described in Example 2 and the ratio of cleaved to uncleaved SNAP-25 was determined by Western Blot analysis. On the X-axis the concentration of the Botulinum Neurotoxin type is given, whereas on the Y-axis the relative amount of cleaved SNAP-25, i.e. the ratio of cleaved to uncleaved SNAP-25 is plotted. The circles symbolize SH-SY5Y cells cultivated without GT1b, the squares

35 symbolize SH-SY5 cells cultivated with 30 μ M GT1b. The cultivation with GT1b led to an increase in sensitivity of about 2-fold.

Figure 3: PC12 cells were cultivated and intoxicated as described in Example 2 and the ratio of cleaved to uncleaved SNAP-25 was determined by Western Blot analysis. On the X-axis the concentration of the Botulinum Neurotoxin type is given, whereas on the Y-axis the relative amount of cleaved SNAP-25, i.e. the ratio of cleaved to uncleaved SNAP-25 is plotted. The circles symbolize PC12 cells cultivated without GT1b, the squares symbolize PC12 cells cultivated with 30 μ M GT1b. The cultivation with GT1b led to an increase in sensitivity of about 1.4-fold.

Figure 4: Neuro2A-cells were cultivated and intoxicated as described in Example 2 and the ratio of cleaved to uncleaved SNAP-25 was determined by Western Blot analysis. On the X-axis the concentration of the Botulinum Neurotoxin type is given, whereas on the Y-axis the relative amount of cleaved SNAP-25, i.e. the ratio of cleaved to uncleaved SNAP-25 is plotted. The circles symbolize Neuro2A cells cultivated without GT1b, the squares symbolize Neuro2A cells cultivated with 30 μ M GT1b. At the given neurotoxin concentrations, no complete dose response curve could be observed as well as no increase in sensitivity with GT1b.

Figure 5: NG108-15-cells were cultivated and intoxicated as described in Example 2 and the ratio of cleaved to uncleaved SNAP-25 was determined by Western Blot analysis. On the X-axis the concentration of the Botulinum Neurotoxin type is given, whereas on the Y-axis the relative amount of cleaved SNAP-25, i.e. the ratio of cleaved to uncleaved SNAP-25 is plotted. The circles symbolize NG108-15-cells cultivated without GT1b, the squares symbolize NG108-15-cells cultivated with 30 μ M GT1b. The cultivation with GT1b led to an increase in sensitivity of about 1.6-fold.

[0052] The invention will now be illustrated by the following examples which shall, however, not be construed as limiting the scope of the present invention.

[0053] Examples:

Example 1:

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 either fresh maintenance medium as described in the user manual or by the same medium supplemented with 30 μ M GT1b.

After further 72 h incubation time, the medium was removed and replaced by fresh medium containing BoNT/A in varying concentrations. If cells were grown on GT1b containing medium the fresh medium also contained 30 μ M GT1b.

- 5 72 h after start of the intoxication, the medium was aspirated and the cells were lysed by addition of 25 μ l SDS sample buffer.

The percentage of cleaved SNAP-25 was determined by SDS-PAGE immunoblot analysis, as described in Pellett et al., 2010 (loc. cit.).

- 10 The EC₅₀ (concentration of BoNT/A yielding half maximum cleavage of SNAP-25) was calculated by plotting the percent cleaved SNAP-25 versus the BoNT/A concentration.

The resulting EC₅₀ values of the different cell batches with and without addition of GT1b are shown in Table 1.

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Table 1:

	EC ₅₀ without GT1b	EC ₅₀ with GT1b
Batch 02	2.84 U/ml	0.65 U/ml
Batch 03	5.37 U/ml	0.89 U/ml
Batch 04	6.40 U/ml	0.77 U/ml
Batch 05	5.47 U/ml	0.68 U/ml
Mean	5.02 U/ml	0.75 U/ml
RSD	30.3%	14.6%

- Despite the higher sensitivity resulting from the addition of GT1b lowering the EC₅₀ from ~5.0 to ~0.75 U/mL, the relative standard deviation of the EC₅₀ values of the batches is
 20 reduced from ~30% to ~15%.

