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(54) Title: MEANS FOR DETECTING OR MEASURING A BIOLOGICAL ACTIVITY OF A BOTULINUM TOXIN

(57) Abstract: The present invention mainly relates to the field of the quality assessment of botulinum toxins for *in vivo* use, and especially for human use. The present invention notably relates to a method for *in vitro* detecting and/or measuring a biological activity of one or more botulinum toxin(s) by implementing one or more membrane vesicle(s), in particular exosomes, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2, synaptotagmin, and fragment(s) thereof.



TITLE

Means for detecting or measuring a biological activity of a botulinum toxin

FIELD OF THE INVENTION

5 The present invention mainly relates to the field of the quality assessment of botulinum toxins for *in vivo* use, and especially for human use.

 The present invention notably relates to a method for *in vitro* detecting and/or measuring a biological activity of one or more botulinum toxin(s) by implementing one or more membrane vesicle(s), in particular exosomes, expressing at their surface one or more botulinum toxin
10 receptor(s) selected in a group comprising SV2, synaptotagmin, and fragment(s) thereof.

BACKGROUND OF THE INVENTION

 Botulinum toxins (or Botulinum neurotoxins (BoNTs)) are neurotoxic proteins produced by the bacterium *Clostridium botulinum* and related species. These botulinum toxins encompass
15 eight serologically toxin types, designated types A to H. These eight serologically distinct toxin types possess different tertiary structures and significant sequence divergence. Moreover, 40
distinct subtypes are now described (Lam KH, Jin R (2015) Current opinion in structural biology 31: 89-95)

 The botulinum neurotoxins A, B and E and F are proteins produced in nature by bacteria
20 and induce at extremely low doses a neuro-paralysis in humans. The BoNTs act on the peripheral nervous system by blocking reversibly the release of acetylcholine in the synaptic cleft, at the motor end plates of neuromuscular junctions.

 The BoNTs are two-chain proteins, each composed of a heavy chain polypeptide joined via disulfide bond to a light chain polypeptide. The heavy chain ensures both a function for
25 recognizing a receptor located on the neuron and for transferring BoNTs in the cytoplasm (translocation), while the light chain has an intracellular enzyme activity responsible for muscle paralysis.

 Despite their dangerousness, the BoNT's are produced by the pharmaceutical industry since the discovery in the 1980s that they could be used in medicine and cosmetics. The BoNTs
30 currently undergoing many clinical indications such as the treatment of muscle hypercontraction (dystonia, strabismus...) or hypersecretion (hyperhidrosis). Locally injected, the BoNT's induce transient immobilization of muscle. On the other hand, the BoNTs have applications in the field of cosmetics for the treatment of expression lines (BoTox).

 The standard test used in the pharmaceutical industry for the validation of therapeutic
35 and cosmetic BoNTs batch is an *in vivo* test in mice (Lindstrom M, Korkeala H (2006) Clinical

microbiology reviews 19: 298-314.)). This *in vivo* mouse lethality assay is the gold standard test for assaying BoNTs activity in that it represents a read out for several aspects of BoNT toxicity, namely (i)- binding to membrane receptors, (ii)- translocation into neuronal cytosol and (iii)- intracellular enzyme activity: symptoms generating, proteolytic cleavage of presynaptic target proteins.

However, this *in vivo* assay is slow, expensive and ethically questionable. Besides, the international regulations strongly encourage the substitution of *in vivo* tests by *in vitro* methods. In addition, this *in vivo* test is a global test that does not allow defining to which biological step a potential problem with a defective batch can be located. Moreover, the conditions of the *in vivo* assay methods may differ from one test laboratory to another, which may lead to distinct conclusions regarding the quality of a given botulinum toxin batch (Brin MF, James C, Maltman J (2014) *Biologics : targets & therapy* 8: 227-241.).

There is therefore a need for rapid and sensitive *in vitro* tests allowing assay of batches produced by the pharmaceutical industry (P Capek, *Toxins* 2010, 2 (1): 24-53 PubMed PMID: 22069545; M Lindstrom, *Clinical Microbiology.. Reviews* 2006, 19 (2).. 298-314 PubMed PMID: 16614251).

Thus, a complete *in vitro* test should be able to measure these 3 parameters. However, the *in vitro* tests currently on the market only allow measuring the enzyme activity. There is no simple *in vitro* test being capable of further measuring the two other above-mentioned parameters, namely the binding of BoNTs to BoNTs-receptor(s) and the translocation through a membrane. This notably results from the complex structure of these BoNTs-receptors as well as their interaction which hinder for instance the development of a simple *in vitro* test to measure BoNT binding as well as its translocation through biological membranes.

SV2 and synaptotagmin proteins are protein receptors for BoNT identified for the BoNT's A, E, F, D (i.e. BoNT/A, BoNT/E, BoNT/F, BoNT/D), and B, G (i.e. BoNT/B and BoNT/G respectively. Moreover, the mosaic native toxin BoNT/DC binds synaptotagmin (Peng L, Berntsson RP, Tepp WH, Pitkin RM, Johnson EA, Stenmark P, Dong M. *J Cell Sci.* **2012** Jul 1;125(Pt 13):3233-42. doi: 10.1242/jcs.103564.). These are membrane proteins, post-translationally modified and which specifically combine with a glycolipid partner to bind BoNT's on the surface of neurons. It is therefore difficult to *in vitro* measure the step of binding of BoNT to their receptors outside of a membrane environment. Thus, the affinity of BoNT/B for the soluble recombinant synaptotagmin is at least 10 times lower than that the affinity measured on biological membranes (Chai Q, Arndt JW, Dong M, Tepp WH, Johnson EA, et al. (2006). *Nature* 444: 1096-1100.). What is more, given the complexity of the structure of SV2 and to a lesser extent synaptotagmin, the membrane insertion of these receptors greatly influences their BoNTs

interaction domains. Most *in vitro* available studies either use partial/minimal soluble domains or full length receptors in detergent micelles. In both cases, the native conformational presentation of these receptors is far from being respected.

There are on the other hand little quantitative methods for analyzing the molecular interactions at the level of native membrane proteins outside a cellular context or extracting receptor containing membrane structures from native tissues implying animal usage.

WO2004/083370, Ferracci *et al.* (Analytical Biochemistry; vol. 334, no. 2, pages 367-375; 2004) and **Ferraci *et al.*** (Biochemical Journal; vol. 391, no. Part 3, pages 659-666; 2005) teach methods for analyzing the effect of compounds on immobilized synaptic vesicles (SV) from brain rat extracts.

However those methods are not fully satisfactory for detecting and/or measuring a biological activity of botulinum toxin. In particular, those methods are not satisfactory for studying toxicity related to the binding of BoNTs to membrane receptors.

Indeed, **WO2004/083370** confirms that the intravesicular domain of synaptotagmin I is not accessible on immobilized synaptic vesicles from such brain rat extracts.

SUMMARY OF THE INVENTION

The present invention relates to an affinity substrate comprising a solid substrate material onto which is/are immobilized one or more membrane vesicles, in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s).

In some embodiments of the said affinity substrate, the one or more botulinum toxin receptors are selected in a group comprising SV2 and synaptotagmin.

In some embodiments of the said affinity substrate, the SV2 botulinum toxin receptor is selected in a group comprising SV2A, SV2B and SV2C

In some embodiments of the said affinity substrate, the synaptotagmin botulinum toxin receptor is selected in a group comprising SYT1 and SYT2.

The invention also concerns a composition comprising *membrane vesicles, in particular exosome-like vesicles*, and still better exosomes, expressing at their surface one or more botulinum toxin receptor(s)

This invention also pertains to a complex between (i) a membrane vesicles, in particular exosome-like vesicles, such as defined above and (ii) one or more botulinum neurotoxin(s).

This invention also relates to a kit for assessing one or more biological activity/ies of a compound, the said kit comprising:

- one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof,

- optionally, at least one solid substrate material at least partially coated with ligand(s) capable of binding to said membrane vesicles, and preferably to one or more botulinum toxin receptor(s) selected in a group comprising SV2 and synaptotagmin, and

- optionally, a composition comprising one or more ganglioside molecule(s).

This invention also concerns a method for *in vitro* detecting and/or measuring a biological activity of one or more botulinum toxin(s), said method comprising the steps of:

10 a) providing one or more membrane vesicles, in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and synaptotagmin,

b) contacting the membrane vesicles provided at step a) with one or more botulinum toxin(s), optionally in presence of ganglioside molecule(s), whereby an assay mixture is provided, and

c) detecting and/or measuring a biological activity of said botulinum neurotoxin(s).

This invention also pertains to a method for *in vitro* detecting the presence of one or more botulinum toxin(s), said method comprising the steps of:

20 - providing one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more botulinum receptor(s) selected in a group comprising SV2 and synaptotagmin (SYT),

- bringing a sample comprising, or suspected of comprising, said botulinum neurotoxin(s) into contact with said membrane vesicle(s), optionally in presence of ganglioside molecule(s), and

25 - detecting the formation of complexes between (i) said membrane vesicle(s) and (ii) said botulinum neurotoxin(s).

More precisely, membrane vesicles, in particular exosome-like vesicles, and still better exosomes, expressing at their surface one or more botulinum toxin receptor(s), can be characterized in that the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s).

Accordingly, the invention relates in particular to an affinity substrate comprising a solid substrate material onto which is/are immobilized one or more membrane vesicles, in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s),

wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s).

It also relates to a composition comprising membrane vesicles, in particular exosome-like vesicles, and still better exosomes, expressing at their surface one or more botulinum toxin receptor(s), wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s).

It also relates to a complex between (i) a membrane vesicles, in particular exosome-like vesicles, such as defined above and (ii) one or more botulinum neurotoxin(s).

It also relates to a kit, notably for assessing one or more biological activity(ies) of a compound, the said kit comprising:

- one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof, wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s),

- optionally, at least one solid substrate material at least partially coated with ligand(s) capable of binding to said membrane vesicles, and preferably to one or more botulinum toxin receptor(s) selected in a group comprising SV2 and synaptotagmin, and

- optionally, a composition comprising one or more ganglioside molecule(s).

It also relates to a method for in vitro detecting and/or measuring a biological activity of one or more botulinum toxin(s), said method comprising the steps of:

- a) providing one or more membrane vesicles, in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and synaptotagmin, wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s),

- b) contacting the membrane vesicles provided at step a) with one or more botulinum toxin(s), optionally in presence of ganglioside molecule(s), whereby an assay mixture is provided, and

- c) detecting and/or measuring a biological activity of said botulinum neurotoxin(s).

It also relates to a method for in vitro detecting the presence of one or more botulinum toxin(s), said method comprising the steps of:

- providing one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more botulinum receptor(s) selected in a group comprising SV2 and synaptotagmin (SYT), wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s),

- bringing a sample comprising, or suspected of comprising, said botulinum neurotoxin(s) into contact with said membrane vesicle(s), optionally in presence of ganglioside molecule(s), and

- detecting the formation of complexes between (i) said membrane vesicle(s) and (ii) said botulinum neurotoxin(s)

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 illustrates the map of the vector used for transfecting cells for producing exosomes expressing one or more botulinum toxin receptors. The nucleic acid sequence encoding the botulinum toxin receptor of interest was introduced between the nucleotide located at position 2974 and the nucleotide located at position 2975 of the said vector, as identified in the figure.

Figure 2 illustrates a Western blot characterizing the expression of botulinum toxin receptors in rat synaptotagmine 2 (rSYT2) and human SV2A (hSV2A) in exosomal preparations.

Figure 3 shows a graph illustrating the specific capture of exosomes expressing rSYT2 on anti synaptotagmin antibodies coated on a Surface Plasmon Resonance (SPR) sensor chip. In X-axis: time in seconds; in ordinate: the resonance units (RU).

Figure 4A shows a graph illustrating SPR detection of BoNT/B binding to exosomes expressing rSYT2, preincubated or not with GT1b. In X-axis: time in seconds; in ordinate: the resonance units (RU).

Figure 4B shows a graph illustrating, by ELISA detection, the binding of BoNT/B to exosomes expressing hSYT1 or rSYT2, preincubated or not with GT1b.

Figure 5 shows a graph illustrating, by ELISA detection, the binding of BoNT/A to exosomes expressing hSV2A, preincubated or not with GT1b.

Figure 6 shows a graph illustrating, by ELISA detection, the binding of BoNT/E to exosomes expressing hSV2A, preincubated or not with GT1b.

Figure 7 shows a graph illustrating the SPR detection of BoNT/B binding, to exosomes expressing hSYT2 in the presence or absence of an anti-BoNT/B antibody. In X-axis: time in seconds; in ordinate: the resonance units (RU). **Curve 1**: binding of BoNT/B to exosomes expressing hSYT2 without the anti-BoNT/B antibody. **Curve 2**: binding of BoNT/B to exosomes expressing hSYT2 in the presence of the anti-BoNT/B antibody.

Figure 8 shows a graph illustrating, by SPR detection, the binding of BoNT/B concentration range to exosomes expressing rSYT2, in order to measure the affinity of BoNT/B to rSYT2 by SPR detection. In X-axis: time in seconds; in ordinate: the resonance units (RU).

Figure 9 shows a graph illustrating, by SPR detection, the binding of BoNT/B concentration range to exosomes expressing hSYT2, in order to measure the affinity of BoNT/B to hSYT2. In X-axis: time in seconds; in ordinate: the resonance units (RU).

Figure 10 illustrates the two distinct topologies of BoNT receptors encountered in synaptic vesicles and exosomes. **A.** At the surface of synaptic vesicles, the topology of BoNT receptors does not allow BoNT binding since the binding site is intravesicular and will be exposed to extracellular side upon synaptic vesicle fusion with neuronal plasma membrane. **B.** On exosomes, the topology of BoNT receptors exposes their BoNT binding site. In our system, BoNT substrates can be targeted to the exosomal lumen or fused to the intra-exosomal side of BoNT receptors. Upon BoNT translocation, BoNT substrate will be cleaved.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is aimed at providing an *in vitro* test allowing easily, rapidly and efficiently detecting / measuring the biological activities of a botulinum toxin.

This invention notably provides an *in vitro* test for detecting or measuring biological activity of a botulinum toxin (BoNT), which encompasses measuring or detecting a biological activity selected in a group comprising (i) the binding of BoNT to its receptor, (ii) the occurrence of a translocation of BoNT complexed to its receptor and (iii) the enzyme activity of BoNT, especially after translocation.

The *in vitro* test provided by the present invention partially replaces, or in some embodiments totally replaces, the existing *in vivo* tests which are based on a measure of the toxicity of a BoNT in mammals and especially in mice.

The present inventors have specifically conceived membrane vesicles, especially exosomes that express at least a botulinum toxin receptor.

The present inventors have also conceived *in vitro* methods making use of these membrane vesicles expressing at least a botulinum toxin receptor, which *in vitro* methods allow detecting the binding of a ligand to the expressed botulinum toxin receptor, and especially allow detecting the binding of a botulinum toxin to its receptor.

As shown in the examples herein, these *in vitro* methods that are described throughout the present specification also allow measuring the binding of a ligand to the expressed botulinum toxin receptor, and especially allow measuring the binding of a botulinum toxin to its receptor.

Particularly, these *in vitro* methods that are described throughout the present specification allow quantifying the ligands, especially botulinum toxins, binding to the expressed botulinum toxin receptor and also allow measuring the affinity of a given ligand, especially of a

given botulinum toxin, to the said receptor expressed at the surface of the membrane vesicles, especially at the surface of the exosomes.

As shown in the examples herein, the inventors have shown the efficiency of an *in vitro* test implementing membrane vesicles, in particular exosome-like vesicles, and still better exosomes, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2, synaptotagmin, and fragment(s) thereof, for easily, rapidly and efficiency detecting or measuring the biological activities of a botulinum toxin, which encompasses (i) the binding of BoNT to its -receptor, (ii) the occurrence of a translocation of BoNT complexed to its receptor and (iii) the enzyme activity of BoNT, especially after translocation.

As shown in the examples herein, the said membrane vesicles, especially exosomes, expressing at their membrane one or more BoNT receptors have allowed the inventors to design and perform *in vitro* methods for detecting the binding of a ligand, especially the binding of a botulinum toxin, to its receptor, which is the first key step in botulinum toxins neuronal attack.

The availability of the membrane vesicles, especially exosomes, expressing at their membrane one or more BoNT receptors have also allowed the inventors to conceive an *in vitro* method for measuring the capacity of a ligand, especially of a botulinum toxin, to undergo membrane translocation.

The availability of the membrane vesicles, especially exosomes, expressing at their membrane one or more BoNT receptors have also allowed the inventors to conceive an *in vitro* method for measuring the enzyme activity of the translocated ligand, especially of the translocated botulinum toxin.

As shown in the examples herein, the use of these membrane vesicles expressing one or more botulinum toxin receptors represents a significant improvement as regards the known *in vivo* tests and in some embodiments offers measuring all aspects of BoNTs activity using their corresponding receptors, without facing the drawback of ethical issues in performing the known *in vivo* assays in animals.

Membrane vesicles, especially exosomes, expressing one or more BoNT receptors encompass native membrane structures collected and enriched from supernatants of cells, especially of cell lines, that have been stably transfected with nucleic acid vectors allowing the expression of the said one or more BoNT receptors.

In addition, these membrane vesicles, and especially exosomes, represent a flexible and robust material that can be stored frozen or even lyophilized, for a stable and long-term storage.

The use of such membrane vesicles expressing BoNT receptor at their membranes allowed the inventors to demonstrate, notably, that the human SYT2 isoform has a moderate

affinity for BoNT/B while other *in vitro* methods using recombinant proteins failed to detect this interaction.

These membrane vesicles, especially exosomes, expressing one or more BoNT receptors are further valuable in that they may be produced and stored in a frozen or freeze-dried form for a stable and long-term storage.

As it is shown in the examples herein, the small size of these membrane vesicles, especially exosomes, notably allows their immobilization on substrates, especially solid substrates, so as to provide sensors allowing to detect and or measure one or more events selected in a group comprising (i) the binding of a ligand, especially BoNT, to the receptors expressed at the membrane surface of the immobilized membrane vesicles, (ii) the translocation of the BoNT receptor(s) complexed with the said bound ligand(s) and (iii) especially when the ligand is a BoNT, the enzyme activity of the translocated ligand.

As it is also shown in the examples herein, the results confirm the orientation of SYT receptors at the surface of the membrane vesicles, in particular exosomes, which is thus identical to their plasma membrane orientation after vesicular fusion and therefore at the surface of said vesicles.

Accordingly, these receptors expose their BoNT binding facet.

Accordingly, the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s).

The expressions “*expose their BoNT binding facet*” and “*exposed outside of said membrane vesicles(s)*” is meant, in particular, to refer to botulinum binding sites which are directly accessible to botulinum toxin in the extra-vesicular space (i.e. accessible to the solvent in which the membrane vesicles are present and/or immobilized).

Affinity substrates onto which these membrane vesicles have been immobilized may be used as optical biosensors biochips, devices having the ability to quantify the binding of BoNT's step. This has the advantage that it can finely measure the affinity of BoNT's for their receptors by fully automated protocols.

Sequence identity

The present description encompasses nucleic acid sequences having specific percentages of nucleic acid identity, with a reference nucleic acid sequence.

For each or the amino acid sequences of interest, reference sequences are described herein. The present description also encompasses amino acid sequences having specific percentages of amino acid identity, with a reference amino acid sequence.

For obvious reasons, in all the present description, a specific nucleic acid sequence or a specific amino acid sequence which complies with, respectively, the considered nucleotide or amino acid identity, should further lead to obtaining a protein which displays the desired biological activity. As used herein, the "percentage of identity" between two nucleic acid sequences or between two amino acid sequences is determined by comparing both optimally aligned sequences through a comparison window.

The portion of the nucleotide or amino-acid sequence in the comparison window may thus include additions or deletions (for example "gaps") as compared to the reference sequence (which does not include these additions or these deletions) so as to obtain an optimal alignment between both sequences.

