

[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, there is provided 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 two of the following steps:
10 (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time of between 4 hours and 96 hours and/or (iii) agitation of the cells during neurotoxin polypeptide exposition, thereby enhancing the specific uptake of the neurotoxin polypeptide into said cells.

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[0009b] In another aspect, there is provided 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 two of
20 the following steps: (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time of between 4 hours and 96 hours and/or (iii) agitation of the cells during neurotoxin polypeptide exposition;
- b) fixing the cells and, optionally, permeabilizing the cells with a detergent;
- 25 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 a neurotoxin-cleaved substrate, under conditions which allow for binding of said capture antibodies to said substrates;
- 30 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
35 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
40 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:

- 5 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
10 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
15 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
25 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
30 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
35 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

Claims

1. 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 two of the following steps: (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time of between 4 hours and 96 hours and/or (iii) agitation of the cells during neurotoxin polypeptide exposition, thereby enhancing the specific uptake of the neurotoxin polypeptide into said cells.
2. The method of claim 1, followed by determining the biological activity of the neurotoxin polypeptide in the cells.
3. 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 two of the following steps: (i) K^+ -mediated depolarization of the cells, (ii) a reduced neurotoxin polypeptide exposition time of between 4 hours and 96 hours 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 a non-cleaved and neurotoxin-cleaved substrate and with at least a second capture antibody specifically binding to the cleavage site of a 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.
4. The method of any of claims 1 to 3, wherein the K^+ -mediated depolarization of the cells is carried out at an additional K^+ concentration of 20 mM to 55 mM, for at least 2 hours.
 5. The method of any of claims 1 to 4, wherein the K^+ -mediated depolarization of the cells and/or the neurotoxin polypeptide exposition is carried out in the presence of the ganglioside GT1b.
 6. The method of any of claims 1 to 5, wherein the ganglioside GT1b is used in a concentration between 15 and 50 μ M, preferably 20 μ M.
 7. The method of any of claims 1 to 6, wherein 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 maximum.
 8. The method of any of claims 1 to 7, 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.
 9. The method of any of claims 1 to 8, wherein the K^+ -mediated depolarization of the cells is carried out at an additional K^+ concentration of at least 20 mM to 55 mM, for at least 2 hours in the presence of 20 μ M ganglioside GT1b and neurotoxin polypeptide, followed by neurotoxin polypeptide exposition for additional 70 hours under agitation.
 10. The method of any of claims 1 to 8, wherein an incubation time without neurotoxin polypeptide precedes the neurotoxin polypeptide exposition.
 11. The method of claim 10, wherein the incubation time without neurotoxin polypeptide is between 16 and 48 hours.

12. The method of any of claims 1 to 11, wherein the method is a fluorescence method.
13. The method of any of claims 1 to 12, wherein the neurotoxin polypeptide is BoNT/A, BoNT/B, BoNT/C1, BoNT/D, BoNT/E, BoNT/F, BoNT/G, BoNT/H, or subtypes thereof.
14. The method of any of claims 1 to 13, wherein the substrate is VAMP/Synaptobrevin, SNAP-25 or Syntaxin.
15. The method of any of claims 1 to 14, 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.