Example 2:

- Cultivation and differentiation of SiMa cells (see Figure 1): A vial containing SiMa-cells
 25 was thawed and re-suspended in culture medium (90% RPMI 1640 + 10% h.i. FBS + 2 mM L-glutamine +/- 30 μ M GT1b) to a final density of 30,000 cells/mL. The cells were seeded on poly-D-lysine coated 96-well microtiter plates at 3,000 cells/well and incubated for 72 hours at 37°C, 95% O₂ / 5 % CO₂ under a saturated water vapor atmosphere. After 72 hours, the medium was exchanged to serum-free medium (MEM + 2% B27 + 1% N2 +
 30 2% Non-essential amino acids + 2 mM L-glutamine +/- 30 μ M GT1b) containing Botulinum neurotoxin type A in concentrations ranging from 1.0*10⁻⁹ to 5.65*10⁻¹⁵ M. After 72 hours of incubation as indicated above, the medium was removed, the cells were

re-suspended in lysis buffer (20mM Tris/HCl, 20 mM NaCl, 2 mM MgCl₂, 0.5% Triton X-100, 5 U/mL benzonase at pH 8.0), mixed with RotiLoad 1 SDS sample buffer and subjected to Western Blot analysis to determine the ratio of cleaved SNAP-25 / uncleaved SNAP-25 as described in Whitemarsh et al. (2012), Toxicol. Sci. 126, 426-35, using an
5 antibody generated in mice (Synaptic Systems SySy111111).

Cultivation and differentiation of SH-SY5Y cells (see Figure 2): A vial containing SH-SY5Y-cells was thawed and re-suspended in culture medium (85% MEM:F12 + 15% h.i. FBS +/-30 μ M GT1b) to a final density of 60,000 cells / mL. The cells were seeded on
10 uncoated 96-well microtiter plates at 6,000 cells/well and incubated for 24 hours at 37°C, 95% O₂ / 5 % CO₂ under a saturated water vapor atmosphere. The medium was then supplemented with Nerve Growth factor (100 ng/ml) and Aphidicoline (0.3 mM) +/- 30 μ M GT1b. This medium was exchanged every 2-3 days. After 17 days of incubation, the medium was exchanged to fresh medium containing Botulinum neurotoxin type A in
15 concentrations ranging from $1.0 \cdot 10^{-9}$ to $5.65 \cdot 10^{-15}$ M. After 72 hours of incubation as indicated above, the medium was removed, the cells were re-suspended in lysis buffer (20mM Tris/HCl, 20 mM NaCl, 2 mM MgCl₂, 0,5% Triton X-100, 5 U/mL benzonase at pH 8.0), mixed with RotiLoad 1 SDS sample buffer and subjected to Western blot analysis to determine the ratio of cleaved SNAP-25 / uncleaved SNAP-25 as described
20 in Whitemarsh et al. (2012), Toxicol. Sci. 126, 426-35, using an antibody produced in mice (Synaptic Systems SySy111111).

Cultivation and differentiation of PC12 cells (see Figure 3): A vial containing PC12 cells was thawed and re-suspended in culture medium (85% RPMI 1640 + 10% horse serum +
25 5% h.i. FBS +/- 30 μ M GT1b) to a final density of 25,000 cells / mL. The cells were seeded on collagen coated 96-well microtiter plates at 2,500 cells/well and incubated for 72 hours at 37°C, 95% O₂ / 5 % CO₂ under a saturated water vapor atmosphere. The medium was then supplemented with Nerve Growth factor (100 ng/ml) +/- 30 μ M GT1b. This medium was exchanged every 2-3 days. After 11 days of incubation, the medium was
30 exchanged to fresh medium containing Botulinum neurotoxin type A in concentrations ranging from $1.0 \cdot 10^{-9}$ to $5.65 \cdot 10^{-15}$ M. After 72 hours of incubation as indicated above, the medium was removed, the cells were re-suspended in lysis buffer (20mM Tris/HCl, 20 mM NaCl, 2 mM MgCl₂, 0,55% Triton X-100, 5 U/mL benzonase at pH 8.0), mixed with RotiLoad 1 SDS sample buffer and subjected to Western blot analysis to determine the
35 ratio of cleaved SNAP-25 / uncleaved SNAP-25 as described in Whitemarsh et al. (2012), Toxicol. Sci. 126, 426-35, using an antibody generated in mice (SYNAPTIC Systems SySy111111).