The identity percentage is calculated by determining the number of positions at which an identical nucleic base, or an identical amino-acid residue, can be noted for both compared sequences, then by dividing the number of positions at which identity can be observed between both nucleic bases, or between both amino-acid residues, by the total number of positions in the comparison window, then by multiplying the result by hundred to obtain the percentage of nucleotide identity between the two sequences or the percentage of amino acid identity between the two sequences.

The comparison of the sequence optimal alignment may be performed by a computer using known algorithms.

Most preferably, the sequence identity percentage is determined using the CLUSTAL W software (version 1.82) the parameters being set as follows: (1) CPU MODE=ClustalW mp; (2) ALIGNMENT="full"; (3) OUTPUT FORMAT="aln w/numbers"; (4) OUTPUT ORDER="aligned"; (5) COLOR ALIGNMENT="no"; (6) KTUP (word size)="default"; (7) WINDOW LENGTH="default"; (8) SCORE TYPE="percent"; (9) TOPDIAG="default"; (10) PAIRGAP="default"; (11) PHYLOGENETIC TREE/TREE TYPE="none"; (12) MATRIX="default"; (13) GAP OPEN="default"; (14) END GAPS="default"; (15) GAP EXTENSION="default"; (16) GAP DISTANCES="default"; (17) TREE TYPE="cladogram" and (18) TREE GRAP DISTANCES="hide".

As described herein, a nucleic acid sequence having at least 70% nucleotide identity with a reference nucleic acid sequence encompasses nucleic acid sequences having at least 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99% nucleotide identity with the said reference nucleic acid sequence.

As described herein, an amino acid sequence having at least 70% amino acid identity with a reference amino acid sequence encompasses amino acid sequences having at least 71%,

72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99% amino acid identity with the said reference amino acid sequence.

5 **Membrane vesicles, especially exosomes, expressing Botulinum toxin receptor(s)**

The present invention is based on the implementation of membrane vesicles, in particular exosome-like vesicles, and still better exosomes, expressing a botulinum toxin receptor and on their use as biological material source, notably for the quantification of biological activity of BoNT's.

10 More precisely, membrane vesicles, in particular exosome-like vesicles, and still better exosomes, expressing at their surface one or more botulinum toxin receptor(s), can be characterized in that the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s).

15 In some preferred embodiments, the botulinum toxin receptor is selected in a group comprising SV2, synaptotagmin and fragments thereof.

In some preferred embodiments, the SV2 botulinum toxin receptor is selected in a group comprising SV2A, SV2B and SV2C.

20 In some preferred embodiments, the synaptotagmin botulinum toxin receptor is selected in a group comprising SYT1 and SYT2.

25 Exosomes are in the form of small spheres bounded by a lipid bilayer. These membrane vesicles are secreted naturally by various cell types, in particular by epithelial cells, tumour cells and certain cells of the immune system, (mastocytes, T and B lymphocytes, dendritic cells, especially Langerhans cells). Therefore, exosomes are distinguished from cells in that they notably do not contain a cell nucleus and in that their size is considerably smaller than the size of a cell. What is more, exosomes are distinguished from other membrane vesicles secreted by the cells notably by their small dimensions (average diameter of 100 nm or less, preferably ranging from 30 to 200 nm, and still better from 50 to 100 nm) and by their membrane protein composition (adhesion, transport, signal transduction molecules and molecules of the major
30 histocompatibility complex, inter alia).

Exosomes might in particular correspond to internal vesicles of multivesicular endosomes (in particular late endosomes) secreted by the cell during fusion of these endosomes with the plasma membrane; multivesicular endosomes are generally involved in the transport of molecules in the lysosomal compartments (the protein degradation pathway), but in certain cells

such as reticulocytes and certain antigen-presenting cells, they may be directed to the plasma membrane with which they fuse to liberate exosomes into the extracellular medium.

The term “exosomes” as used in the present application means nanovesicles as defined above. These exosomes may be purified from culture supernatants of cells by differential centrifugation, by ultrafiltration or by adsorption onto a support or by any other method. This aspect belongs to the general knowledge of a man skilled in the art.

Exosomes are capable of exposing at their surface exogenic proteins either in the form of native full length proteins or in the form of peptides associated with MHC I and II molecules. The exposition of an exogenic protein at the surface of exosomes is similar to the exposition of the said exogenous protein at the membrane surface of a cell. However, since exosomes are neither alive nor infectious, they have the advantage of being manipulated like a normal substance without confinement precautions having to be taken, as would be the case with a mammalian cell.

The protocol/method for obtaining membrane vesicles according to the invention, in particular exosome-like vesicles, and more particularly exosomes, belongs to the general knowledge of a man skilled in the art (Petersen KE, Manangon E, Hood JL, Wickline SA, Fernandez DP, et al. (2014) *Analytical and bioanalytical chemistry* 406: 7855-7866).

A method for obtaining recombinant membrane vesicles, in particular recombinant exosome-like vesicles, and more particularly recombinant exosomes, may consist of a method such as described in WO 2009/115561 and WO 2011/036416 whose respective teaching is herein incorporated by reference.

Hence, a protocol considered for making membrane vesicles, in particular exosome-like vesicles, and more particularly exosomes, especially recombinant exosomes, according to the present invention may be based on the technology described in WO 2009/115561 or WO 2011/036416.

In some embodiments, a protocol for obtaining a membrane vesicle, in particular an exosome-like vesicle, and more particularly an exosome, expressing at its surface one or more botulinum toxin receptor(s) selected in a group comprising SV2, synaptotagmin, and fragment(s) thereof, require the surface expression of a BoNT receptor polypeptide comprising or consisting of the following domains:

(i) a polypeptide comprising the extracellular domain of a botulinum toxin receptor selected in a group comprising SV2, synaptotagmin, and fragment(s) thereof;

(ii) a membrane domain having the capacity to become anchored in the lipid bilayer of a cell membrane, which membrane domain may comprise, or may consist of, the extracellular domain(s) of the said botulinum toxin receptor, and

(iii) a cytoplasmic domain (CD) which may comprise, or which may consist of, the cytoplasmic domain of the said botulinum toxin receptor, or alternatively which may comprise, or which may consist of the cytoplasmic domain of another membrane protein.

The cytoplasmic domain, in eukaryotic cells, enables addressing said BoNT receptor polypeptide to the membrane vesicles, in particular to exosome-like vesicles, and/or to the cell compartment(s) involved in the formation of the membrane vesicles, and in particular the exosome-like vesicles, or a mutated derivative of said CD domain, the mutated domain being defined by substitution, deletion and/or insertion of one or several residue(s) in the sequence of the reference CD domain and said mutated derivative maintaining the addressing capacity of the reference CD domain.

Said BoNT receptor polypeptide is in particular capable of being secreted in association with membrane vesicles, in particular with exosomes, when it is expressed in appropriate eukaryotic cells.

The BoNT receptor polypeptide may comprise at least one membrane domain positioned between the polypeptide comprising the extracellular domain of a botulinum toxin and the CD domain or its mutated derivative.

According to a preferred embodiment, domains (i) to (iii) are positioned in succession in the following order, from the N-terminal end to the C-terminal end in the polypeptide comprising the extracellular domain of a botulinum toxin—membrane domain—CD domain or its mutated derivative.

Alternatively, domains (i) to (iii) are positioned in the following order, for example: CD domain or its mutated derivative—membrane domain—polypeptide comprising the extracellular domain of a botulinum toxin.

The expression “secreted in association with membrane vesicles, in particular with exosomes” as used in the present application means that a BoNT receptor polypeptide of the invention and/or at least one of its degradation products is secreted into the extracellular space, not in the soluble form, but in a form which is anchored in the membrane of the membrane vesicles, in particular exosomes. This anchoring is accomplished via the membrane domain(s) of said BoNT receptor polypeptide. In a BoNT receptor polypeptide that is so anchored in the membrane of a membrane vesicle (in particular an exosome), the polypeptide comprising the extracellular domain of a botulinum toxin is exposed (completely or partially) outside said membrane vesicle.

For reference, **figure 10** illustrates the opposite topologies of botulinum toxin receptors (related to their botulinum toxin (BoNT) binding site) when expressed either at the surface of exosomes, or synaptic vesicles.

According to a particular embodiment, the BoNT receptor polypeptide of the invention is produced with and is integrated into the exosomes before they leave the cell.

The secretion of a peptide or polypeptide in association with membrane vesicles (in particular exosomes) requires in particular (1) addressing said peptide or polypeptide to the location(s) for formation of membrane vesicles and in particular exosomes, and (2) vesicular budding from the membrane in which said peptide or polypeptide is anchored.

The term “addressing”, also termed “targeting”, “sorting” or “intracellular routing” as used in the present application refers to the process which allows a peptide the synthesis of which commences in the cytosol to reach the compartments involved in the budding of membrane vesicles, in particular exosome-like vesicles, and/or of reaching membrane vesicles, in particular exosome-like vesicles.

Addressing a peptide or a polypeptide to the location(s) for formation of the membrane vesicles and in particular to the location(s) for formation of exosomes may in particular require that said peptide or polypeptide should include a signal peptide for importation into the endoplasmic reticulum, so that said peptide or polypeptide can be inserted into a cell membrane, the membrane anchoring function being provided by the membrane domain.

The expression “transmembrane protein” denotes any polypeptide chain which passes entirely through a cell membrane at least once, in particular the plasma membrane of a cell.

The or at least one of the membrane domain(s) of the BoNT receptor polypeptide may in particular be that of one and the same membrane protein, or a mutated derivative of that domain. Said mutated derivative is defined by substitution, deletion and/or insertion of one or several residue(s) in the sequence of the reference membrane domain and conserves the capacity of this reference domain to become anchored in the lipid bilayer of a cell membrane. Said mutated derivative may, for example, be obtained by replacing a portion of the sequence of the reference domain by a sequence derived from the membrane domain of another membrane protein.

The term “cytoplasmic domain” (CD) as used in the present application means a particular cytoplasmic domain which is capable of being addressed to the membrane vesicles, in particular to the exosome-like vesicles, or to the cell compartment(s) involved in the formation of the membrane vesicles, and in particular the exosome-like vesicles in eukaryotic cells; this domain may thereby be secreted into the extracellular space in association with exosomes, when it is expressed in appropriate eukaryotic cells.

Preferably, the CD domain may comprise an amino acid sequence having at least 80% amino acid identity, preferably at least 90 % amino acid identity with the amino acid sequence of SEQ ID N°25).

In view of the above, said membrane vesicle(s), in particular exosomes, are particularly advantageous in view of the orientation of SYT and/or SV2 receptors at their surface which is identical to their plasma membrane orientation after vesicular fusion and therefore at the surface of exosomes these proteins expose their BoNT binding facet.

5 In this regard, an exosome according to the present invention presents BoNT receptors in the same configuration as they are at the surface of synaptosomes prepared directly from native tissues. However, beside storage flexibility and availability of exosomal preparations, this system allows the expression of a particular receptor that belongs to a particular species and most interestingly human receptors, while synaptosomes contain a mixture of several receptors (types,
10 isoforms) of the animal species from which the synaptosomes are prepared.

A membrane vesicle according to the present invention, and in particular exosome, expresses at least partially at its surface a botulinum toxin receptor selected in a group comprising SV2 and/or synaptotagmin, and fragment(s) thereof. Therefore, a membrane vesicle according to the present invention, and in particular exosomes, may be characterized as being a recombinant
15 membrane vesicle, and in particular a recombinant exosome, owing to the fact of the expressing at its surface of a recombinant botulinum toxin receptor selected in a group comprising SV2 and/or synaptotagmin, and fragment(s) thereof.

Indeed, these membrane vesicles, especially exosomes, when secreted naturally by various types of cells, do not express any botulinum toxin receptor.

BoNTs receptors

SV2 (synaptic vesicle glycoprotein 2) mediates the uptake, notably, of BoNT/A and /E, whereas SYT (synaptotagmin) is responsible for the endocytosis of BoNT/B and /G.

SYT receptors

25 Synaptotagmins (SYT) I and II (Nishiki T, Kamata Y, Nemoto Y, Omori A, Ito T, et al. (1994). J Biol Chem 269: 10498-10503.) are homologous synaptic vesicle membrane proteins thought to function as Ca^{2+} -sensors for exocytosis (Chapman ER (2002) Nature reviews Molecular cell biology 3: 498-508; Schiavo G, Stenbeck G (1998) Molecular analysis of neurotransmitter
30 release. Essays in biochemistry 33: 29-41). SYT I and II were reported to bind BoNT/B in the presence of gangliosides (Nishiki T, Tokuyama Y, Kamata Y, Nemoto Y, Yoshida A, et al. (1996) FEBS letters 378: 253-257). BoNT/A and E have also been reported to bind SYT I, albeit in a ganglioside independent manner (Li L, Singh BR (1998) Journal of natural toxins 7: 215-226).

It is known in the art that the function and amino acid sequences of SYT I and II are
35 conserved across animal *species*. Although the disclosure here is based on the rat and human SYT

receptors, the present invention applies to SYT receptors of all animal *species* that have conserved SYT I or II binding domains for BoNTs and/or ganglioside.

In the context of the present invention, the receptor synaptotagmin is selected from a group comprising SYT 1, SYT 2, a fragment thereof, and a combination thereof.

5 In some embodiments of a synaptotagmin receptor, within the meaning of the present invention, the BoNT receptor polypeptide comprises the extracellular domain of the said synaptotagmin receptor, and more particularly the region of the considered synaptotagmin receptor involved in the binding with the BoNTs, the said extracellular domain being fused to at least one transmembrane polypeptide allowing the anchoring of the synaptotagmin BoNT receptor
10 in the lipid bilayer of the membrane vesicles, especially the lipid bilayer of the exosomes. The transmembrane domain may originate from a polypeptide distinct from the natural transmembrane domain of the said synaptotagmin receptor.

In some embodiments, the SYT receptor is identical to the corresponding native receptor and thus comprises the extracellular domain, the transmembrane domain and the cytoplasmic
15 domain of the native SYT receptor.

In some embodiments, the SYT receptor comprises an amino acid sequence having at least 70% amino acid identity, preferably at least 80 % amino acid identity, and still better at least 90% amino acid identity, with an amino acid sequence selected from the group comprising SEQ ID N° 14 (rat SYT1), SEQ ID N° 17 (human SYT1), SEQ ID N° 20 (rat SYT2), SEQ ID N° 23
20 (human SYT2), and combination thereof.

Also, a fragment of a SYT receptor according to the present invention comprises an amino acid sequence having at least 70% amino acid identity, preferably at least 80 % amino acid identity, and still better at least 90 % amino acid identity, with an amino acid sequence selected from the group comprising SEQ ID N° 15 (comprising the extra-cellular domain of rat SYT1),
25 SEQ ID N° 18 (comprising the extra-cellular domain of human SYT1), SEQ ID N° 21 (comprising the extra-cellular domain of rat SYT2), SEQ ID N° 24 (comprising the extra-cellular domain of human SYT2), and combination thereof.

In some embodiments, the SYT receptor comprises (i) an extracellular domain having a high amino acid identity with the extracellular domain of a native SYT receptor and (ii) a
30 transmembrane domain and a cytoplasmic domain having a lower identity with the transmembrane domain and the cytoplasmic domain of a native SYT receptor.

According to these embodiments, the SYT receptor may possess 70% or more amino acid identity with a reference native SYT receptor, provided that the extracellular domain of the said SYT receptor possesses a high amino acid identity with the extracellular domain of the

reference native SYT receptor, e.g. 90% or more amino acid identity with the extracellular domain of the reference native SYT receptor.

SYT receptors according to the present invention encompass polypeptides selected in a group comprising:

- 5 - a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 14 and comprising a polypeptide having at least 90% amino acid identity with the polypeptide of SEQ ID N° 15,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 17 and comprising a polypeptide having at least 90% amino acid identity with the polypeptide of
- 10 SEQ ID N° 18,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 20 and comprising a polypeptide having at least 90% amino acid identity with the polypeptide of SEQ ID N° 21,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 23
- 15 and comprising a polypeptide having at least 90% amino acid identity with the polypeptide of SEQ ID N° 24,

SYT receptors according to the present invention also encompass polypeptides selected in a group comprising:

- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 14
- 20 and comprising the polypeptide of SEQ ID N° 15,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 17 and comprising the polypeptide of SEQ ID N° 18,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 20 and comprising the polypeptide of SEQ ID N° 21,
- 25 - a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 23 and comprising the polypeptide of SEQ ID N° 24,

For obvious reasons, a specific amino acid sequence of a synaptotagmin receptor or a fragment thereof has to lead to obtaining a protein which displays the desired biological activity, notably the capacity of bonding to one or more botulinum toxin.

30

SV2 receptors

SV2 is a highly glycosylated integral membrane protein (Buckley K, Kelly RB (1985) The Journal of cell biology 100: 1284-1294.). Sequence analysis revealed that the protein is highly homologous to a group of 12 transmembrane domain prokaryotic and eukaryotic

35 transporters including the human glucose transporter (Bajjalieh SM, Peterson K, Shinghal R,

Scheller RH (1992) Science 257: 1271-1273; Feany MB, Lee S, Edwards RH, Buckley KM (1992) Cell 70: 861-867; Gingrich JA, Andersen PH, Tiberi M, el Mestikawy S, Jorgensen PN, et al. (1992) FEBS letters 312: 115-122). It seems likely that SV2 functions as a transporter, although its substrate(s) has not been identified, and additional channel or enzymatic functions have not been excluded.

The SV2 receptor comprises three isoforms in mammals (i.e. SV2A, B and C). SV2 contains twelve transmembrane domains with one large luminal domain (the fourth luminal domain, L4) between the seventh and eighth transmembrane domains. SV2 is a proteoglycan on synaptic vesicles and is heavily glycosylated, possibly through three putative N-glycosylation sites within the L4 luminal domain. It is also suggested that SV2 mediates the uptake of BoNT/D and /F, and tetanus toxin.

In the context of the present invention, the receptor SV2 is selected from a group comprising SV2A, SV2B, SV2C, or a fragment thereof, and a combination thereof.

In some embodiments of a SV2 receptor, within the meaning of the present invention, the BoNT receptor polypeptide comprises the extracellular domain of the said SV2 receptor, and more particularly the region of the considered SV2 receptor involved in the binding with the BoNTs, the said extracellular domain being fused to at least one transmembrane polypeptide allowing the anchoring of the SV2 BoNT receptor in the lipid bilayer of the membrane vesicles, especially the lipid bilayer of the exosomes. The transmembrane domain may originate from a polypeptide distinct from the natural transmembrane domain of the said SV2 receptor.

In some embodiments, the receptor SV2 is identical to the corresponding native receptor and thus comprises the extracellular domain, the transmembrane domain and the cytoplasmic domain of the native SV2 receptor.

In some embodiments, the receptor SV2 may comprise an amino acid sequence having at least 70% amino acid identity, preferably at least 80 % amino acid identity, and still better at least 90% amino acid identity, with an amino acid sequence selected from the group comprising SEQ ID N° 2 (human SV2A), SEQ ID N° 5 (SV2A comprising a T7 Tag peptide), SEQ ID N° 8 (SV2B), SEQ ID N° 11 (SV2C) and combination thereof.

Also, a fragment of a receptor SV2 according to the present invention may comprise an amino acid sequence having at least 70% amino acid identity, preferably at least 80 % amino acid identity, and still better at least 90 % amino acid identity, with an amino acid sequence selected from the group comprising SEQ ID N° 3 (comprising the extra-cellular domain of human SV2A), SEQ ID N° 6 (SV2A comprising the extracellular domain of the embodiment of human SV2A comprising a T7 Tag peptide), SEQ ID N° 9 (comprising the extra-cellular domain of human

SV2B), SEQ ID N° 12 (comprising the extra-cellular domain of human SV2C), and combination thereof.

In some embodiments, the SV2 receptor comprises (i) an extracellular domain having a high amino acid identity with the extracellular domain of a native SV2 receptor and (ii) a transmembrane domain and a cytoplasmic domain having a lower identity with the transmembrane domain and the cytoplasmic domain of a native SV2 receptor.