Cultivation and differentiation of Neuro2A cells (see Figure 4): A vial containing Neuro2A cells was thawed and re-suspended in culture medium (90% DMEM + 10% h.i. FBS +/- 30 μ M GT1b) to a final density of 20,000 cells / mL. The cells were seeded on 96-well microtiter plates at 2,000 cells/well and incubated for 24 hours at 37°C, 95% O₂ / 5 % CO₂ under a saturated water vapor atmosphere. The medium was exchanged by serum-free DMEM +/- 30 μ M GT1b followed by 3 days of incubation at 37°C. Then the medium was exchanged to fresh serum-free medium containing 0.2% BSA +/- 30 μ M GT1b and Botulinum neurotoxin type A in concentrations ranging from $1.0 \cdot 10^{-9}$ to $5.65 \cdot 10^{-15}$ M. After 72 hours of incubation as indicated above, the medium was removed, the cells were re-suspended in lysis buffer (20mM Tris/HCl, 20 mM NaCl, 2 mM MgCl₂, 0,5% Triton X-100, 5 U/mL benzonase at pH 8.0), mixed with RotiLoad 1 SDS sample buffer and subjected to Western blot analysis to determine the ratio of cleaved SNAP-25 / uncleaved SNAP-25 as described in Whitemarsh et al. (2012), Toxicol. Sci. 126, 426-35, using an antibody produced in mice (SYNAPTIC Systems SySy111111).

Cultivation and differentiation of NG108-15 cells (see Figure 5): A vial containing SH-SY5Y cells was thawed and re-suspended in culture medium (90% DMEM + 10% h.i. FBS +/- 30 μ M GT1b) to a final density of 60,000 cells / mL. The cells were seeded on 96-well microtiter plates at 6,000 cells/well and incubated for 72 hours at 37°C, 95% O₂ / 5 % CO₂ under a saturated water vapor atmosphere. The medium was then supplemented with dibutyryl-cAMP (1 mM) +/- 30 μ M GT1b. This Medium was exchanged every 2-3 days. After 5 days of incubation, the medium was exchanged to fresh medium containing Botulinum neurotoxin type A in concentrations ranging from $1.0 \cdot 10^{-9}$ to $5.65 \cdot 10^{-15}$ M. After 72 hours of incubation as indicated above, the medium was removed, the cells were re-suspended in lysis buffer (20mM Tris/HCl, 20 mM NaCl, 2 mM MgCl₂, 0,5% Triton X-100, 5 U/mL benzonase at pH 8.0), mixed with RotiLoad 1 SDS sample buffer and subjected to Western blot analysis to determine the ratio of cleaved SNAP-25 / uncleaved SNAP-25 as described in Whitemarsh et al. (2012), Toxicol. Sci. 126, 426-35 using an antibody generated in mice (Synaptic Systems SySy111111).

Claims

- 5 1. A method for standardizing the sensitivity of induced pluripotent stem cell (iPS)-
derived neurons to a neurotoxin polypeptide, comprising the steps of:
a) cultivating different batches of induced pluripotent stem cell-derived neurons in
a cell culture medium comprising GT1b for at least 3 hours;
b) contacting the different batches of induced pluripotent stem cell-derived neurons
10 of step a) with a neurotoxin polypeptide;
c) cultivating the different batches of induced pluripotent stem cell-derived neurons
of step b) for at least 24 hours in the presence of GT1b under conditions which
allow for the neurotoxin polypeptide to exert its biological activity;
thereby standardizing the sensitivity of the induced pluripotent stem cell-derived
15 neurons to a neurotoxin polypeptide.
2. A method for the generation of induced pluripotent stem cell (IPS)-derived neurons
having a standardized sensitivity to a neurotoxin polypeptide, comprising the steps
of:
20 a) providing different batches of induced pluripotent stem cell-derived neurons;
b) cultivating the different batches of induced pluripotent stem cell-derived neurons
of step a) in a cell culture medium comprising GT1b for at least 3 hours,
thereby standardizing the sensitivity of the induced pluripotent stem cell-derived
neurons to a neurotoxin polypeptide.
- 25 3. The method of claim 2, further comprising:
c) contacting the different batches of induced pluripotent stem cell-derived neurons
of step b) with a neurotoxin polypeptide; and
d) cultivating the different batches of induced pluripotent stem cell-derived neurons
30 of step c) for at least 24 hours in the presence of GT1b under conditions which
allow for the neurotoxin polypeptide to exert its biological activity.
4. A method for determining the biological activity of a neurotoxin polypeptide,
comprising the steps of:
35 a) cultivating induced pluripotent stem cell-derived neurons in a cell culture
medium comprising GT1b for at least 3 hours;

b) contacting the induced pluripotent stem cell-derived neurons of step a) with a neurotoxin polypeptide;

c) cultivating the induced pluripotent stem cell-derived neurons of step b) for at least 24 hours in the presence of GT1b under conditions which allow for the neurotoxin polypeptide to exert its biological activity; and

d) determining the biological activity of the neurotoxin polypeptide in said cells.

5. The method of claim 4, wherein different batches of induced pluripotent stem cell-derived neurons are used.

6. The method of claim 1 or 3, further comprising determining the biological activity of the neurotoxin polypeptide in the different batches of induced pluripotent stem cell-derived neurons.

7. The method of any of claims 1 to 3, wherein standardizing the sensitivity is a reduction in the variability of the sensitivity of the different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide, in comparison to control batches of induced pluripotent stem cell-derived neurons treated without GT1b.

8. The method of claim 7, wherein the reduction in the variability of the sensitivity of the different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide is an at least 1.5-fold or at least 2-fold reduction.

9. The method of any of claims 1 to 8, wherein the induced pluripotent stem cell-derived neurons are human induced pluripotent stem cell-derived neurons, preferably iCell[®] neurons (Cellular Dynamics International).

10. The method of any of claims 1 to 3 and 5 to 9, wherein the different batches of induced pluripotent stem cell-derived neurons differ in the number of passages, the number of freeze/thaw cycles, the cultivation conditions, the storage time, the growth time, the differentiation conditions, or combinations thereof.

11. The method of claim 1, 2 or 4, wherein the cell culture medium comprises Neurobasal medium, B27 Supplement (2%), and Glutamin or Glutamax (1%).

12. The method of any of claims 1 to 11, wherein GT1b is added in a concentration of 1 to 300 μ M, preferably 30 μ M.
13. The method of any of claims 1 to 12, wherein the neurotoxin polypeptide is
5 BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/H or TeNT, or a subtype thereof.
14. The method of claim 4 or 6, wherein the biological activity of the neurotoxin polypeptide is determined by quantification of the neurotoxin-cleaved substrate by
10 Immuno-Western blot analysis, SDS-PAGE Immunoblot analysis or ELISA.
15. Use of GT1b for
- a) standardizing the sensitivity of different batches of induced pluripotent stem cell-derived neurons to a neurotoxin polypeptide; or
- 15 b) reducing the variability of the sensitivity of different batches of induced pluripotent stem cell-derived neurons to a Neurotoxin polypeptide.