According to these embodiments, the SV2 receptor may possess 70% or more amino acid identity with a reference native SV2 receptor, provided that the extracellular domain of the said SV2 receptor possesses a high amino acid identity with the extracellular domain of the reference native SV2 receptor, e.g. 90% or more amino acid identity with the extracellular domain of the reference native SV2 receptor.

SV2 receptors according to the present invention encompass polypeptides selected in a group comprising:

- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 2 and comprising a polypeptide having at least 90% amino acid identity with the polypeptide of SEQ ID N° 3,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 5 and comprising a polypeptide having at least 90% amino acid identity with the polypeptide of SEQ ID N° 6,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 8 and comprising a polypeptide having at least 90% amino acid identity with the polypeptide of SEQ ID N° 9,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 11 and comprising a polypeptide having at least 90% amino acid identity with the polypeptide of SEQ ID N° 12,

SV2 receptors according to the present invention also encompass polypeptides selected in a group comprising:

- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 2 and comprising the polypeptide of SEQ ID N° 3,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 5 and comprising the polypeptide of SEQ ID N° 6,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 8 and comprising the polypeptide of SEQ ID N° 9,
- a polypeptide having at least 70% amino acid identity with the polypeptide of SEQ ID N° 11 and comprising the polypeptide of SEQ ID N° 12,

For obvious reasons, a specific amino acid sequence of a SV2 receptor or a fragment thereof has to lead to obtaining a protein which displays the desired biological activity, notably the capacity of bonding to one or more botulinum toxin.

According to a particular embodiment, the BoNTs receptors SV2 and/or SYT intended
5 to be expressed at least partially at the surface of exosomes may comprise one or more epitope tag(s) or labeling molecule(s), such as hereinafter defined.

An epitope tag is useful for the labelling and detection of proteins using notably immunoblotting, immunoprecipitation, and immunostaining techniques. Because of their small size, they are unlikely to affect the tagged protein's biochemical properties.

10 In the context of the present invention, said epitope tag or labeling molecule is preferably inserted in an extra-exosomal region of the considered BoNTs receptors SV2 and/or SYT.

In some embodiments wherein the membrane vesicles, especially exosomes, express an SV2 receptor the SV2 polypeptide most preferably comprises an epitope tag, e.g. a T7 tag, which
15 has been inserted therein, so as to facilitate the capture of SV2-containing exosomes.

In some embodiments wherein the membrane vesicles, especially exosomes, express a SYT receptor the SYT polypeptide most preferably comprises an epitope tag, e.g. a T7 tag, which has been inserted therein, so as to facilitate the capture of SYT-containing exosomes.

In some embodiments wherein the membrane vesicles, especially exosomes, express an
20 SV2 receptor and does not express a SYT receptor, the SV2 polypeptide most preferably comprise an epitope tag, e.g. a T7 tag, which has been inserted therein, so as to facilitate the capture of SV2-containing exosomes.

In some embodiments wherein the membrane vesicles, especially exosomes, express a
25 SYT receptor and does not express a SV2 receptor, the SYT polypeptide most preferably comprise an epitope tag, e.g. a T7 tag, which has been inserted therein, so as to facilitate the capture of SYT-containing exosomes.

Irrespective of the BoNT receptor which is concerned, the tag epitope, e.g. the T7 tag is most preferably inserted outside the extracellular domain of the said BoNT receptor, and is most preferably inserted in the cytoplasmic domain of the said BoNT receptor, as illustrated notably by
30 the human SV2A receptor comprising a T7 tag peptide within its cytoplasmic domain which is referred herein as SEQ ID NO.5.

The T7 tag peptide is referred herein as SEQ ID NO.26.

Botulinum toxins (BoNTs)

A botulinum toxin according to the present invention may be selected in a group comprising botulinum toxin type A, botulinum toxin type B, botulinum toxin type C, botulinum toxin type D, botulinum toxin type E, botulinum toxin type F, botulinum toxin type G, botulinum toxin type H and combinations thereof.

Preferably, the botulinum toxin may be selected in a group comprising botulinum neurotoxin type A, botulinum neurotoxin type B, botulinum neurotoxin type E and combinations thereof.

According to a particular embodiment, a botulinum toxin may be also represented by a fragment thereof or a hybrid thereof, or a chemically-modified botulinum toxin.

In the context of the present invention, said fragment and hybrid of botulinum toxins and said chemically-modified botulinum toxin are such that the property of binding with one or more botulinum receptor(s) selected in a group comprising SV2 and/or synaptotagmin (SYT), or fragment(s) thereof, is identical or at least similar to the corresponding native botulinum toxin.

Preferably, a fragment of a botulinum toxin may be characterized by the heavy chain of the considered botulinum toxin.

Preferably, a hybrid of a botulinum toxin may represent recombinant toxin composed of parts of different BoNTs isoforms (Pickett 2011, Dolly 2011).

Preferably, a chemically-modified botulinum toxin (or chimera) may represent a botulinum toxin or a fragment thereof further comprising at least one labeling molecule such as a fluorescent dye, a fluorophore or a fluorescent derivative binding tag.

This variant of realization may be useful to detect the presence, and the case arising the behavior, of a biomolecule of interest in a biological system.

All method implementing such a labeling molecule may be used herein. The most commonly labelled molecules are antibodies, proteins, amino acids and peptides, which are then, used as specific probes for detection of a particular target. In particular, the labeling molecule may be a heavy isotope labeling, a radioactive isotope labeling or a fluorescent labeling molecule (or a fluorescent tag).

Fluorescent labeling is known for its non-destructive nature and high sensitivity. This has made it one of the most widely used methods for labeling and tracking biomolecules. Several techniques of fluorescent labeling can be utilized depending on the nature of the target, including notably the enzymatic labeling (i.e. fluorescein or biotin), the chemical labeling and the protein labeling (i.e. biarsenical tags, Histidine tags, and FLAG tags).

AFFINITY SUBSTRATE

The present invention relates to an affinity substrate comprising a solid substrate material onto which is/are at least partially immobilized one or more membrane vesicles, in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising a SV2 receptor, a synaptotagmin receptor, and fragment(s) thereof.

In some embodiments, the membrane vesicles are bound on the solid substrate material by non-covalent bonds, which include hydrophobic interactions between the said exosomes and the said solid substrate. Illustratively, the inventors have shown that the exosomes expressing one or more botulinum toxin receptors as described herein may be non-covalently immobilized on a hydrophobic solid substrate such as a polystyrene or polyethylene substrate. Illustratively, the membrane vesicles expressing one or more botulinum toxin receptors as described herein may be non-covalently immobilized on the solid substrate of microtiter plates which are conventionally used for performing ELISA assays.

In some other embodiments, the solid substrate material is at least partially coated with one or more ligand(s) and the said membrane vesicle(s) is/are immobilized onto the said solid substrate by binding to the said ligand(s).

According to a first variant, said ligand(s) consist of antibodies directed against the botulinum toxin receptor(s) present at the surface of the membrane vesicles.

According to a second variant, said ligand(s) consist of antibodies directed against proteins present at the surface of the membrane vesicles but which are distinct from the botulinum toxin receptor(s). For example, such antibodies may be antibodies directed against an exosomal protein marker selected in a group comprising CD63, CD9, CD81 or Hsp70.

In particular, the solid substrate material may be selected in a group comprising resins, polymer beads, magnetic beads, paramagnetic beads, ELISA plates, substrate materials of filter membranes, substrate materials of sensor chips and polymer materials.

In an affinity substrate according to the invention, the receptor(s) SV2 and/or synaptotagmin is/are such as above defined.

According to a particular variant, in an affinity substrate according to the invention, the said botulinum toxin receptor(s) may be complexed with one or more botulinum toxin(s), a fragment thereof, a hybrid thereof, or a chemically-modified botulinum toxin. Modified botulinum toxins are notably those disclosed by Pickett and Perrow (2011, Toxins, Vol. 3: 63-81).

According to another variant, in an affinity substrate according to the invention, said membrane vesicles may further comprise ganglioside molecule(s) such as above defined.

Gangliosides may be selected in a group comprising GD1a, GD1b, GT1b, GQ1b, GM1a and GD3, which are well known from the one skilled in the art.

For SV2 receptors, gangliosides selected in a group comprising GD1a, GD1b, GT1b and GQ1b are preferred.

5 In some embodiments, the ganglioside molecule is selected from a group comprising aNeu5Ac(2-3)bDGalp(1-3)bDGalNAc(1-4)[aNeu5Ac(2-8)aNeu5Ac(2-3)]bDGalp(1-4)bDGlc(1-1)Cer (GT1b), aNeu5Ac(2-3)bDGalp(1-3)bDGalNAc(1-4)[aNeu5Ac(2-3)]bDGalp(1-4)bDGlc(1-1)Cer (GD1a), bDGalp(1-3)bDGalNAc(1-4)[aNeu5Ac(2-8)aNeu5Ac(2-3)]bDGalp(1-4)bDGlc(1-1)Cer (GD1b), and a mixture thereof.

10 In an affinity substrate as described herein, the said membrane vesicle(s), especially the said exosome(s), has/have an average diameter of 100 nm or less, and preferably has/have an average diameter ranging from 30 to 200 nm, and still better from 50 to 100 nm.

COMPOSITION COMPRISING EXOSOMES EXPRESSING BOTULINUM

15 TOXINS RECEPTORS

The present invention relates to a composition comprising membrane vesicles, in particular exosome-like vesicles, and still better exosomes, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2, synaptotagmin, and fragment(s) thereof.

20 Preferably, a composition according to the invention may be a suspension, a solution or an emulsion, and preferably a suspension.

Preferably, a composition according to the invention may further comprise one or more formulatary agent(s), notably such as suspending, stabilizing and/or dispersing agents.

25 Preferably, a composition according to the invention may further comprise one or more ganglioside molecule(s) such as above defined.

Preferably, a composition according to the invention may be is in a frozen or a lyophilized form.

COMPLEX BETWEEN EXOSOMES EXPRESSING BOTULINUM TOXIN

30 RECEPTORS AND RECEPTOR LIGANDS

In view of the above, the present invention also relates to a complex between (i) an membrane vesicles, in particular exosome-like vesicles, such as above defined and (ii) one or more receptor ligands, and especially botulinum neurotoxin(s) such as above defined.

35 According to a particular variant, in such a complex, said membrane vesicles may further comprise ganglioside molecule(s), such as above defined.

KIT

In view of the above, the present invention further relates to a kit, notably for assessing one or more biological activity(ies) of a compound, the said kit comprising:

- one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more receptor(s) selected in a group comprising SV2, synaptotagmin, or fragment(s) thereof,

- optionally, at least one solid substrate material at least partially coated with ligand(s) capable of binding to said membrane vesicles, and preferably to one or more botulinum toxin receptor(s) selected in a group comprising SV2, synaptotagmin, or fragment(s) thereof, and

- optionally, a composition comprising one or more ganglioside molecule(s).

According to a particular variant, said membrane vesicle(s) may be in the form of a composition of exosomes expressing one or more botulinum toxin receptors such as above-defined. When said membrane vesicles are in the form of a composition such as above-defined in a kit according to the present invention, then said kit does not comprise the above-mentioned solid substrate material.

METHOD FOR DETERMINING A BIOLOGICAL ACTIVITY OF BOTULINUM TOXINS

The present invention further relates to a method for *in vitro* detecting and/or measuring a biological activity of one or more botulinum toxin(s), said method comprising the steps of:

a) providing one or more membrane vesicles, in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2, synaptotagmin, or fragment(s) thereof,

b) contacting the membrane vesicles provided at step a) with one or more botulinum toxin(s), optionally in presence of ganglioside molecule(s), whereby an assay mixture is provided, and

c) detecting and/or measuring a biological activity of said botulinum neurotoxin(s).

More particularly, said biological activity is selected from a group comprising:

(i) the binding of said botulinum toxin(s) with said receptor(s) SV2 and/or synaptotagmin, or fragment(s) thereof,

(ii) the translocation of said botulinum toxin(s) within said membrane vesicles, and/or

(iii) the enzyme activity of said botulinum toxin(s) with one or more enzyme substrate(s) capable of reacting with said botulinum toxin(s).

(i) binding of said botulinum toxin(s) with said receptor(s) SV2 and/or synaptotagmin, or fragment(s) thereof

Any method known to one of ordinary skill in the art for measuring protein-protein interaction can be used to measure the binding between BoNTs and the receptor(s) SV2 and/or synaptotagmin, or fragment(s) thereof expressed in exosomes. For example, ELISA assays, co-immunoprecipitation and affinity columns are commonly used methods. Another method that can be used is surface plasmon resonance (SPR) measurements. SPR uses changes in refractive index to quantify binding and dissociation of macromolecules to ligands covalently linked onto a thin gold chip within a micro flow cell. This technique has been used to study protein-protein interactions in many systems, including the interactions of analytes with immobilized synaptic vesicles (Ferracci et al., Anal.Biochem., 2004, 334, 367-375).

It provides high sensitivity and accuracy, the ability to observe binding and release in real time, and consumption of only minute quantities of protein. Besides, the equilibrium dissociation constant (K_d), on- and off-rate constants (k_a and k_d) may also be measured, according to any of the methods which are well known to the one skilled in the art.

(ii) the translocation of said botulinum toxin(s) within said membrane vesicles, and/or

Exosomes expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof may be also used to monitor the translocation step after pre-incubation with BoNTs and acidification.

On neuronal membranes, after binding of the Botulinum toxins to their receptors, the toxin/receptor complex is endocytosed and the toxin is trapped in the luminal part of endocytotic vesicles. The physiological acidification of these vesicles trigger a conformational change of the toxin and the heavy chain is thought to form a translocation channel through the membrane that allows the light chain to escape towards the cytoplasm where it can exert its proteolytic activity on its specific presynaptic targets. After the binding of BoNTs to the exosomes-like structures, acidification should induce the desired conformational change and light chain translocation.

Any method known to one of ordinary skill in the art permitting the quantification of BoNT associated with exosomes in experimental conditions allowing BoNT to translocate into exosomes for example following medium acidification. In particular methods permitting to discriminate BoNT associated with the extracellular domain of SYT or SV2 and BoNT that penetrated partially or totally the lumen of the exosome. After BoNT binding and acidification, the exosomal preparation can be treated with proteases as "proteinase K" in order to eliminate extraexosomal proteins. Partially or totally inserted light chain will therefore be protected from proteolytic activity can then be revealed for example by its enzymatic activity or by mass

spectrometry. Also, exosomes bearing BoNT receptors can be fused with planar bilayer and used to monitor (using electrophysiological methods for example) acidic pH induced pore formation properties of BoNTs.

- 5 (iii) the enzyme activity of said botulinum toxin(s) with one or more enzyme substrate(s) capable of reacting with said botulinum toxin(s)

Exosomes expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof may be also used to monitor the enzyme activity of said botulinum toxin(s) with one or more enzyme substrate(s)
10 capable of reacting with said botulinum toxin(s).

According to a first variant, this step is carried out with membrane vesicles of the invention, especially exosomes, wherein one or more enzyme substrate(s) is/are present within said membrane vesicles.

In such a variant, BoNT substrates, such as for example VAMP and/or SNAP25, may be
15 co-addressed with BoNT receptors in the same membrane vesicles and notably in using the same technology as described in WO 2009/115561 and WO 2011/036416.

Alternatively, the BoNT substrates may be characterized by a compound which is naturally fluorescent, this fluorescence being lost when said compound is cleaved by one or more BoNTs, or conversely.

20 An example of such a substrate could be the use of an opposite technology to the known fluorescent complementation (Ghosh A, Singh A, Ramteke PW, Singh VP (2000) Biochemical and biophysical research communications 272: 6-11; Hu CD, Kerppola TK (2003). Nat Biotechnol 21: 539-545). or FRET-based methods to detect substrate cleavage using engineered GFP or YFP molecules containing BoNTs target cleavage sequences (Basavanna U, Muruvanda
25 T, Brown EW, Sharma SK (2013) International journal of microbiology 2013: 593219; Ruge DR, Dunning FM, Piazza TM, Molles BE, Adler M, et al. (2011). Anal Biochem 411: 200-209).

According to a second variant, this step involve an additional step d) comprising lysing the membrane vesicles of the assay mixture provided at the above-mentioned step b), and then
30 bringing the resulting lysate into contact with the enzyme substrate(s).

This variant may implement substrates and devices described in Leveque, C. et al. (Leveque C. et al. Appl Microbiol Biotechnol (2015); Leveque, C. et al. Biosens Bioelectron 57, 207-12 (2014); Leveque, C. et al. Biosens Bioelectron 49, 276-81 (2013) or Ferracci, G. et al. Anal Biochem 410, 281-8 (2011)).

Besides, a method such as above-defined may be notably particularly interesting for assessing the properties of fragments of botulinum toxins, of hybrids of botulinum toxins or of *chemically-modified botulinum toxin*.

In view of the above, the present invention also encompasses the use of one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof, optionally in presence of ganglioside molecule(s), for *in vitro* detecting and/or measuring one or more biological activity(ies) of one or more botulinum toxin(s) in a sample comprising said botulinum toxin(s).

METHOD FOR DETECTING THE PRESENCE OF BoNT's

The present invention further relates to a method for *in vitro* detecting the presence of one or more botulinum toxin(s), said method comprising the steps of:

- providing one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more botulinum receptor(s) selected in a group comprising SV2 and/or synaptotagmin (SYT), or fragment(s) thereof,
- bringing a sample comprising, or suspected of comprising, said botulinum neurotoxin(s) into contact with said membrane vesicle(s), optionally in presence of ganglioside molecule(s), and
- detecting the formation of complexes between (i) said membrane vesicle(s) and (ii) said botulinum neurotoxin(s).

Said detection may be carried out with all method well known of a man skilled the art and notably with the methods previously cited.

In view of the above, the present invention also encompasses the use of one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof, optionally in presence of ganglioside molecule(s), for *in vitro* detecting the presence of one or more botulinum neurotoxin(s) in a sample comprising, or suspected of comprising, said botulinum toxin(s).

METHOD FOR SCREENING COMPOUNDS WITH RESPECT TO BoNT RECEPTOR

The present invention further relates to a method for *in vitro* screening one or more candidate compounds different from a botulinum toxin binding to one or more botulinum toxin receptor(s), said method comprising the steps of:

- providing one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof,

- bringing a sample comprising one or more candidate compound(s) into contact with said membrane vesicle(s), optionally in presence of ganglioside molecule(s), and

- detecting and/or measuring the binding of said candidate compound(s) with said receptor(s).

Appropriate detection and/or measurement methods belong to the general knowledge of the man skilled the art, and are notably previously cited.

This method according to the present invention is also advantageous in that it further allows *in vitro* screening compounds other than botulinum toxins.

In particular, it is known that SV2 is the receptor for Levetiracetam (LEV), an antiepileptic drug (Lynch, B.A. et al. Proceedings of the National Academy of Sciences of the United States of America 101, 9861-9866 (2004)).

Exosomes according to the present invention may thus be implemented in the above-mentioned method for measuring the LEV binding but also for screening libraries of compounds in order to identify new antiepileptic drugs.

In particular, said candidate compound may be an antibody.

METHOD FOR SCREENING COMPOUNDS WITH RESPECT TO BoNT

The present invention further relates to a method for *in vitro* screening one or more candidate compounds binding to one or more botulinum toxin(s), said method comprising the steps of:

- providing one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof,

- bringing a sample comprising one or more candidate compound(s) and one or more botulinum toxin(s) into contact with said membrane vesicle(s), optionally in presence of ganglioside molecule(s), and

- detecting and/or measuring the binding of said botulinum toxin(s) with said receptor(s).

Appropriate detection and/or measurement methods belong to the general knowledge of the man skilled the art, and are notably previously cited.

This method is notably appropriate for *in vitro* screening inhibitors of botulinum toxin(s).

In particular, said candidate compound may be an antibody.

In view of the above, the present invention also encompasses the use of one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof, optionally in presence of ganglioside molecule(s), for *in vitro* screening one or more candidate compounds binding to one or more botulinum toxin receptor(s).