Fig. 1

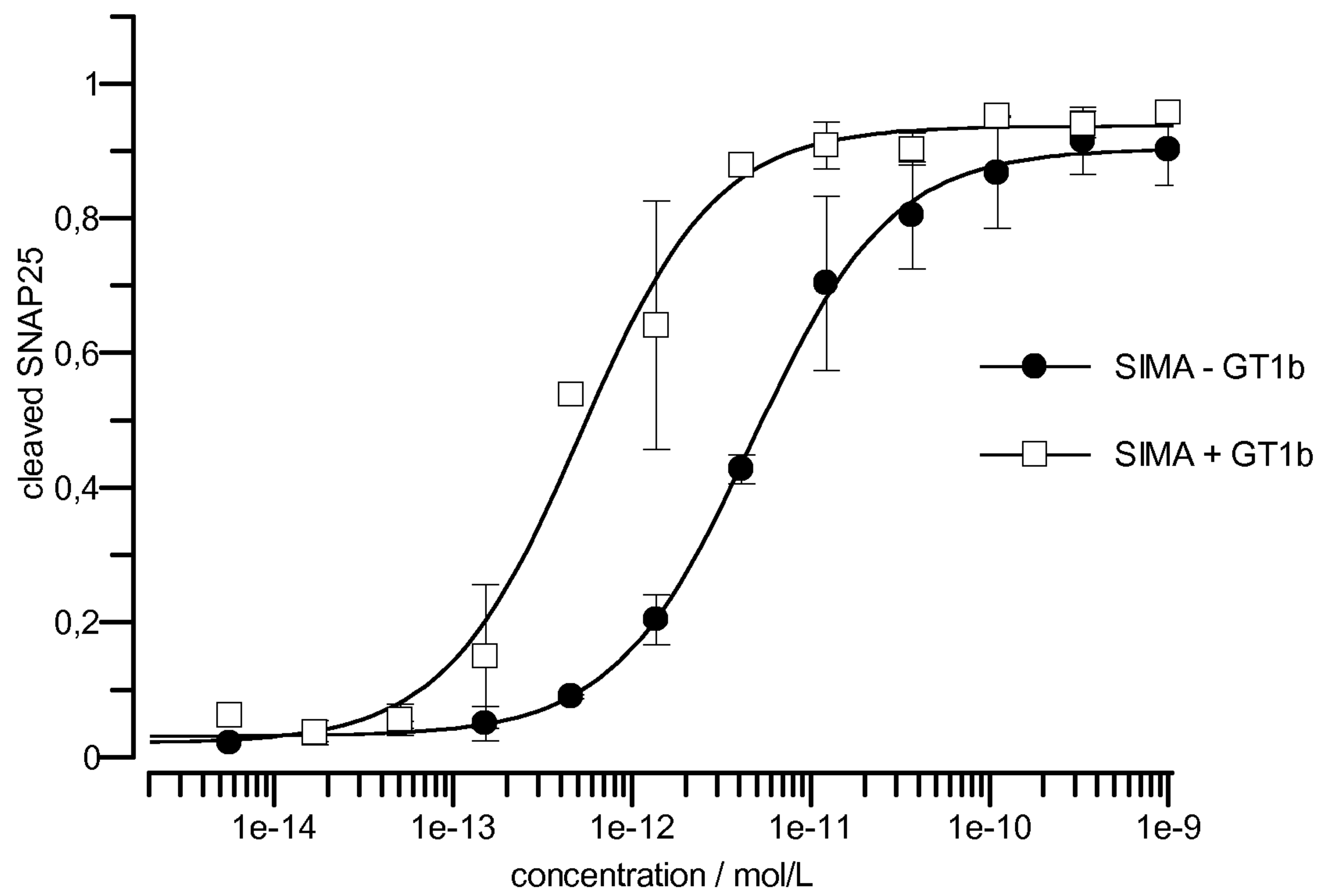


Fig. 2

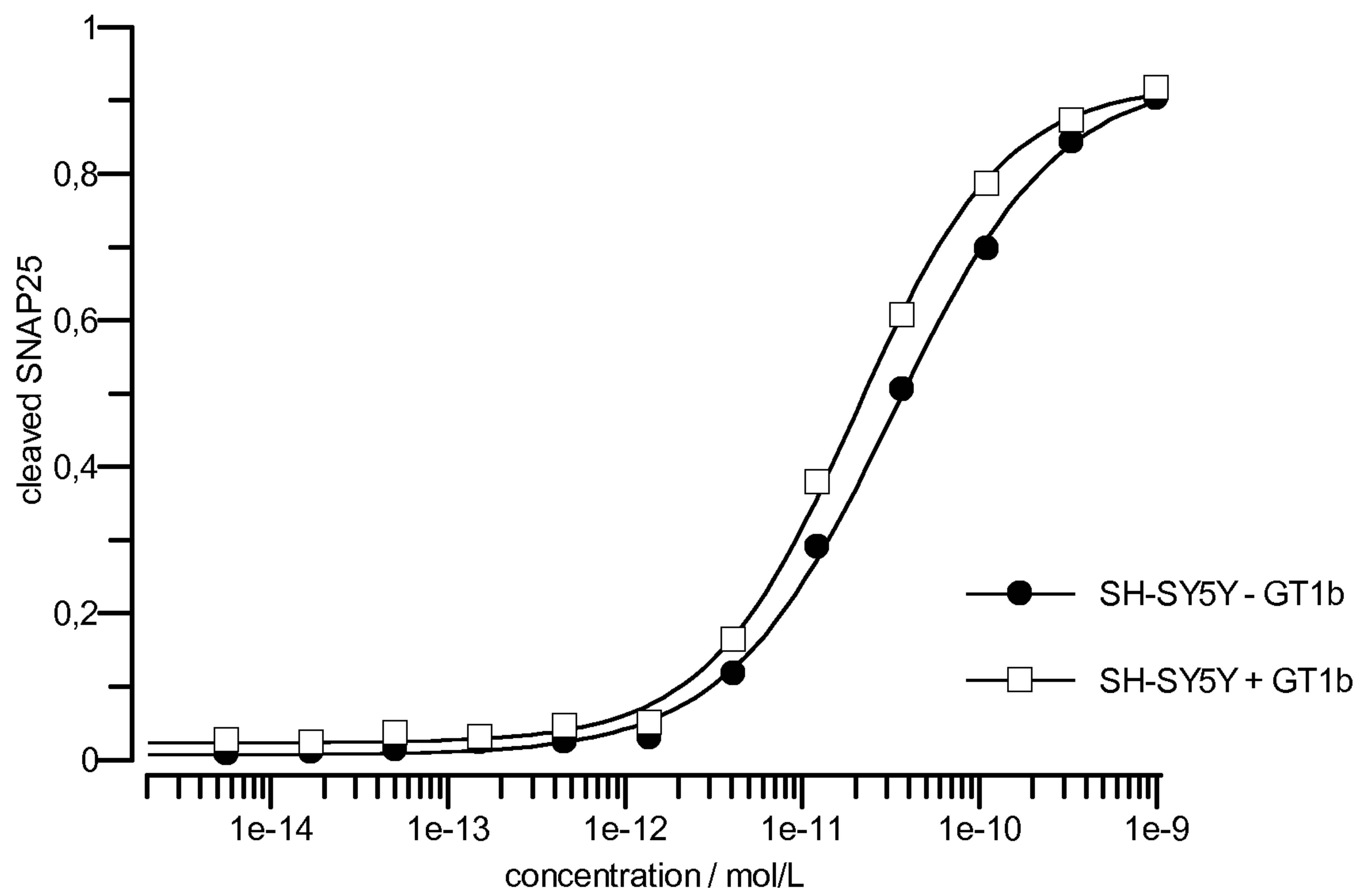