METHOD FOR MONITORING THE LEVEL OF ANTIBODIES ANTI-BoNT's

This invention also relates to a method for *in vitro* monitoring the level of antibodies directed against one or more botulinum neurotoxin(s) in a subject, preferably a mammal, said method comprising the steps of:

- providing blood sample of a subject previously treated with one or more botulinum neurotoxin(s),
- bringing said sample into contact with a botulinum toxin,
- measuring the binding of the said botulinum toxin to membrane vesicles expressing one or more botulinum receptors as described herein; especially to one or more exosomes(s) expressing at their surface one or more receptor(s) SV2 and/or synaptotagmin, or fragment(s) thereof, optionally in presence of one or more ganglioside(s), and
- determining the presence or absence of an inhibition of the binding of the said botulinum toxin to the said membrane vesicles, especially to the said exosomes.

This invention also relates to a method for *in vitro* monitoring the level of antibodies directed against the receptor SV2 or the receptor complex SYT/SV2 in a subject, preferably a mammal, said method comprising at least the steps consisting in:

- providing blood sample of a subject,
- bringing said sample into contact with one or more recombinant exosomes(s) expressing at their surface one or more receptor(s) SV2 and/or synaptotagmin, or fragment(s) thereof, optionally in presence of one or more ganglioside(s), and
- detecting the presence or the absence of said antibodies associated with exosomes.

The amount of exosome bound auto-antibodies can be followed by labelling exosomes by classical methods as fluorescence, radioactivity...

This invention further relates to a method for *in vitro* monitoring the innocuity of a vaccine against a botulinum toxin using denaturated toxin preparations, said method comprising at least the steps consisting of:

- providing a sample comprising one or more denaturated botulinum neurotoxin(s),

- bringing said sample into contact with one or more exosomes(s) expressing at their surface one or more receptor(s) SV2 and/or synaptotagmin, or fragment(s) thereof, optionally in presence of one or more ganglioside(s), and

- detecting the presence or the absence of binding between the receptor(s) SV2 and/or synaptotagmin, or fragment(s) thereof, and said denatured BoNT.

Throughout the description, including the claims, the expression "comprising a" should be understood as being synonymous with "comprising at least one", unless otherwise specified.

The terms "between... and..." and "ranging from... to..." should be understood as being inclusive of the limits, unless otherwise specified.

The examples and figures which follow are presented by way of illustration and without implied limitation of the invention.

Table 1: Sequences

<u>SEQ ID NO</u>	<u>Type</u>	<u>Description</u>
<u>1</u>	<u>nucleic acid</u>	<u>human SV2A</u>
<u>2</u>	<u>amino acid</u>	<u>human SV2A</u>
<u>3</u>	<u>amino acid</u>	<u>human SV2A extracellular loop 4 domain and adjacent transmembrane domains delimiting this loop</u>
<u>4</u>	<u>nucleic acid</u>	<u>human SV2A comprising a T7 Tag peptide</u>
<u>5</u>	<u>amino acid</u>	<u>human SV2A comprising a T7 Tag peptide</u>
<u>6</u>	<u>amino acid</u>	<u>human SV2A (comprising a T7 Tag peptide) extracellular loop 4 domain and adjacent transmembrane domains delimiting this loop</u>
<u>7</u>	<u>nucleic acid</u>	<u>human SV2B</u>
<u>8</u>	<u>amino acid</u>	<u>human SV2B</u>
<u>9</u>	<u>amino acid</u>	<u>human SV2B extracellular loop 4 domain and adjacent transmembrane domains delimiting this loop</u>
<u>10</u>	<u>nucleic acid</u>	<u>human SV2C</u>
<u>11</u>	<u>amino acid</u>	<u>human SV2C</u>
<u>12</u>	<u>amino acid</u>	<u>human SV2C extracellular loop 4 domain and adjacent transmembrane domains delimiting this loop</u>
<u>13</u>	<u>nucleic acid</u>	<u>rat SYT1</u>

<u>SEQ ID NO</u>	<u>Type</u>	<u>Description</u>
<u>14</u>	<u>amino acid</u>	<u>rat SYT1</u>
<u>15</u>	<u>amino acid</u>	<u>rat SYT1 extracellular domain and transmembrane domain</u>
<u>16</u>	<u>nucleic acid</u>	<u>human SYT1</u>
<u>17</u>	<u>amino acid</u>	<u>human SYT1</u>
<u>18</u>	<u>amino acid</u>	<u>human SYT1 extracellular domain and transmembrane domain</u>
<u>19</u>	<u>nucleic acid</u>	<u>rat SYT2</u>
<u>20</u>	<u>amino acid</u>	<u>rat SYT2</u>
<u>21</u>	<u>amino acid</u>	<u>rat SYT2 extracellular domain and transmembrane domain</u>
<u>22</u>	<u>nucleic acid</u>	<u>human SYT2</u>
<u>23</u>	<u>amino acid</u>	<u>human SYT2</u>
<u>24</u>	<u>amino acid</u>	<u>human SYT2 extracellular domain and transmembrane domain</u>
<u>25</u>	<u>amino acid</u>	<u>exosome targeting peptide</u>
<u>26</u>	<u>amino acid</u>	<u>T7 Tag peptide</u>
<u>27</u>	<u>nucleic acid</u>	<u>BoNT receptor recipient expression vector</u>

EXAMPLES

Material and methods

Recombinant exosomes: the protocol considered for making exosomes according to the present invention is based on the technology described in WO 2009/115561 or WO 2001/036416, with any adequate adaptations

In this regard, the signal peptide implemented is the one represented by the amino acid sequence SEQ ID N° 25.

The nature of the considered SV2 and/or SYT receptor(s) is specified in the hereinafter examples. The corresponding amino acid sequence is also defined in the sequence listing.

1. Exosomes preparations : exosomes expressing SV2 and/or SYT, optionally further tagged with an epitope tag such the T7 Tag Peptide (see amino acid sequences SEQ ID N° 2, 5, 14,17), were produced from stably transfected HEK cell lines.

In the hereinafter described examples, human SV2 tagged with the T7 Tag Peptide (hSV2A), rat synaptotagmin2 (rSYT2) and rat synaptotagmin 1 (rSYT1) receptors are more particularly considered. The corresponding amino acid sequences are displayed in the hereinafter listing sequences.

5 Exosomes expressing human synaptotagmin 2 (hSYT2) were obtained from transient expression in HEK.

When the BoNT receptor is the SV2 receptor, the above-mentioned T7 Tag Peptide, when it is present, is inserted as illustrated by the amino acid sequence SEQ ID N°5).

10 **2. Exosomes purification:** 8L of culture medium of cells producing exosomes are clarified by:

- a centrifugation at 2000 rpm (Jouan) for 10 min at 4 ° C;
- a centrifugation at 10 000 rpm (JA14 rotor, Beckman) for 15 min at 4 ° C;
- a filtration 0.22µm filters (Millipore TM Stericup filtration system, polyethersulfone membrane).

15 The clarified medium is subjected to a step of ultrafiltration on a membrane of cut-off of 300kDa (Sartorius Stedim system), until a final volume of about 40ml, which is then dialyzed by diafiltration with 2 liters of PBS.

The concentrate obtained is ultra-centrifuged for 2 h at 24000rpm (100000 g) in tubes of 12ml of a SW 41 rotor (Beckman) per fractions of 10 ml deposited on a double cushion of sucrose
20 5% in D2O (400µl) + sucrose 30% in D2O (800µl).

After ultracentrifugation, the cushion of sucrose at 30% and the lower half of the cushion at 5% are recovered. The different samples are combined and then subjected to 2 successive dialysis against 1L buffer (PBS 1X; BSA 10µg/ml; 5% (ww/v) sucrose) overnight at 4 ° C.

The sample is then concentrated by centrifugation on Macrosep 100K membrane
25 (Sartorius) until a volume less than 1 ml.

The sample is subjected to an exclusion chromatography (1000 kDa). The excluded collected fraction contains exosomes, it is collected.

This final fraction of about 3 ml corresponds to the name "exosome preparations" which will be used in all hereinafter described experiments (ELISA, SPR ...).

30

3. Western blot: exosomes of the present invention (1-5 µg of total proteins) were denatured in Laemmli buffer in presence of reductant (10 mM Dithiothreitol). Samples were loaded on a 10% SDS-PAGE and transferred to a nitrocellulose membrane at 100 V during 1 hour. After incubation in TBS (25 mM Tris/NaOH pH 7.4, 150 mM NaCl) containing 5% nonfat
35 milk, membranes were washed in washing buffer (TBS tween 0.005%) and incubated with

antibodies against the N-terminal domain (1-20 aa) of SYT2 (8G2B MBL) or antibodies against the cytosolic part of SV2A (from Developmental Studies Hybridoma Bank, Ref AB 2315387).

Membranes were washed again four times for 15 min and incubated with a 1:10000 dilution of horseradish peroxidase-conjugated anti-mouse antibodies (*Jackson ImmunoResearch Europe Ltd*, Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) ; 115-035-166 for 1 h. Blots were washed with washing buffer four times and developed with the ECL system (Pierce or Amersham Biosciences) according to the manufacturer's indications.

4. Surface Plasmon Resonance (SPR) measurements: SPR detection is based on an optical phenomenon that reports changes in bound mass at the surface of a sensor chip that was functionalized in the present invention with antibodies. A plot of the SPR signal against time is called sensorgram. Experiments were performed at 25° C on a Biacore 3000 biosensor using HEPES buffered saline (HBS: 150 mM NaCl, 10 mM HEPES / NaOH pH 7.4) as running buffer. Anti-rat synaptotagmin 2 monoclonal antibody (5B7A obtained by collaboration with Masami Takahashi, Japan) was amine coupled at pH 5 to a CM3 sensor chip (GE Healthcare) according to the manufacturer's instructions. Recombinant exosomes expressing the SYT and/or SV2 receptor(s), and notably rSYT2, hSYT2, rSYT2 and/or hSV2A were loaded (0.2-0.4 mg/ml during about 30 min) at 1 µl/min flow rate. The amount of exosomes captured on the chip was monitored and injection stopped when 2000-4000 Resonance Units (RU) of exosomes were bound. Control flow cell was functionalized or not with recombinant exosomes transfected with non-relevant receptor. GT1b (4.5 µM) diluted in running buffer was injected or not at 10 µl/min during 2 minutes leading to an increment of signal. BoNT/B was injected and wet monitored the binding of the neurotoxins. All sensorgrams show data in which the non-specific signal obtained from the control surface is subtracted from the signal obtained from the surface bearing exosomes expressing botulinum neurotoxin receptors. Chips could be regenerated by injecting detergent as n-Octyl-β-D-glucopyranoside (100 mM) as described (Ferracci et al., *Anal.Biochem.*, 2004, 334, 367-375) and reused several times.

5. ELISA: exosomes of the present invention (1 µg of total protein) were directly adsorbed overnight in bicarbonate buffer pH 8.9 on Maxisorp 96 wells plates. Wells were washed with TBS containing BSA 0.1% (TBSA 0.1%) and blocked 1 hour with TBSA 3%. After washing, wells received or not GT1b (4.5 µM) in TBS during 30 min. Plates were washed again and incubated 1h at 37° C with 10 nM botulinum neurotoxins, notably BoNTs A, B or E (complexed forms obtained from Metabionics). Plates were washed again and 100 µl/well of primary polyclonal antibodies against BoNT/A, B or E (appropriate antibodies are commercially

available) were added and incubated as before. After washing 100 µl/well with TBSA 0.1%, goat anti-rabbit-HRP conjugated antibodies (Jackson ImmunoResearch, 1/15 000 in TBSA 0.1%), were added and incubated for 1 h at 37°C. The reaction was revealed with TMB (3,3',5,5-tetramethylbenzidine, Sigma) reagent during 20 min. Reaction was stopped by adding H₂SO₄ and the yellow color quantified at 450 nm.

Example 1: preparation of exosomes co-expressing at their surface the botulism toxin (BoNT) receptor(s) SYT and/or SV2

The exosomes were prepared according to the methods disclosed in the PCT applications n° WO 2009/115561 and WO 2011/036416, using the vector disclosed in Figure 1, which has the sequence of SEQ ID N0. 27, wherein the relevant nucleic acid sequences encoding the botulinum toxin receptor of interest was inserted.

Example 2: assessing of the expression of botulism toxin (BoNT) receptor(s) SYT and/or SV2 at the surface of exosomes

This example starts from recombinant exosomes resulting from the implementation of above-defined protocols (see items entitled “Recombinant exosomes”, “Exosomes preparations”, and “Exosomes purification”, and example 1).

The exosomes co-express at their surface, as toxin (BoNT) receptors, the rat synaptotagmin 2 (rSYT2) (i.e. amino acid sequence SEQ ID N°20) and human SV2A (hSV2A) (i.e. amino acid sequence SEQ ID N°6).

A western blot, such as above-defined is carried out. Especially, approximately three micrograms of crude exosomes co-expressing hSV2A and rSYT2 were denatured in Laemmli buffer in the presence of reductant and subjected to polyacrylamide gel electrophoresis. Proteins were then transferred to a nitrocellulose membrane and the expression of both rSYT2 and hSV2A was probed respectively using mAb 8G2B and anti-SV2A mAb from developmental studies hybridoma bank.

The results of this western blot are represented in the figure 2.

The receptors hSV2A and rSYT2 in these exosomal preparations were also detected by mass spectrometry using the following protocol:

10µg of the exosomal preparation containing SYT2 and SV2 was digested with trypsin in the presence of reducing agent. The peptide mixture was injected on a nano UHPLC (C18 column) coupled to a mass spectrometer (Q-Exactive, ThermoFisher). Peptides from SYT2 and SV2 were detected using the MASCOT software. Absolute or relative SV2 and SYT quantifications might also be performed using this technology.

In parallel, a Surface Plasmon Resonance (SPR) measurements according to the above-defined protocol were carried out with recombinant exosomes expressing at their surface the receptor rSYT2 (i.e. having the amino acid sequence SEQ ID N° 20). In this regard, the exosomes were injected at 1µl / min on a flow cell functionalized with anti SYT2 mAb (5B7A) antibodies.

5 RU increase indicates exosomes capture.

Injection of the ganglioside GT1b (the co-receptor for botulinum neurotoxin B) induces an increment in RU and thus show that immobilized synaptotagmin 2 expressing exosomes capture GT1b.

10 The results of this SPR are represented in the figure 3. Therefore, exosomes expressing the Synaptotagmin receptor can be efficiently captured on Surface Plasmon Resonance (SPR) sensor chip functionalized with anti-synaptotagmin antibodies. What is more, in this figure 2, the increments in RU level following injection of the ganglioside GT1b show the capture of GT1b on synaptotagmin expressing exosomes.

15

Conclusion

The above-mentioned results confirm the orientation of SYT receptors at the surface of the exosomes, which is thus identical to their plasma membrane orientation after vesicular fusion and therefore at the surface of exosomes these proteins expose their BoNT binding facet.

20 The exosomes thus represent a serious alternative tests to the in vivo mouse lethality assay which is today the gold standard test for assaying BoNTs activity.

Indeed, the exosomes offer measuring all aspects of BoNTs activity using native receptors without facing the drawback of ethical issues in using animals. Indeed, exosomes-like structures are native membrane structures collected and enriched from supernatants of stably
25 transfected cell lines. In addition, they represent a flexible and robust material that can be stored frozen or even lyophilized.

Example 3: assessing of the binding between the botulism toxin B (BoNT/B) and exosomes expressing the receptor SYT

30 This example starts from recombinant exosomes resulting from the implementation of above-defined protocols (see items entitled “Recombinant exosomes”, “Exosomes preparations”, and “Exosomes purification”, and example 1).

The exosomes express at their surface, as toxin (BoNT) receptors, either the rat synaptotagmin 2 (rSYT2) (i.e. amino acid sequence SEQ ID N°20...) or both human SYT1
35 (hSYT1) (i.e. amino acid sequence SEQ ID N°17...).

A Surface Plasmon Resonance (SPR) measurement according to the above-defined protocol was carried out with the recombinant exosomes expressing at their surface only the receptor rSYT2. More particularly, these exosomes were captured on 2 independent flow cells of a biosensor. GT1b was injected in one flow cell. 3 nM BoNT/B was then injected on connected
5 flow cells and binds only to rSYT2/GT1b exosomes.

The figure 4A shows the binding of BoNT/B on synaptotagmin/GT1b containing exosomes. No BoNT/B binding was detected in the absence of GT1b.

An ELISA according to the above-defined protocol was carried out with the recombinant
10 exosomes expressing at their surface hSYT1 or rSYT2. More particularly, 1 µg of control exosomes or exosomes expressing hSYT1 or rSYT2 were coated overnight in « Nunc Maxisorp » 96 well plates. BoNT/B (10nM) was then added and incubated for 1 h at 37°C and its binding revealed using anti-BoNT/B antibodies and horseradish peroxidase coupled secondary antibodies. The coated exosomes are incubated or not in presence of GT1b before BoNT/B incubation.

15 The figure 4B shows that GT1b dependent binding of BoNT/B can also be detected using a classical ELISA assay, such as above-defined. Under these conditions, two different isoforms of synaptotagmin (1 & 2) were differentially expressed in exosomes and showed GT1b dependent binding to BoNT/B.

Therefore, BoNT/B at 10 nM binds specifically to exosomes expressing hSYT1 or
20 rSYT2 only in presence of GT1b.

Example 4: assessing of the binding between the botulism toxin A (BoNT/A) and exosomes expressing the receptor hSV2A

This example starts from recombinant exosomes resulting from the implementation of
25 above-defined protocols (see items entitled “Recombinant exosomes”, “Exosomes preparations”, and “Exosomes purification”, and example 1).

The exosomes express at their surface, as toxin (BoNT) receptors, the human SV2A (hSV2A) (i.e. amino acid sequence SEQ ID N°2).

An ELISA according to the above-defined protocol was carried out with the recombinant
30 exosomes expressing at their surface hSV2A. More particularly, 1 µg of control exosomes or exosomes expressing hSV2A were coated overnight in « Nunc Maxisorp » 96 well plates. BoNT/A (10nM) was then added and incubated for 1 h at 37°C and its binding revealed using anti-BoNT/A antibodies and horseradish peroxidase coupled secondary antibodies. The coated exosomes are incubated or not in presence of GT1b before BoNT/A incubation.

The figure 4 shows a representative ELISA assay where exosomes expressing hSV2A interact with BoNT/A. Notably, these results indicate that BoNT/A binding to SV2 is not strictly dependent on the presence of GT1b. However, GT1b potentiates these interactions.

5 **Example 5: assessing of the binding between the botulism toxin E (BoNT/E) and exosomes expressing the receptor hSV2A**

This example starts from recombinant exosomes resulting from the implementation of above-defined protocols (see items entitled “Recombinant exosomes”, “Exosomes preparations”, and “Exosomes purification”, and example 1).

10 The exosomes express at their surface, as toxin (BoNT) receptors, the human SV2A (hSV2A) (i.e. amino acid sequence SEQ ID N°2).

An ELISA according to the above-defined protocol was carried out with the recombinant exosomes expressing at their surface hSV2A. More particularly, 1 µg of control exosomes collected from non-transfected HEK cells or exosomes expressing SV2A were coated overnight in
15 « Nunc Maxisorp » 96 well plates.

BoNT/E (10nM) was then added and incubated for 1 h at 37°C and its binding revealed using anti-BoNT/E antibodies and horseradish peroxidase coupled secondary antibodies. The coated exosomes are incubated or not in presence of GT1b before BoNT/E incubation.

The figure 6 shows a representative ELISA assay where exosomes expressing SV2A
20 interact with BoNT/E. What is more, these results indicate that BoNT/E binding to SV2 is dependent on the presence of GT1b.

Example 6: screening of inhibitors of botulism toxin B (BoNT/B) with exosomes expressing the receptor hSYT2

25 This example starts from recombinant exosomes resulting from the implementation of above-defined protocols (see items entitled “Recombinant exosomes”, “Exosomes preparations”, and “Exosomes purification”, and example 1).

The exosomes express at their surface hSYT2 as toxin (BoNT) receptor (i.e. amino acid sequence SEQ ID N° 23).