Fig. 3

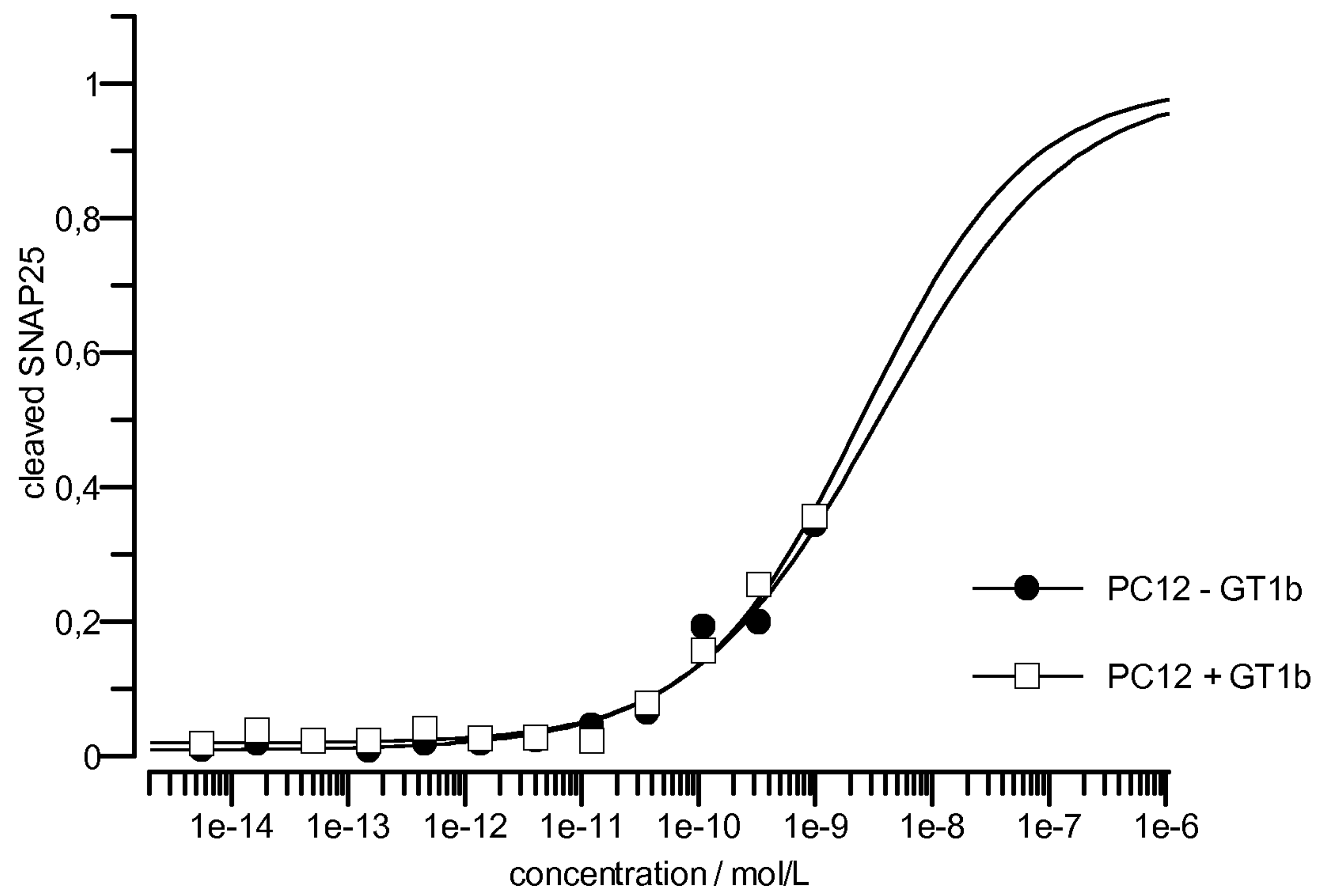


Fig. 4