30 A Surface Plasmon Resonance (SPR) measurement according to the above-defined protocol was carried out with these recombinant exosomes. More particularly, hSYT2 presenting exosomes were captured on a sensor chip and loaded with GT1b. BoNT/B pre-incubated or not with anti-BoNT/B IgG (obtained by collaboration with Mr Michel Popoff, Pasteur Institute, Paris, France) used for seroneutralization assays was then injected.

As displayed in figure 7, the fact that neutralizing anti BoNT/B antibodies, used in sero-neutralization test in the mouse bio assay, are able to block the binding of BoNT/B to human SYT2/GT1b presenting exosomes is verified. Therefore, exosomes according to the present invention can be used to monitor the efficacy of neutralizing BoNTs by anti-toxin sera.

In other words, this example confirms the suitability of the exosomal system to screen for inhibitors of botulinum neurotoxin binding to native receptors.

Example 7: affinity measurement and quality control of botulinum neurotoxin BoNT/B pharmaceutical batches using exosomes expressing rSYT2

This example starts from recombinant exosomes resulting from the implementation of above-defined protocols (see items entitled “Recombinant exosomes”, “Exosomes preparations”, and “Exosomes purification”, and example 1).

The exosomes express at their surface rSYT2 as toxin (BoNT) receptor (i.e. amino acid sequence SEQ ID N°23...).

A Surface Plasmon Resonance (SPR) measurement according to the above-defined protocol was carried out with these recombinant exosomes. More particularly, a range of BoNT/B concentrations (0.7-12 nM) was injected over exosomes rSYT2/GT1b immobilized on a flow cell of a Biacore 3000 biosensor (2400 RU). A K_d of 0.4 nM was measured using the Biacore software ($k_{on} = 6.9 \cdot 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{off} = 2.9 \cdot 10^{-4} \text{ s}^{-1}$).

As displayed in figure 8, there is a clear relation between the amplitude of the observed signal and the BoNT/B concentration.

This example confirms the suitability of the exosomal system of the present invention for affinity measurement and the quality control of botulinum neurotoxin pharmaceutical batches.

Example 8: exosomes of the invention as a research tool

Preliminarily, it is underlined that current in vitro methods using recombinant proteins failed to detect the interaction between the human SYT2 isoform and the BoNT/B (Peng L, Berntsson RP, Tepp WH, Pitkin RM, Johnson EA, Stenmark P, Dong M.J Cell Sci. 2012 Jul 1;125(Pt 13):3233-42. doi: 10.1242/jcs.103564

This example starts from recombinant exosomes resulting from the implementation of above-defined protocols (see items entitled “Recombinant exosomes”, “Exosomes preparations”, and “Exosomes purification”, and example 1).

The exosomes express at their surface hSYT2 as toxin (BoNT) receptor (i.e. amino acid sequence SEQ ID N° 23).

A Surface Plasmon Resonance (SPR) measurement according to the above-defined protocol was carried out with these recombinant exosomes. More particularly, a range of BoNT/B concentrations (2-32 nM) was injected over exosomes hSYT2/GT1b immobilized on a flow cell of a Biacore 3000 biosensor (3000 RU). A KD of 4.3 nM was measured using the Biacore software ($k_{on} = 6.2 \cdot 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{off} = 2.7 \cdot 10^{-3} \text{ s}^{-1}$).

As displayed in figure 8, the implemented exosomal system allows detecting the presence of the BoNT/B but further a clear relation between the power of the observed signal and BoNT/B concentration.

This example attests that the exosomal system according to the present invention allows demonstrating that the human SYT2 isoform has an affinity for BoNT/B while other in vitro methods using recombinant proteins failed to detect this interaction. Therefore, in contrast to published data, the exosomal system demonstrates that the human synaptotagmin 2 is a receptor for botulinum neurotoxin/B.

In other words, this example confirms the suitability of the exosomal system of the present invention as a research tool.

SEQUENCE LISTING**SEQ ID NO 1** (*nucleic acid sequence human SV2A*)

ATGGAAGAAGGGTTTCGCGATAGGGCTGCGTTTATCAGAGGAGCCAAAGACATCGCT
5 AAGGAGGTGAAGAAGCATGCCGCAAAGAAAGTGGTGAAAGGGCTCGATAGAGTGCA
AGACGAGTACTCACGAAGGTCTTACAGCCGATTCGAGGAGGAAGATGACGATGATG
ACTTCCCTGCGCCAAGCGACGGCTACTACAGGGGTGAGGGCACTCAGGATGAGGAA
GAGGGTGGAGCATCCTCCGATGCAACGGAAGGGCATGACGAGGACGACGAGATTTA
CGAAGGCGAATATCAGGGGATTCCACGTGCTGAGTCAGGAGGGAAGGGCGAACGGA
10 TGGCTGATGGAGCTCCATTAGCCGGAGTCCGTGGTGGCCTGAGTGATGGCGAGGGTC
CTCCTGGCGGCAGAGGGCGAAGCCCAACGGCGCAAAGAACGCGAAGAGTTAGCCCAG
CAGTATGAGGCAATCCTTCGGGAATGCGGGCATGGACGGTTTCAGTGGACACTCTAC
TTTGTGCTCGGACTGGCCCTGATGGCTGACGGGGTAGAAGTGTTTCGTTGTCGGATTCC
TCCTACCGAGTGCCGAGAAAGACATGTGCCTGTCTGACAGTAACAAGGGGATGTTGG
15 GTCTAATCGTGTATCTGGGCATGATGGTTGGTGCCTTTCTTTGGGGTGGGCTGGCAGA
TCGCTTGGGAAGGAGGCAGTGTCTCCTTATTAGCCTGTCAGTGAATAGCGTCTTTGCC
TTCTTCTCCAGCTTCGTCCAAGGTTATGGCACCTTTCTCTTTTGTGCGCTGTTGTCAGG
GGTAGGCATCGGCGGATCTATTCCCATCGTTTTTCAGCTATTTTCAGCGAGTTTCTGGCT
CAGGAGAAGAGAGGGCGAGCACCTGTCTTGGCTTTGCATGTTCTGGATGATAGGAGGA
20 GTTTACGCAGCAGCCATGGCTTGGGCTATCATACCCCACTATGGCTGGTCCTTCCAGA
TGGGGTCTGCCTACCAGTTCCACTCCTGGAGAGTGTTTCGTACTGGTGTGTGCCTTTCC
CAGTGTGTTTGCCATTGGCGCGCTCACTACACAGCCCGAATCTCCACGATTCTTCCTG
GAAAACGGTAAGCACGACGAAGCTTGGATGGTGCTCAAACAGGTTACGATACCAAT
ATGAGAGCCAAAGGCCATCCGGAACGCGTTTTTCAGCGTGACTCACATCAAGACAATC
25 CATCAGGAGGATGAGCTTATCGAGATTCAGTCTGACACAGGAACCTGGTATCAGCGC
TGGGGAGTCAGAGCACTATCCCTCGGTGGCCAAGTGTGGGGCAATTTTCTGTCCTGTT
TCGGGCCTGAGTATCGGCGGATAACCCTTATGATGATGGGAGTCTGGTTCACGATGA
GCTTTAGCTACTATGGGTTGACCGTGTGGTTTCCCGATATGATTCGCCATTTGCAAGC
CGTTGATTACGCTTCCAGGACTAAAGTCTTTCCAGGCGAAAGGGTCGAGCATGTGAC
30 ATTTAACTTCACTCTCGAGAATCAGATCCACAGAGGCGGGCAGTACTTCAATGACAA
GTTCAATTGGCTTAAGGCTCAAGTCTGTAAGCTTTGAGGATTCTCTGTTTGAAGAGTGC
TATTTTGAGGATGTGACTAGTTCCAACACGTTCTTTTCGTAATTGTACGTTCAATTAACA
CAGTTTTCTACAATAACCGATCTCTTTGAGTACAAGTTTGTGAACTCACGGCTGATTAA
CAGCACCTTTCTGCACAACAAAGAGGGGTGTCCACTGGACGTTACCGGAACCGGTGA
35 AGGGGCATACATGGTGTACTTCGTGTCATTCTTGGGCACACTGGCCGTA CTCCCTGGT

AACATAGTGTCTGCCTTGCTGATGGACAAGATCGGTAGACTTCGGATGCTTGCCGGA
 TCAAGTGTGATGAGTTGCGTGTCTGTTTCTTCCTGTCATTTCGGCAATAGCGAGAGTG
 CGATGATAGCACTGCTGTGCCTTTTCGGAGGGGTGTCAATTGCCTCCTGGAATGCTCT
 CGACGTACTAACTGTGGAAGTGTATCCCAGTGACAAACGAACAAGTGCCTTTGGCTT
 5 CCTGAACGCGCTGTGCAAGTTAGCTGCCGCTTGGGGATCAGCATCTTTACCAGCTTT
 GTCGGAATCACCAAGGCTGCCCCATTCTGTTTGCCTCCGCAGCTTTAGCACTGGGCA
 GCTCTTTGGCCCTAAACTGCCCGAAACAAGGGGACAAGTCCTTCAGTCTCGCGCTG
 GcgcgcccCACTTCCCTGAAATCTCCTTCCCCCCTAAACCCGATTCTGATTATCAGGCCTT
 GCTACCATCCGCGCCAGAGATCTACTCTCACCTCTCCCCACCAAACCCGATTACATC
 10 AACCTTCGACCGGCGCCCTAa

SEQ ID NO 2 (*amino acid sequence human SV2A*)

MEEGFRDRAAFIRGAKDIAKEVKKHAAKKVVKGLDRVQDEYSRRSYSRFEEDDDDDDFP
 15 APSDGYRGEQTQDEEEGGASSDATEGHDEDEIYEGEYQGIPRAESGGKGERMADGAP
 LAGVRGGLSDGEGPPGGRGEAQRRKEREELAQQYEAILRECGHGRFQWTLYFVLGLAL
 MADGVEVFVVGFLPSAEKDMCLSDSNKGMLGLIVYLGMMVGAFLWGGLADRLGRRQ
 CLLISLSVNSVFAFFSSFVQGYGTFLFCRLLSGVGIGGSIPIVFSYFSEFLAQEKRGHLSWL
 CMFWMIGGVYAAAMAWAIIPHYGWSFQMGSAYQFHSWRVFVLVCAFPSVFAIGALTQ
 20 PESPRFFLENGKHDEAWMVLKQVHDTNMRAKGHPERVFSVTHIKTIHQEDELIEIQSDTG
 TWYQRWGVRAISLGGQVWGNFLSCFGPEYRR***ITLMMMGVWFTMSFSYYGLTVWFPD***
MIRHLQAVDYASRTKVFPGERVEHVTFNFTLENQIHRGGQYFNDKFIGLRLKSVSFEDSLFEEC
YFEDVTSSNTFFRNCTFINTVFYNTDLFEYKFVNSRLINSTFLHNKEGCPLDVTGTGEGAYMVY
FVSFLGTLAVLPGNIVSALLMDKIGRLRMLAGSSVMSCVSCFFLSFGNSESAMIALLCFLG
 25 GVSIAWNALDVLTVELYPSDKRTTAFGFLNALCKLA AVLGISIFTSFVGITKAAPILFAS
 AALALGSSLALKLPETRQVLQSRAGAPHFPEISFPPKPDSDYQALLPSAPEIYSHLSPT
KPDYINLRPAP

In italics: the fourth extracellular loop and surrounding transmembrane domains

30 **In bold and italics; transmembrane domains**

In bold characters: Exosome targeting sequence

SEQ ID NO 3 Fourth extracellular loop of human SV2A + surrounding transmembrane domains

ITLMMMGVWFTMSFSYYGLTVWFPDMIRHLQAVDYASRTKVFPGERVEHVTFNFTLEN
QIHRGGQYFNDKFIGLRLKSVSFEDSLFEECYFEDVTSSNTFFRNCTFINTVFYNTDLFEYK
 5 *FVNSRLINSTFLHNKEGCPLDVTGTGEGAYMVYFVSFLGTLAVLPGNIVSALLMD*

In bold and italics ; transmembrane domains

SEQ ID NO 4 (nucleic acid sequence human SV2A comprising a T7 Tag Peptide)

ATGGAGGAAGGATTCCGGGACCGGGCTGCATTCATTCGGGGAGCAAAAGACATTGCT
 10 AAGGAAGTGAAGAAACATGCAGCAAAGAAAGTGGTGAAGGGACTcGACAGAGTGCA
 AGATGAGTATTCCAGAAGAAGCTATTCCAGGTTCGAAGAAGAGGATGACGATGACG
 ATTTTCCTGCTCCAAGCGATGGATATTACAGAGGaGAGGGCACACAGGATGAaGAaGA
 aGGaGGCGCAAGCAGCGACGcACAGAAGGGCACGACGAGGATGATGAAATCTACGA
 aGGaGAATACCAGGGAATCCCAAGaGcTGAAGCGGaGGCAaAGGAGAGagaATGGCtG
 15 ATGGGGCTCCTCTGGCaGGAGTGagaGGAGGGCTGTCCGAtGGAGAAAGGCCCTCCcG
 GgGGAaGaGGAGAAGCACAGCGGAGGAAaGAAAGGGAGGAGCTGGCTCAGCAGTATG
 AGGcTATCCTGCGGGAATGCGGGCATGGAAGATTCCAATGGACTCTGTATTTCTGTGCT
 cGGaCTGGCACTGATGGCTGACGGaGTCGAaGTGTTtGTGGTcGGaTTtGTGCTGCCaTCC
 GCTGAGAAAGACATGTGtCTGGGAGCtGGaATGGCCTCCATGACaGGaGGACAGCAGAT
 20 GGGAGCTTCCAGCGATTCCAACAAGGGCATGCTGGGACTGATtGTGTAtCTGGGaATG
 ATGGTTCGGcGCCTTTCTcTGGGGaGGACTGGCtGACAGaCTGGGCAGAAaGGCAATGtCTc
 CTcATCTCCCTGAGCGTgAATtcCGTCTTTGCCTTCTTCTCCTCCTTCGTcCAaGGCTACG
 GCACTTTCTGTTCTGtcGcCTGCTGAGCGGCGTGGGAATTGGAGGGTCCATCCCAATT
 GTGTTCTCCTATTTCTCCGAATTCCTGGCCCAAGAAAAGAGGGGCGAGCATCTGAGCT
 25 GGCTGTGTATGTTTTGGATGATCGGCGGAGTGTACGCCGCCGAATGGCCTGGGCTA
 TTATCCCACACTATGGCTGGTCCTTTCAAATGGGCAGCGCCTACCAATTCCATTCTG
 GAGAGTCTTTGTCCTGGTGTGTGCATTTCCCAGCGTGTtTGCCATTGGCGCTCTGACC
 ACCCAGCCCGAATCCCCACGGTTCTTTCTGGAAAATGGGAAACACGATGAGGCTTGG
 ATGGTGCTGAAACAGGTGCATGATACTAACATGAGAGCTAAAGGCCATccggAACGCG
 30 TTTTCAGCGTGACTCACATCAAGACAATCCATCAGGAGGATGAGCTTATCGAGATTC
 AGTCTGACACAGGAACCTGGTATCAGCGCTGGGGAGTCAGAGCACTATCCCTCGGTG
 GCCAAGTGTGGGGCAATTTTCTGTCCTGTTTCGGGCCTGAGTATCGGCGGATAACCCT
 TATGATGATGGGAGTCTGGTTCACGATGAGCTTTAGCTACTATGGGTGACCGTGTGG
 TTTCCCGATATGATTGCGCATTTGCAAGCCGTTGATTACGCTTCCAGGACTAAAGTCT
 35 TTCCAGGCGAAAGGGTCGAGCATGTGACATTTAACTTCACTCTCGAGAATCAGATCC

ACAGAGGCGGGCAGTACTTCAATGACAAGTTCATTGGCTTAAGGCTCAAGTCTGTAA
 GCTTTGAGGATTCTCTGTTTGAAGAGTGCTATTTTGAGGATGTGACTAGTTCCAACAC
 GTTCTTTCGTAATTGTACGTTCAATTAACACAGTTTTCTACAATACCGATCTCTTTGAGT
 ACAAGTTTGTGAACTCACGGCTGATTAACAGCACCTTTCTGCACAACAAAGAGGGGT
 5 GTCCACTGGACGTTACCGGAACCGGTGAAGGGGCATACATGGTGTACTTCGTGTCAT
 TCTTGGGCACACTGGCCGTACTCCCTGGTAACATAGTGTCTGCCTTGCTGATGGACAA
 GATCGGTAGACTTCGGATGCTTGCCGGATCAAGTGTGATGAGTTGCGTGTCTGTTTC
 TTCCTGTCATTCGGCAATAGCGAGAGTGCGATGATAGCACTGCTGTGCCTTTTCGGAG
 GGGTGTCAATTGCCTCCTGGAATGCTCTCGACGTACTAACTGTGGAAGTGTATCCCAG
 10 TGACAAACGAACAACACTGCGTTTGGCTTCCTGAACGCGCTGTGCAAGTTAGCTGCCGT
 CTTGGGGATCAGCATCTTTACCAGCTTTGTCTCGGAATCACCAAGGCTGCCCCATTCTG
 TTTGCCTCCGCAGCTTTAGCACTGGGCAGCTCTTTGGCCCTAAAACTGCCCCGAAACAA
 GGGGACAAGTCCTTCAGTCTCGCGCTGGGcggccCACTTCCCTGAAATCTCCTTCCCCC
 CTAAACCCGATTCTGATTATCAGGCCTTGCTACCATCCGCGCCAGAGATCTACTCTCA
 15 CCTCTCCCCACCAAACCCGATTACATCAACCTTCGACCGGCGCCCTAa

SEQ ID NO 5 (*amino acid sequence human SV2A comprising a T7 Tag Peptide*)

MEEGFRDRAAFIRGAKDIAKEVKKHAAKKVVKGLDRVQDEYSRRSYSRFEEEDDDDDFP
 20 APSDGYRGEQTQDEEEGGASSDATEGHDEDEIYEGEYQGIPRAESGGKGERMADGAP
 LAGVRGGLSDGEGPPGGRGEAQRRKEREELAQQYEAILRECGHGRFQWTLYFVLGLAL
 MADGVEVFVVGFLPSAEKDMCLGAGMASMTGGQQMGASSDSNKGMLGLIVYLGMM
 VGAFLWGGLADRLGRRQCLLISLSVNSVFAFFSSFVQGYGTFLFCRLLSGVGIGGSIPVFS
 YFSEFLAQEKRGELSWLCMFWMIGGVYAAAMAWAIIPHYGWSFQMGSAYQFHSWRV
 25 FVLVCAFPSVFAIGALTTPESPRFFLENGKHDEAWMLKQVHDTNMRAKGHPERVFVS
 THIKTIHQEDELIEIQSDTGTWYQRWGVRLSLGGQVWGNFLSCFGPEYRR***ITLMMM******GV***
WFTMSFSYYGLTVWFPDMIRHLQAVDYASRTKVFPGERVEHVTFNFTLENQIHRGGQYFND
KFIGLRLKSVSFEDSLFEECYFEDVTSSNTFFRNCTFINTVFYNTDLFEYKFVNSRLINSTFLHNK
EGCPLDVTGTGEGAYMVYFVSFLGTLAVLPGNIVSALLMDKIGRLRMLAGSSVMSCVSCF
 30 FLSFGNSESAMIALLCFLGGVSIASWNALDVLTVELPSDKRTTAFGFLNALCKLAAVLGI
 SIFTSFVGITKAAPILFASAALALGSSLALKLPETRQVLQSRAGAP***HFPEISFPPKPDSDY***
QALLPSAPEIYSHLSPTKPDYINLRPAP

Underlined : T7 Tag peptide

In italics: the fourth extracellular loop and surrounding transmembrane domains

35 In bold and italics: transmembrane domains

In bold characters: Exosome targeting sequence

SEQ ID NO 6 Fourth extracellular loop of human SV2A and surrounding transmembrane domains

5

ITLMMMGVWFTMSFSYYGLTVWFPDMIRHLQAVDYASRTKVFPGERVEHVTFNFTLEN
QIHRGGQYFNDKFIGRLKSVSFEDSLFEECYFEDVTSSNTFFRNCTFINTVFYNTDLFEYK
FVNSRLINSTFLHNKEGCPLDVTGTGEGAYMVYFVSFLGTLAVLPGNIVSALLMD

In bold and italics: transmembrane domains

10

SEQ ID NO 7 (nucleic acid sequence human SV2B)

ATGGATGACTACAAGTATCAGGACAATTATGGGGGCTATGCTCCCAGTGATGGCTAT
TACCGCGGCAATGAGTCCAACCCAGAAGAAGATGCACAGAGTGATGTCACCGAAGG
CCATGATGAGGAAGACGAGATCTATGAGGGCGAGTACCAGGGTATCCCTCACCCAGA
15 TGATGTCAAGGCCAAGCAGGCCAAGATGGCGCCCTCCAGAATGGACAGCCTTCGGGG
CCAGACAGACCTGATGGCTGAGAGGCTGGAAGATGAGGAGCAGTTGGCCCACCACT
ACGAGACCATCATGGATGAGTGTGGCCATGGCCGCTTCCAGTGGATCCTCTTTTTCGT
CTTGGGTTTGGCCCTGATGGCCGATGGGGTGGAAGTGTTTCGTGGTGAGTTTGGCCCTG
CCCAGTGCAGAGAAGGACATGTGTCTGTCCAGTTCCAAAAAAGGAATGCTAGGGATG
20 ATAGTCTACTTGGAATGATGGCGGGCGCCTTCATCCTGGGAGGCCTGGCTGATAAG
CTGGGAAGGAAGCGAGTCCTCAGCATGTCTCTGGCCGTCAATGCCTCCTTCGCCTCCC
TCTCTTCCTTCGTGCAGGGATATGGAGCCTTCCTCTTCTGCCGACTCATCTCAGGCATC
GGTATTGGGGGTGCTCTACCGATTGTTTTTGCCTATTTTTCTGAATTCTTGTCTCGGGA
GAAGCGAGGAGAACACCTCAGTTGGCTGGGCATCTTCTGGATGACTGGGGGCCTGTA
25 CGCATCTGCCATGGCCTGGAGCATCATCCCACTATGGCTGGGGCTTCAGCATGGG
GACCAATTACCACTTCATAGCTGGAGAGTGTTTGTTCATCGTCTGTGCTCTGCCCTGC
ACCGTGTCCATGGTGGCCCTGAAGTTCATGCCAGAGAGCCCAAGGTTTCTGCTAGAG
ATGGGCAAACATGATGAAGCCTGGATGATTCTCAAGCAAGTCCATGACACCAACATG
AGAGCTAAGGGGACCCAGAGAAAGTGTTACGGTTTCCAACATCAAACTCCCAAG
30 CAAATGGATGAATTCATTGAGATCCAAAGTTCAACAGGAACCTGGTACCAGCGCTGG
CTGGTCAGATTCAAGACCATTTTCAAGCAGGTCTGGGATAATGCCCTGTACTGTGTG
ATGGGGCCCTACAGAATGAATACACTGATTCTGGCCGTGGTTTGGTTTGCCATGGCAT
TCAGTTACTATGGACTGACAGTTTGGTTTCTGATATGATCCGCTATTTTCAAGATGA
AGAATAACAAGTCTAAAATGAAGGTGTTTTTGGTGAGCATGTGTACGGCGCCACAAT
35 CAACTTCACGATGGAAAATCAGATCCACCAACATGGGAACTTGTGAATGATAAGTT

CACAAGAATGTACTTTAAACATGTACTCTTTGAGGACACATTCTTTGACGAGTGCTAT
 TTTGAAGACGTAACATCAACAGATACCTACTTCAAAAATTGTACCATTGAATCAACC
 ATCTTTTACAACACAGACCTCTACGAGCACAAGTTCATCAACTGTCGGTTTATCAACT
 CCACCTTCCTGGAGCAGAAGGAGGGCTGCCACATGGACTTGGAGCAAGATAATGACT
 5 TCCTGATTTACCTCGTCAGCTTCCTGGGCAGCCTGTCTGTCTTACCCGGGAACATCAT
 TTCTGCCCTGCTCATGGATAGAATTGGAAGGCTCAAGATGATTGGTGGCTCCATGCTA
 ATCTCTGCAGTCTGCTGCTTCTTCCTGTTTTTTGGCAACAGTGAGTCTGCAATGATCGG
 CTGGCAGTGCCTGTTCTGTGGGACAAGCATTGCAGCCTGGAATGCTCTGGATGTGATC
 ACAGTGGAGCTGTATCCCACCAACCAGAGAGCAACAGCCTTCGGCATTCTCAATGGA
 10 TTATGCAAATTTGGCGCCATCCTGGGAAACACCATCTTTGCTTCTTTTGTGGGATAA
 CCAAAGTGGTCCCCATCCTTCTGGCTGCTGCTTCTCTGGTTGGGGGTGGCCTGATTGC
 CCTTCGACTGCCAGAGACTCGAGAACAGGTCCTGATGTGA

SEQ ID NO 8 (*amino acid sequence human SV2B*)

15 MDDYKYQDNYGGYAPSDGYRGNESNPEEDAQSDVTEGHDEEDEIYEGEYQGIPHPDD
 VKAKQAKMAPSRMDSLRGQTDLMAERLEDEEQLAHQYETIMDEC GHGRFQWILFFVLG
 LALMADGVEVFVVSFALPSAEKDMCLSSSKKGM LGMIVYLGMMAGAFILGGLADKLGR
 KRVLSMSLAVNASFASLSSFVQGYGAFLFCRLISGIGIGGALPIVFAYFSEFLSREKRGEHL
 SWLGIFWMTGGLYASAMAWSIIPHYGWGFSMGTYHFHSWRV FVIVCALPCTVSMVAL
 20 KFMPE SPRFLLEMKGHDEAWMILKQVHDTNMRAKGTPEKVFTVSNIKTPKQMDEFIEIQ
 SSTGTWYQRWLVRFKTIFKQVWDN ***ALYCVMPYRMNTLILAVVWFAMAFSYYGLTVWF***
PDMIRYFQDEEYKSKMKVFFGEHVYGATINFTMENQIHQHGKLVNDKFTRMYFKHVLFE
DTFFDECYFEDVTSTDYFKNCTIESTIFYNTDLYEHKFINCRFINSTFLEQKEGCHMDLEQDNDFL
IYLVSFLGSLVLPGNIISALLMDRIGRLKMIGGSMLISAVCCFFLFFGNSESAMIGWQCLF
 25 CGTSIAAWNALDVITVELYPTNQRATAFGILNGLCKFGAILGNTIFASFVGITKVVPILLAA
 ASLVGGGLIALRLPETREQVLM

In italics: the extracellular domain and surrounding transmembrane domains

In bold and italics: transmembrane domains

30

SEQ ID NO 9 **Fourth extracellular loop of human SV2B and surrounding transmembrane domains**

ALYCVMPYRMNTLILAVVWFAMAFSYYGLTVWFPDMIRYFQDEEYKSKMKVFFGEHV
YGATINFTMENQIHQHGKLVNDKFTRMYFKHVLFE
DTFFDECYFEDVTSTDYFKNCTIE

STIFYNTDLYEHKFINCRFINSTFLEQKEGCHMDLEQDNDFLIYLVSFLGSLSVLPGNIISA
LLM

In bold and italics: transmembrane domains

5 **SEQ ID NO 10** (*nucleic acid sequence human SV2C*)

ATGGAAGACTCTTACAAGGATAGGACTTCACTGATGAAGGGTGCCAAGGACATTGCC
AGAGAGGTGAAGAAACAAACAGTAAAGAAGGTGAATCAAGCTGTGGACCGAGCCCA
GGATGAATACACCCAGAGGTCCTACAGTCGGTTCCAAGATGAAGAAGATGATGATGA
CTACTACCCGGCTGGAGAAACCTATAATGGTGAGGCCAACGATGACGAAGGCTCAAG
10 TGAAGCCACTGAGGGGCATGATGAAGATGATGAGATCTATGAGGGGGAGTATCAGG
GCATCCCCAGTATGAACCAAGCGAAGGACAGCATCGTGTCAGTGGGGCAGCCCAAG
GGCGATGAGTACAAGGACCGGCGGGAGCTGGAATCAGAAAGGAGAGCTGACGAGGA
AGAGTTAGCCCAGCAGTATGAGCTGATAATCCAAGAATGCGGTCATGGTCGTTTTCA
GTGGGCCCTTTTCTTCGTCTCTGGGCATGGCTCTTATGGCAGACGGTGTAGAGGTGTTT
15 GTCGTTGGCTTCGTGTTACCCAGTGCTGAGACAGACCTCTGCATCCCAAATTCAGGAT
CTGGATGGCTAGGCAGCATAGTGTACCTCGGGATGATGGTGGGGGCGTTCTTCTGGG
GAGGACTGGCAGACAAAGTGGGAAGGAAACAGTCTCTTCTGATTTGCATGTCTGTCA
ACGGATTCTTTGCCTTCCTTTCTTCATTTGTCCAAGGTTATGGCTTCTTTCTTCTGTG
GCTTACTTTCTGGATTCTGGGATTGGAGGAGCCATACCCACTGTGTTCTCGTACTTTGC
20 TGAAGTCCTGGCCCCGGGAAAAGCGGGGCGAACACTTGAGCTGGCTCTGCATGTTCTG
GATGATCGGTGGCATCTACGCCTCTGCCATGGCCTGGGCCATCATCCCGCACTACGG
GTGGAGCTTCAGCATGGGATCGGCCTACCAGTTTCACAGTTGGCGTGTGTTTGTCTC
GTCTGTGCACTCCCCTGTGTCTCCTCCGTGGTGGCCCTCACATTCATGCCTGAAAGCC
CACGATTCTTGTTGGAGGTTGGAAAACATGATGAAGCTTGGATGATTCTGAAGTTAA
25 TTCATGACACCAACATGAGAGCCCCGGGGTCAGCCTGAGAAGGTCTTCACGGTAAACA
AAATAAAAACCTCCTAAACAAATAGATGAGCTGATTGAAATTGAGAGTGACACAGGA
ACATGGTATAGGAGGTGTTTTGTTTCGGATCCGCACCGAGCTGTACGGAATTTGGTTGA
CTTTTATGAGATGTTTCAACTACCCAGTCAGGGATAATACAATAAAGCTTACAATTGT
TTGGTTACCCCTGTCCTTTGGGTACTATGGATTATCCGTTTGGTTCCCTGATGTCATTA
30 AACCTCTGCAGTCCGATGAATATGCATTGCTAACCAGAAATGTGGAGAGAGATAAAT
ATGCAAATTTCACTATTAACCTTTACAATGGAAAATCAGATTCATACTGGAATGGAAT
ACGACAATGGCAGATTCATAGGGGTCAAGTTCAAATCTGTAACCTTTCAAAGACTCTG
TTTTTAAGTCCTGCACCTTTGAGGATGTAACCTTCAGTGAACACCTACTTCAAGAACTG
CACATTTATTGACACTGTTTTTGACAACACAGATTTTGAGCCATATAAATTCATTG

ACAGTGAATTTAAAACTGCTCGTTTTTTCACAACAAGACGGGATGTCAGATTACCTT
 TGATGATGACTATAGTGCCTACTGGATTTATTTTGTCAACTTTCTGGGGACATTGGCA
 GTATTGCCAGGGAACATTGTGTCTGCTCTGCTGATGGACAGAATTGGGCGCTTAACA
 ATGCTAGGTGGCTCTATGGTGCTTTCGGGGATCAGCTGTTTCTTCCTTTGGTTCGGCA
 5 CCAGTGAATCCATGATGATAGGCATGCTGTGTCTGTACAATGGATTGACCATCTCAGC
 CTGGAACCTCTCTTGACGTGGTCACTGTGGAACCTGTACCCACAGACCGGAGGGCAAC
 AGGCTTTGGCTTCTTAAATGCGCTATGCAAGGCAGCAGCCGTCCTGGGAAACTTAAT
 ATTTGGCTCTCTGGTCAGCATCACCAAATCAATCCCCATCCTGCTGGCTTCTACTGTG
 CTCGTGTGTGGAGGACTCGTTGGGCTGTGCCTGCCTGACACACGAACCCAGGTTCTG
 10 ATGTAA

SEQ ID NO 11 (*amino acid sequence human SV2C*)

MEDSYKDRTSLMKGAKDIAREVKKQTVKKVNQAVDRAQDEYTQRSYSRFQDEEDDDD
 YYPAGETYNGEANDDEGSSEATEGHDEDDEIYEGEYQGIPSMNQAKDSIVSVGQPKGDE
 15 YKDRRELESERRADEEELAQQYELIIQECGHGRFQWALFFVLGMALMADGVEVFVVGFFV
 LPSAETDLCIPNSGSGWLGSIVYLGMMVGAFWGGGLADKVGRKQSLICMSVNGFFAFL
 SSFVQGYGFFLFCRLLSGFGIGGAIPTVFSYFAEVLAREKRGEHL**SWLCMFWMIGGIYAS**
 AMAWAIIPHYGWSFSMGSAYQFHSWRVVFVIVCALPCVSSVVALTFMPESPRFLLEVKGH
 DEAWMILKLIHDTNMRARGQPEKVFTVNKIKTPKQIDELIEIESDTGTWYRRCFVRIRTEL
 20 YGIWLTF**MRCFNYPVRDNTIKLTIVWFTLSFGYYGLSVWFPDVIKPLQSDEYALLTRNVERD**
KYANFTINFTMENQIHTGMEYDNGRFIGVKFKSVTFKDSVFKSCTFEDVTSVNTYFKNCTFIDT
VFDNTDFEPYKFIDSEFKNCSFFHNKTGCQITFDDDYSAWYIFVNFLGTLAVLPGNIVSAL
 LMDRIGRLTMLGGSMVLSGISCFWLWFGTSESMIGMLCLYNGLTISAWNSLDVVTVEL
 YPTDRRATGFGFLNALCKAAAVLGNLIFGSLVSITKSIPILLASTVLVCGGLVGLCLPDTRT
 25 QVLM

In italics: the fourth extracellular loop and surrounding transmembrane domains

In bold and italics: transmembrane domains

30 **SEQ ID NO 12** **Fourth extracellular loop of human SV2C and surrounding transmembrane domains**

MRCFNYPVRDNTIKLTIVWFTLSFGYYGLSVWFPDVIKPLQSDEYALLTRNVERDK
YANFTINFTMENQIHTGMEYDNGRFIGVKFKSVTFKDSVFKSCTFEDVTSVNTYFKNCTFI
DTVFDNTDFEPYKFIDSEFKNCSFFHNKTGCQITFDDDYSAWYIFVNFLGTLAVLPGNIVS
 35 ***A***

In bold and italics: transmembrane domains**SEQ ID NO 13** (*nucleic acid sequence rat SYT1*)

ATGGTGAGTGCCAGTCATCCTGAGGCCCTGGCCGCCCCTGTCACCACTGTTGCGACCC
5 TTGTCCCACACAATGCCACTGAGCCAGCCAGTCCTGGGGAAGGGAAGGAAGATGCCT
TTTCCAAGCTGAAGCAGAAGTTTATGAATGAGCTGCATAAAATTCCATTGCCACCGT
GGGCCTTAATAGCCATAGCCATAGTTGCGGTCCTTTTAGTCGTAACCTGCTGCTTTTG
TGTCTGTAAGAAATGTTTGTTCAAAAAGAAAAACAAGAAGAAGGGGAAGGAAAAGG
GAGGAAAGAACGCCATTAACATGAAAGACGTGAAAGACTTAGGGAAGACCATGAAG
10 GATCAGGCCCTTAAGGATGACGATGCTGAAACCGGACTGACTGATGGAGAAGAAAA
GGAAGAGCCCAAGGAAGAGGAGAACTGGGAAAGCTCCAATATTCCTGGACTATG
ACTTCCAGAATAACCAGCTGTTGGTGGGAATCATCCAGGCTGCTGAACTGCCCCGCC
TGGACATGGGGGGTACATCCGATCCATACGTCAAAGTCTTCCTGCTGCCTGACAAAA
AGAAGAAATTTGAGACTAAAGTCCACCGGAAAACCCTCAATCCAGTCTTCAATGAAC
15 AATTTACTTTCAAGGTGCCCTACTCGGAATTAGGTGGCAAACCCTGGTGATGGCTGT
GTATGACTTTGATCGCTTCTCCAAGCATGACATCATCGGAGAGTTCAAAGTTCCTATG
AACACCGTGGATTTTGGCCATGTGACCGAGGAGTGGCGTGATCTCCAGAGCGCTGAG
AAAGAAGAGCAAGAGAACTGGGTGACATCTGCTTCTCCCTCCGCTACGTCCCTACT
GCCGGCAAACCTGACTGTTGTCATTCTGGAAGCCAAGAACCTGAAGAAGATGGATGTG
20 GGTGGCTTATCTGATCCCTACGTGAAGATTACCTGATGCAGAACGGTAAGAGGCTG
AAGAAGAAAAAGACGACGATTAAGAAGAACACACTCAACCCCTACTACAACGAGTC
CTTCAGCTTTGAAGTTCCGTTTCGAGCAAATCCAGAAAGTGCAAGTGGTGGTAACGTG
TTGGACTATGACAAGATTGGCAAGAACGACGCCATCGGCAAAGTCTTCGTTGGTTAC
AACAGCACTGGGGCGGAGCTGCGACACTGGTCAGACATGCTGGCCAACCCCCGGCG
25 ACCCATCGCACAGTGGCACACTCTGCAGGTAGAGGAGGAGGTTGATGCCATGCTGGC
TGTCAGAAGTAA

SEQ ID NO 14 (*amino acid sequence rat SYT1*)

MVSASHPEALAAPVTTVATLVPHNATEPASPGEKGEDAFSKLKQKFMNELHKIPLPPWALIAI
30 AIVAVLLVVTCCFCVCKKCLFKKKNNKKKGKEKGGKNAINMKDVKDLGKTMKDQALKD
DDAETGLTDGEEKEEPKEEEKLGKLQYSLDYDFQNNQLLVGIIQAAELPALDMGGTSDP
YVKVFLLPDKKKKFETKVHRKTLNPVFNEQFTFKVPYSELGGKTLVMAVYDFDRFSKHD
IIGEFKVPMTNVDVFGHVTEEWRDLQSAEKEEQEKLGDICFSLRYVPTAGKLTVVILEAKN
LKKMDVGGLSDPYVKIHLMQNGKRLKKKKTTIKKNTLNPYYNESFSFEVPFEQIQKVQV

VVTVLDYDKIGKND AIGKV FVGYNSTGAELRHWS DMLANPRRPIAQWHTLQVEEEVDA
MLAVKK

In italics: the extracellular domain and surrounding transmembrane domain

5 **In bold and italics: transmembrane domain**

SEQ ID N° 15 Extracellular domain of rat SYT1 and transmembrane domain

MVSASHPEALAAPVTTVATLVPHNATEPASPGE GKEDAFSKLKQKFMNELHKIPLPP***WAL***
IAIAIVAVLLVVTCCFCVC

10

In bold and italics: transmembrane domain

SEQ ID NO 16 (nucleic acid sequence human SYT1)