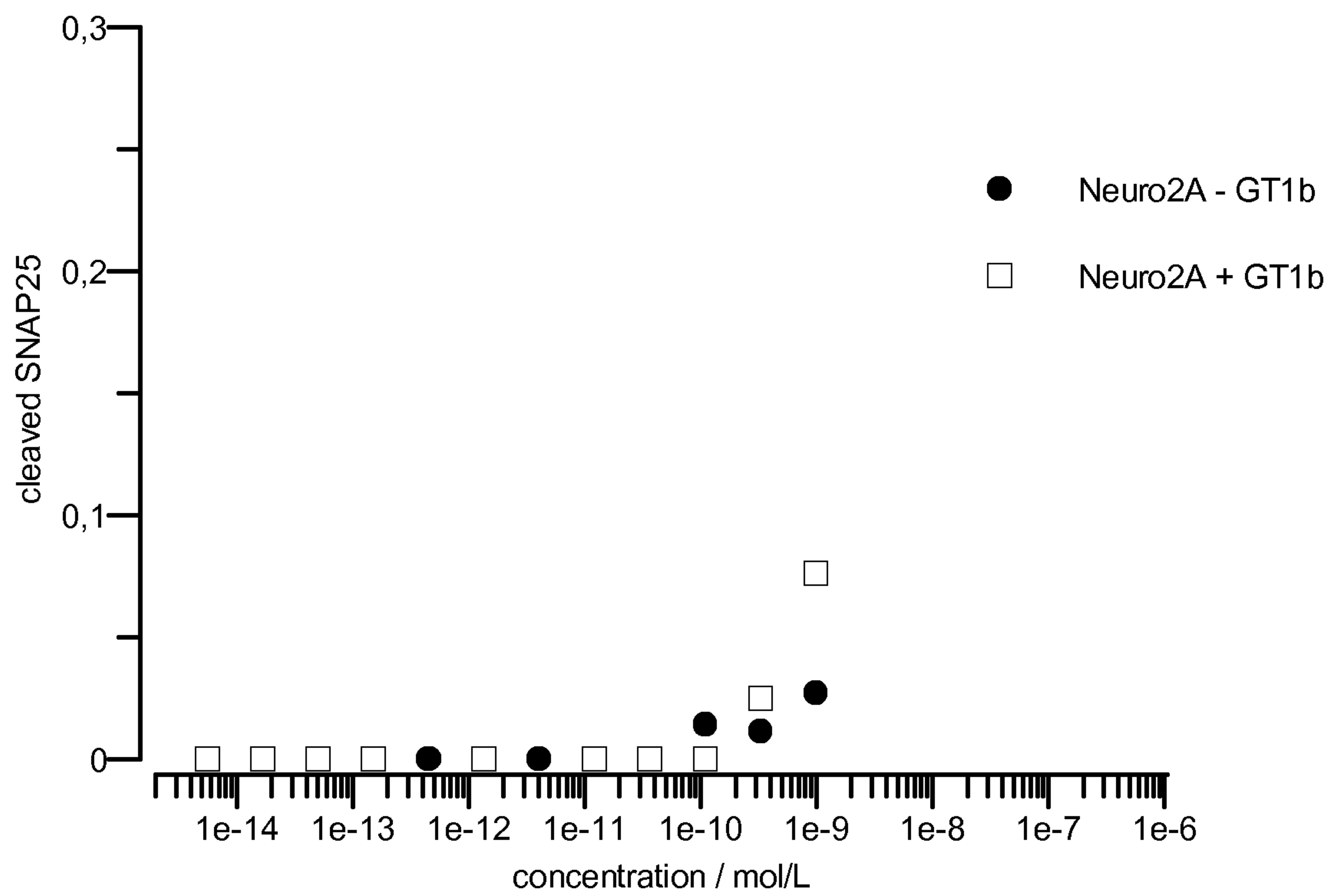


Fig. 5