ATGGTGTCCGAaTCCCATCATGAAGCTCTGGCtGCACCACCAGTCACTACTGTCTGCGCAA
15 CAGTGCTGCCTTCCAAtGCTACaGAGCCAGCTTCCCCTGGGGAGGGGAAGGAGGATGC
TTTCAGCAAActGAAGGAGAAGTTCATGAACGAActGCACAAGATCCCTCTGCCACC
ATGGGCCCTGATTGCTATCGCCATTGTCTGCAGTGCTGCTGGTCCTGACCTGTTGTTTCT
GTATTTGCAAGAAGTGTCTGTTCAAGAAGAAGAATAAGAAGAAGGGAAAGGAGAAA
GGCGGAAAGAACGCTATCAATATGAAGGACGTCAAGGATCTGGGaAAGACTATGAA
20 GGACCAgGCtCTGAAGGACGACGACGCTGAAACAGGCCTGACAGACGGCGAAGAGA
AGGAAGAGCCAAAGGAGGAGGAGAAGCTGGGGAAGCTGCAGTATTCCCTGGACTAT
GACTTCCAGAACAACCAGCTGCTGGTCGGaATTATTCAAGCTGCAGAACTGCCAGCtC
TGGACATGGGCGGCACCAGCGACCCTTACGTGAAGGTCTTTCTGCTGCCAGATAAGA
AGAAGAAATTTGAGACC AAAGTCCACaGGAAAACCCTGAACCCAGTGTTCAATGAGC
25 AGTTCAC TTTCAAAGTGCCATACAGCGAGCTGGGCGGAAAGACTCTGGTCATGGCTG
TGTATGATTTTCGATAGGTTCTCCAAGCACGACATTATCGGAGAATTCAAGGTGCCAAT
GAATACTGTGGATTTTCGGGCACGTCACTGAAGAATGGaGATCTGCAGAGCGCTGA
AAAGGAAGAACAAGAGAACTGGGCGACATCTGCTTTAGCCTGAGaTATGTCCCTACC
GCTGGCAAGCTGACCGTcGTcAttCTGGAGGCTAAGAATCTGAAGAAGATGGACGTGG
30 GAGGACTGTCCGACCCATATGTCAAGATCCATCTGATGCAAAATGGCAAGaGGCTGA
AGAAGAAGAAGACTACTATTAAGAAGAACACTCTGAACCCCTACTATAACGAATCCT
TCAGCTTTGAAGTCCCATTTCGAGCAGATTCAAAAGGTCCAAGTCGTCGTCACTGTCTCT
GGATTACGACAAGATTGGAAAGAACGATGCCATCGGGAAGGTGTTCTGTCGGCTACAA
CAGCACTGGCGCAGAACTGAGACATTGGAGCGACATGCTGGCTAATCCTcGGcgg

CCTATCGCaCAgTGGCATAcCTGCAAGTGGAGGAGGAAGTCGATGCTATGCTGGCTG
TGAAGAAAtctagagcgcgcccCACTTCCCTGAAATCTCCTTCCCCCTAAACCCGATTCTG
ATTATCAGGCCTTGCTACCATCCGCGCCAGAGATCTACTCTCACCTCTCCCCACCAA
ACCCGATTACATCAACCTTCGACCGGCGCCCTAa

5

SEQ ID NO 17 (*amino acid sequence human SYT1*)

MVSESHHEALAAPPVTTVATVLP SNATEPASPGEKGEDAFSKLKEKFMNELHKIPLPPWALIAI
AIVAVLLVLTCCFCICKKCLFKKKNNKKKGKEKGGKNAINMKDVKDLGKTMKDQALKDD
DAETGLTDGEEKEEPKEEEKLGLKQYSLDYDFQNNQLLVGIIQAAELPALDMGGTSDPY
10 VKVFLLPDKKKKFETKVHRKTLNPFVNEQFTFKVPYSELGGKTLVMAVYDFDRFSKHDI
IGEFKVP MNTVDFGHVTEEWRDLQSAEKEEQEKLGDICFSLRYVPTAGKLTVVILEAKNL
KKMDVGGLSDPYVKIHLMQNGKRLKKKKTTIKNTLNPYYNESFSFEVPFEQIQKVQVV
VTVLDYDKIGKND AIGKV FVGYNSTGAELRHWS DMLANPRRPIAQWHTLQVEEEVDAM
LAVKKSRGAPHFPEISFPKPDS DYQALLPSAPEIYSHLSPTKPDYINLRPAP

15

In italics: the extracellular domain and surrounding transmembrane domain

In bold and italics: transmembrane domain

In bold characters: Exosome targeting sequence

20

SEQ ID NO 18 **Extracellular domain and surrounding transmembrane domain**

MVSESHHEALAAPPVTTVATVLP SNATEPASPGEKGEDAFSKLKEKFMNELHKIPLPPWA
LIAIAIVAVLLVLTCCFCIC

In bold and italics: transmembrane domain

25

SEQ ID NO 19 (*nucleic acid sequence rat SYT2*)

ATGAGGAACATCTTCAAGAGAAcCAGGAACCTATTGTGGCACCaGCTACCACTACTGC
AACaATGCCTCTCGCTCCaGCAGCCCCTGCTGACAATAGCACTGAATCCACaGGaACTG
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30 AAaATCCCTCTgCCACCTTGGGcTCTCATCGCCATGGCAGTCGTGGCCGGCCTGCTCCT
CCTCACTTGCTGCTTCTGTATCTGCAAGAAGTGTTGTTGCAAGAAGAAGAATAA
GAAAGAGAAGGGCAAGGGaATGAAGAACGCCATGAATATGAAGGACATGAAaGGCG
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GGAGAAGGAACCAGAAAATTTAGGTAAACTCCAGTTCAGCCTGGACTACGATTTCCA
35 AGCTAACCAATTAACCGTTGGCGTACTTCAGGCTGCGGAACCTCCCAGCCTTGGACAT

GGGTGGAACGTCAGACCCCTACGTCAAGGTCTTTCTTTTGCCAGATAAGAAGAAGAA
 GTATGAAACAAAGGTCCATAGAAAGACCTTAAACCCAGCTTTCAATGAAACATTTAC
 CTTTAAAGTCCCCTATCAGGAGTTAGGAGGGAAAACACTTGTTATGGCTATTTATGAT
 TTCGACAGATTTTCTAAGCACGACATTATCGGGGAAGTCAAAGTGCCTATGAATACT
 5 GTAGATCTTGGGCAGCCAATTGAAGAGTGGCGAGACCTTCAAGGCGGCGAAAAGGA
 AGAACCAGAGAAGTTGGGAGACATCTGCACCTCACTCCGCTATGTGCCTACTGCCGG
 CAAGCTAACTGTTTGCATCTTGGAAGCTAAGAATTTGAAGAAAATGGACGTGGGAGG
 GTTGAGTGATCCATATGTGAAAATACACCTAATGCAAAACGGGAAACGTCTTAAGAA
 GAAGAAAACAACGGTAAAGAAGAAGACGTTGAACCCATATTTCAATGAATCATTCTC
 10 CTTTCGAGATACCTTTTCGAGCAGATCCAGAAAGTGCAAGTAGTGGTGACAGTATTGGA
 CTACGACAAGCTGGGAAAGAACGAGGCTATAGGGAAGATATTCGTCGGTTCGAATGC
 GACAGGCACAGAGTTACGGCATTGGTCCGATATGTTGGCCAATCCGAGAAGACCGAT
 AGCTCAATGGCACTCTCTGAAACCAGAAGAGGAGGTGGATGCCTTACTTGGCAAGAA
 CAAGTCTCGCGCTGGGcgcgcccCACTTCCCTGAAATCTCCTTCCCCCCTAAACCCGATTCT
 15 GATTATCAGGCCTTGCTACCATCCGCGCCAGAGATCTACTCTCACCTCTCCCCACCA
AACCCGATTACATCAACCTTCGACCGGCGCCCTAa

SEQ ID NO 20 (amino acid sequence rat SYT2)

MRNIFKRNQEPIVAPATTTATMPLAPAAPADNSTESTGTGESQEDMFAKLKDKFFNEINKIPLP
 20 *PWALIAMAVVAGLLLLTCCFCICKKCCCKKKKNKKEKGKGMKNAMNMKDMKGGQDD*
DDAETGLTEGEGEGEEKEPENLGKLQFSLDYDFQANQLTVGVLQAAELPALDMGGTSD
PYVKVFLLPDKKKKYETKVHRKTLNPAFNETFTFKVPYQELGGKTLVMAIYDFDRFSKH
DIIGEVKVPMTVDLGQPIEEWRDLQGGEKEEPEKLGDICTSLRYVPTAGKLTVCILEAK
NLKKMDVGGLSDPYVKIHLMQNGKRLKKKKTTVKKKTLNPFNFESFSFEIPFEIQIKVQ
 25 *VVVTVLDYDKLGKNEAIGKIFVGSNATGTELRHWSMLANPRRPIAQWHSCLKPEEEVDA*
LLGKNKSRAGAPHFPEISFPPKPDSDYQALLPSAPEIYSHLSPTKPDYINLRPAP

In italics: the extracellular domain and surrounding transmembrane domain

In bold and italics: transmembrane domain

30 **In bold characters: Exosome targeting sequence**

SEQ ID NO 21 Extracellular domain of rat SYT2 +transmembrane domain

MRNIFKRNQEPIVAPATTTATMPLAPAAPADNSTESTGTGESQEDMFAKLKDKFFNEINKI
PLPPWALIAMAVVAGLLLL
 35 ***TCCFCIC***

In bold and italics: transmembrane domain

SEQ ID NO 22 (*nucleic acid sequence human SYT2*)

5 ATGCGTAATATCTTCAAGCGAAACCAGGAGCCCATAGTCGCACCAGCCACAACAACG
GCAACTATGCCAATAGGCCCAGTAGATAATTTCGACAGAGTCAGGAGGCGCCGGAGA
ATCACAAGAGGATATGTTCCGCAAGCTCAAGGAGAAGCTGTTCAATGAAATCAATAA
GATTCCGCTCCCTCCTTGGGCCCTCATTGCCATTGCGGTAGTGGCCGGATTGTTGCTG
CTCACTTGTTGCTTCTGCATCTGTAAGAAGTGTTGTTGCAAGAAGAAGAACAAG
10 AAAGAGAAGGGAAAGGGGATGAAGAATGCAATGAATATGAAGGATATGAAGGGAG
GCCAAGATGATGACGATGCGGAAACCGGTCTCACCGAGGGTGAGGGCGAGGGTGAA
GAGGAGAAGGAACCAGAAAATTTAGGTAAACTCCAGTTCAGCCTGGACTACGATTTT
CAAGCTAACCAATTAACCGTTGGCGTACTTCAGGCTGCGGAACTCCCAGCCTTGAC
ATGGGTGGAACGTCAGACCCCTACGTCAAGGTCTTTCTTTTGCCAGATAAGAAGAAG
15 AAGTATGAAACAAAGGTCCATAGAAAGACCTTAAACCCAGCTTTCAATGAAACATTT
ACCTTTAAAGTCCCCTATCAGGAGTTAGGAGGGAAAACACTTGTTATGGCTATTTATG
ATTTTCGACAGATTTTCTAAGCACGACATTATCGGGGAAGTCAAAGTGCCTATGAATA
CTGTAGATCTTGGGCAGCCAATTGAAGAGTGGCGAGACCTTCAAGGCGGCGAAAAG
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25 ATGCGACAGGCACAGAGTTACGGCATTGGTCCGATATGTTGGCCAATCCGAGAAGAC
CGATAGCTCAATGGCACTCTCTGAAACCAGAAGAGGAGGTGGATGCCTTACTTGGCA
AGAACAAGTCTCGCGCTGG**GcgG**CCCCACTTCCCTGAAATCTCCTTCCCCCTAAACCC
GATTCTGATTATCAGGCCTTGCTACCATCCGCGCCAGAGATCTACTCTCACCTCTCCC
CCACCAAACCCGATTACATCAACCTTCGACCGGCGCCCTAG

30

SEQ ID NO 23 (*amino acid sequence human SYT2*)

MRNIFKRNQEPIVAPATTTATMPIGPVDNSTESGGAGESQEDMFAKLKEKLFNEINKIPLPPWA
LIAIAVVAGLLLLTCC**FC**ICKKKCKKKKKNKKEKGKGMKNAMNMKDMKGGQDDDDAE
TGLTEGEGEGEEKEPENLGKLQFSLDYDFQANQLTVGVLQAAELPALDMGGTSDPYVK
35 VFLLPDKKKKYETKVHRKTLNPAFNETFTFKVPYQELGGKTLVMAIYDFDRFSKHDIIGE

VKVPMNTVDLGQPIEEWRDLQGGEKEEPEKLGDICTSLRYVPTAGKLTVCILEAKNLKK
MDVGGLSDPYVKIHLMQNGKRLKKKKTTVKKKTLNPFNFESFSFEIPFEIQKVQVVVT
VLDYDKLGKNEAIGKIFVGSNATGTELRHWSMLANPRRPIAQWHSKPEEEVDALLGK
NKS RAGAPHFPEISFPPKPDSDYQALLPSAPEIYSHLSPTKPDYINLRPAP

5

In italics: the extracellular domain and surrounding transmembrane domain

In bold and italics: transmembrane domain

In bold characters: Exosome targeting sequence

10 **SEQ ID NO 24** Extracellular domain of human SYT2 **+transmembrane domain**

MRNIFKRNQEPIVAPATTTATMPIGPVDNSTESGGAGESQEDMFAKLKEKLFNEINKIPLP
PWALIAIAVVAGLLLLTCCFCIC

In bold and italics: transmembrane domain

15

SEQ ID NO 25 (*amino acid sequence of the exosome targeting peptide*)

SRAGAPHFPEISFPPKPDSDYQALLPSAPEIYSHLSPTKPDYINLRPAP

SEQ ID NO 26 (*amino acid sequence of T7 Tag Peptide*)

20

MASMTGGQMG

SEQ ID N° 27 Nucleic sequence of the recipient vector

CTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCCGGCTGCGGCGAGCG
GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCA
25 GGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG
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CTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCC
TGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCC
GCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCA
30 GTTCGGTGTAGGTCGTTGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTTCAGCC
CGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGG
CGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGT
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ACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCA
CGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGC
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10 CATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTAGCTCCGGTTCCCAACGA
TCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTC
CTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGC
ACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAG
TACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGG
15 CGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTG
GAAAACGTTCTTCGGGGCGAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTT
CGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTACCAGCGT
TTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGA
CACGGAAATGTTGAATACTCATACTCTTCCTTTTCAATATTATTGAAGCATTTATCA
20 GGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT
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AGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGA
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25 CCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACT
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GGTGCCACTCCCACTGTCTTTTCTTAATAAAATGAGGAAATTGCATCGCATTGTCTGA
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AAAGGAGAGCCTGTAAGCGGGCACTCTCCGTGGTCTGGTGGATAAATTTCGAAGGG
TATCATGGCGGACGACCGGGGTTCGAACCCCGGATCCGGCCGTCCGCCGTGATCCAT
GCGGTTACCGCCCGCGTGTCTGAACCCAGGTGTGCGACGTCAGACAACGGGGGAGCGC
30 TCCTTTTGGCTTCCTTCCGTCTGA

CLAIMS

1. An affinity substrate comprising a solid substrate material onto which is/are immobilized one or more membrane vesicles, in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s), wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s).

2. The affinity substrate according to claim 1, wherein the one or more botulinum toxin receptors are selected in a group comprising SV2 and synaptotagmin.

3. The affinity substrate according to claim 2, wherein the SV2 botulinum toxin receptor is selected in a group comprising SV2A, SV2B and SV2C.

4. The affinity substrate according to claim 2, wherein the synaptogamin botulinum toxin receptor is selected in a group comprising SYT1 and SYT2.

5. The affinity substrate according to anyone of the preceding claims, wherein the SV2 receptor or a fragment thereof comprises an amino acid sequence having at least 70% amino acid identity, preferably at least 80 % amino acid identity, and still better at least 90% amino acid identity, with an amino acid sequence selected from the group comprising SEQ ID N° 3, SEQ ID N° 6, SEQ ID N° 9, SEQ ID N° 12, and combination thereof.

6. The affinity substrate according to anyone of the preceding claims, wherein the synaptotagmin receptor or a fragment thereof comprises an amino acid sequence having at least 70% amino acid identity, preferably at least 80 % amino acid identity, and still better at least 90% amino acid identity, with an amino acid sequence selected from the group SEQ ID N° 14, SEQ ID N° 17, SEQ ID N° 20, SEQ ID N° 23, and combination thereof.

7. The affinity substrate according to anyone of the preceding claims, wherein the said membrane vesicles further comprise ganglioside molecule(s).

8. The affinity substrate according to the preceding claim, wherein the ganglioside molecule is selected in a group comprising GD1a, GD1b, GT1b, GQ1b, GM1a and GD3.

9. The affinity substrate according to anyone of the preceding claims, wherein said membrane vesicle(s) has/have an average diameter of 100 nm or less, and preferably has/have an average diameter ranging from 30 to 200 nm, and still better from 50 to 100 nm.

10. A composition comprising membrane vesicles, in particular exosome-like vesicles, and still better exosomes, expressing at their surface one or more botulinum toxin receptor(s), wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s).

11. The composition according to claim 10, wherein the said one or more botulinum toxin receptors are selected in a group comprising SV2 and synaptotagmin.

12. The composition according to anyone of the claims 10 and 11, wherein said composition further comprises ganglioside molecule(s).

5 13. A complex between (i) a membrane vesicles, in particular exosome-like vesicles, such as defined in anyone of claims 1 to 9 and (ii) one or more botulinum neurotoxin(s).

14. The complex according to the preceding claim, wherein the said membrane vesicles further comprises ganglioside molecule(s) such as defined in claims 7 or 8.

10 15. A kit, notably for assessing one or more biological activity(ies) of a compound, the said kit comprising:

- one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more receptor(s) selected in a group comprising SV2 and/or synaptotagmin, or fragment(s) thereof, wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s),

15 - optionally, at least one solid substrate material at least partially coated with ligand(s) capable of binding to said membrane vesicles, and preferably to one or more botulinum toxin receptor(s) selected in a group comprising SV2 and synaptotagmin, and

- optionally, a composition comprising one or more ganglioside molecule(s).

20 16. A method for in vitro detecting and/or measuring a biological activity of one or more botulinum toxin(s), said method comprising the steps of:

- a) providing one or more membrane vesicles, in particular exosome-like vesicles, expressing at their surface one or more botulinum toxin receptor(s) selected in a group comprising SV2 and synaptotagmin, wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s),

25 b) contacting the membrane vesicles provided at step a) with one or more botulinum toxin(s), optionally in presence of ganglioside molecule(s), whereby an assay mixture is provided, and

- c) detecting and/or measuring a biological activity of said botulinum neurotoxin(s).

30 17. The method according to the preceding claim, wherein said biological activity is selected from a group comprising:

- (i) the binding of said botulinum toxin(s) with said receptor(s) SV2 and/or synaptotagmin, or fragment(s) thereof,

- (ii) the translocation of said botulinum toxin(s) within said membrane vesicles, and/or

(iii) the enzyme activity of said botulinum toxin(s) with one or more enzyme substrate(s) capable of reacting with said botulinum toxin(s).

18. The method according to the claim 17, wherein, when the biological activity is (iii) the enzyme activity, the enzyme substrate(s) is/are present within said membrane vesicles.

5 19. The method according to the claim 17, wherein, when the biological activity is (iii) the enzyme activity, said method further comprises a step d) comprising lysing the membrane vesicles of the assay mixture provided at step b) and then bringing the resulting lysate into contact with the enzyme substrate(s).

10 20. A method for in vitro detecting the presence of one or more botulinum toxin(s), said method comprising the steps of:

- providing one or more membrane vesicle(s), in particular exosome-like vesicles, expressing at their surface one or more botulinum receptor(s) selected in a group comprising SV2 and synaptotagmin (SYT), wherein the botulinum toxin (BoNT) binding site of said receptor(s) is exposed outside of said membrane vesicle(s),
- 15 - bringing a sample comprising, or suspected of comprising, said botulinum neurotoxin(s) into contact with said membrane vesicle(s), optionally in presence of ganglioside molecule(s), and
- detecting the formation of complexes between (i) said membrane vesicle(s) and (ii) said botulinum neurotoxin(s).

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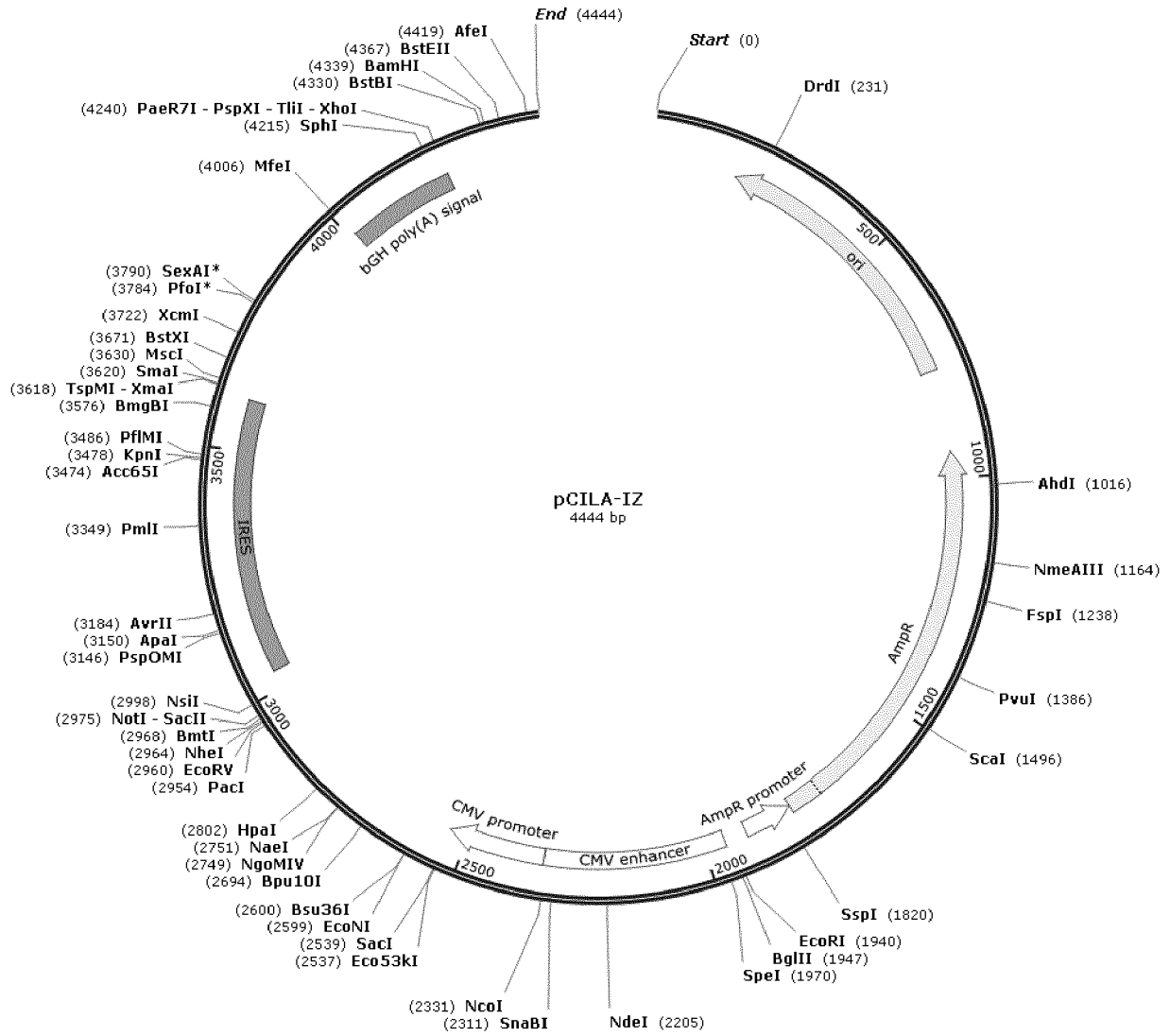


FIGURE 1

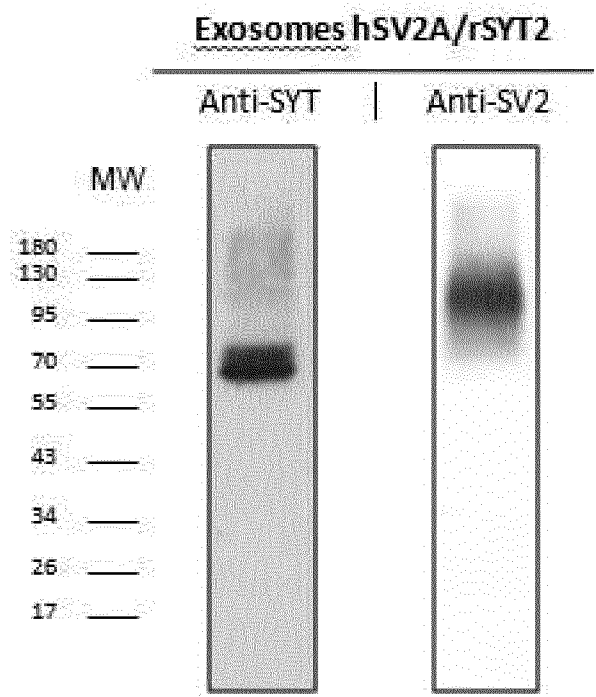


FIGURE 2

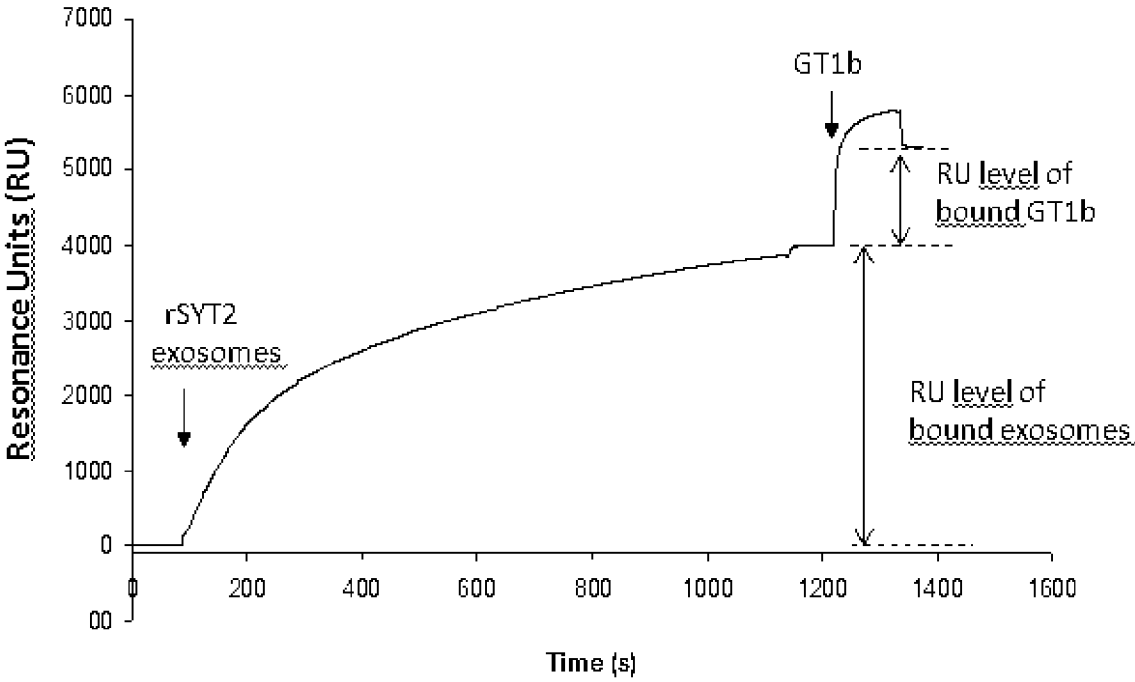


FIGURE 3

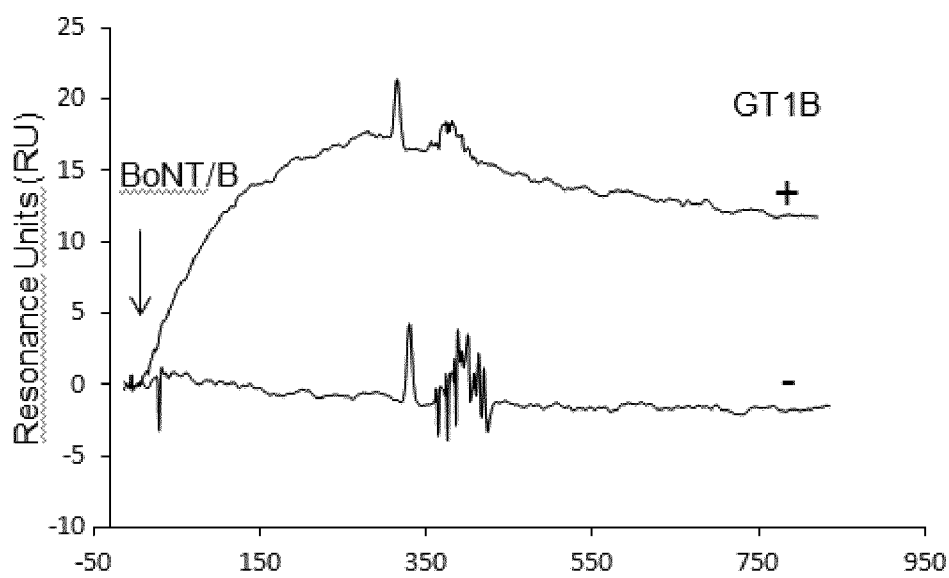


FIGURE 4A

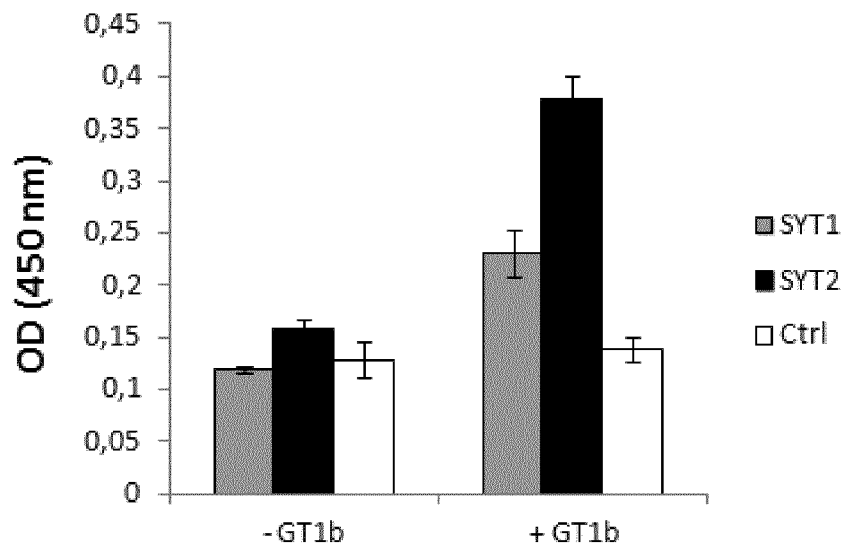
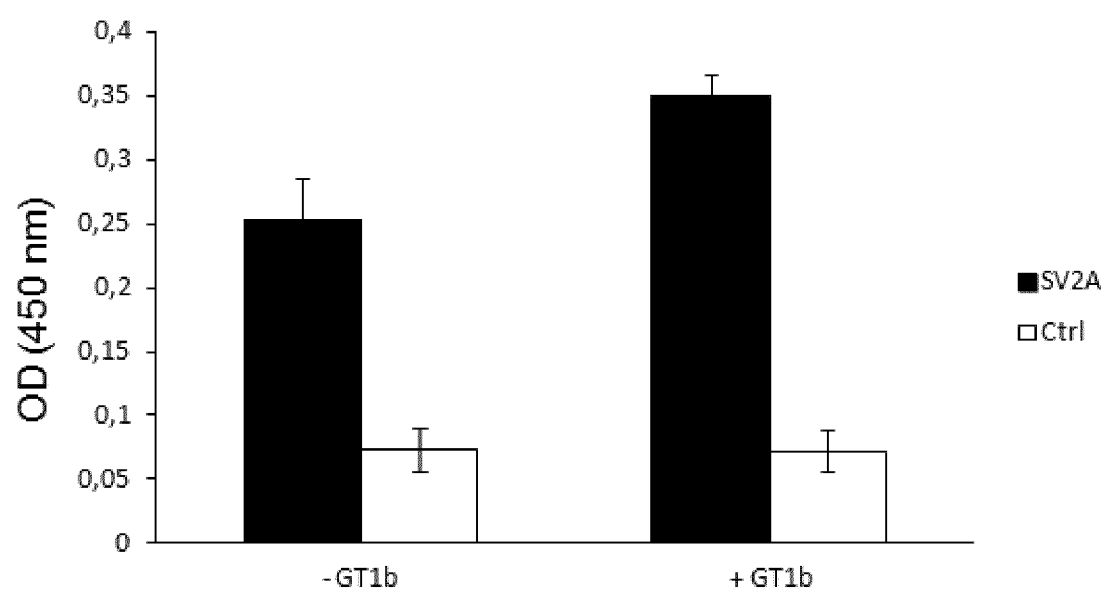
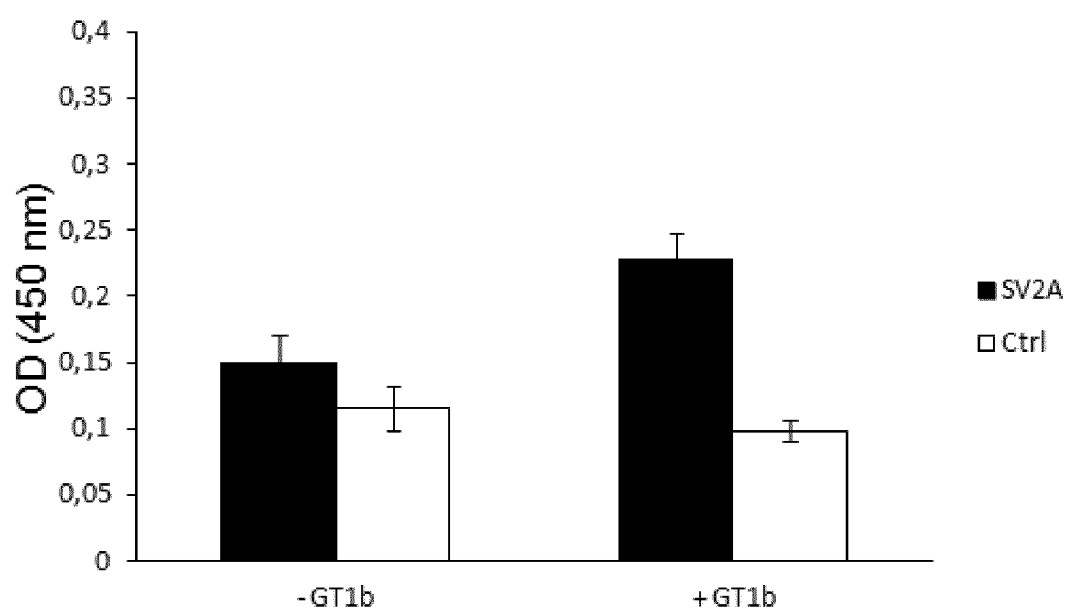
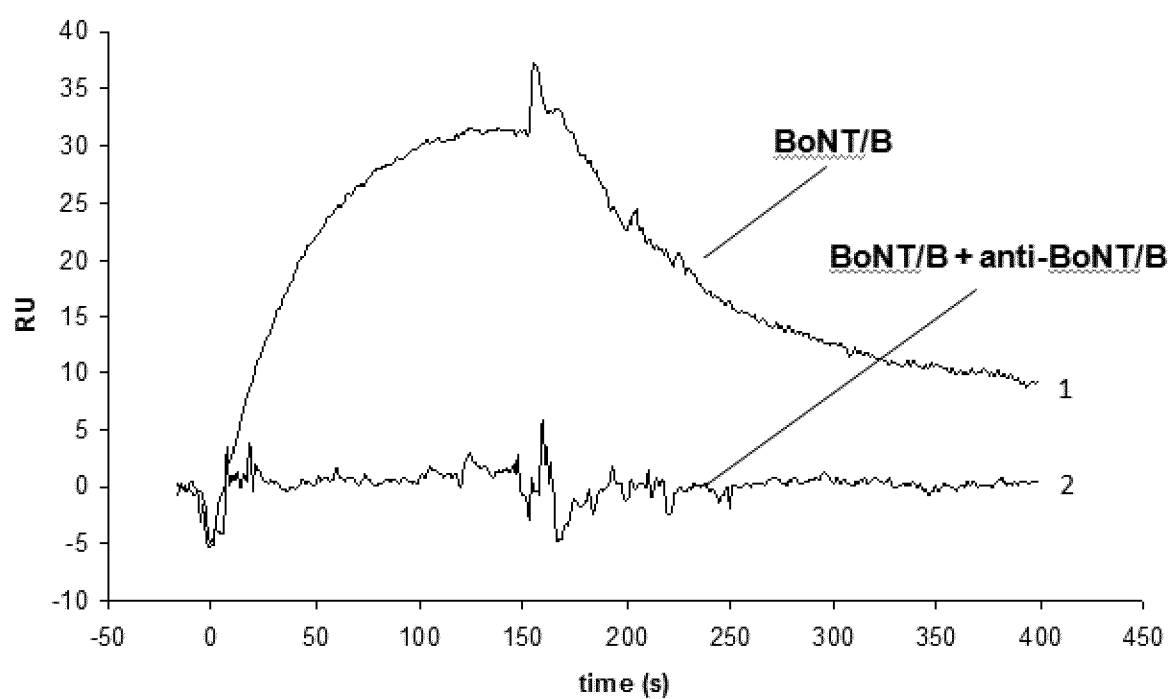
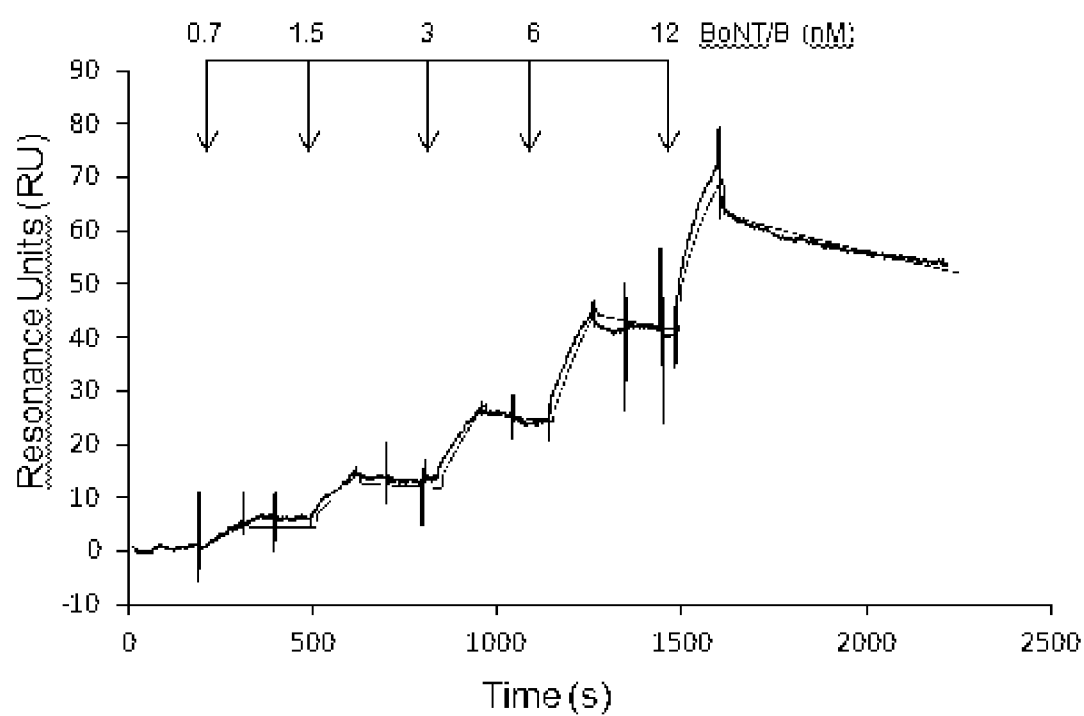


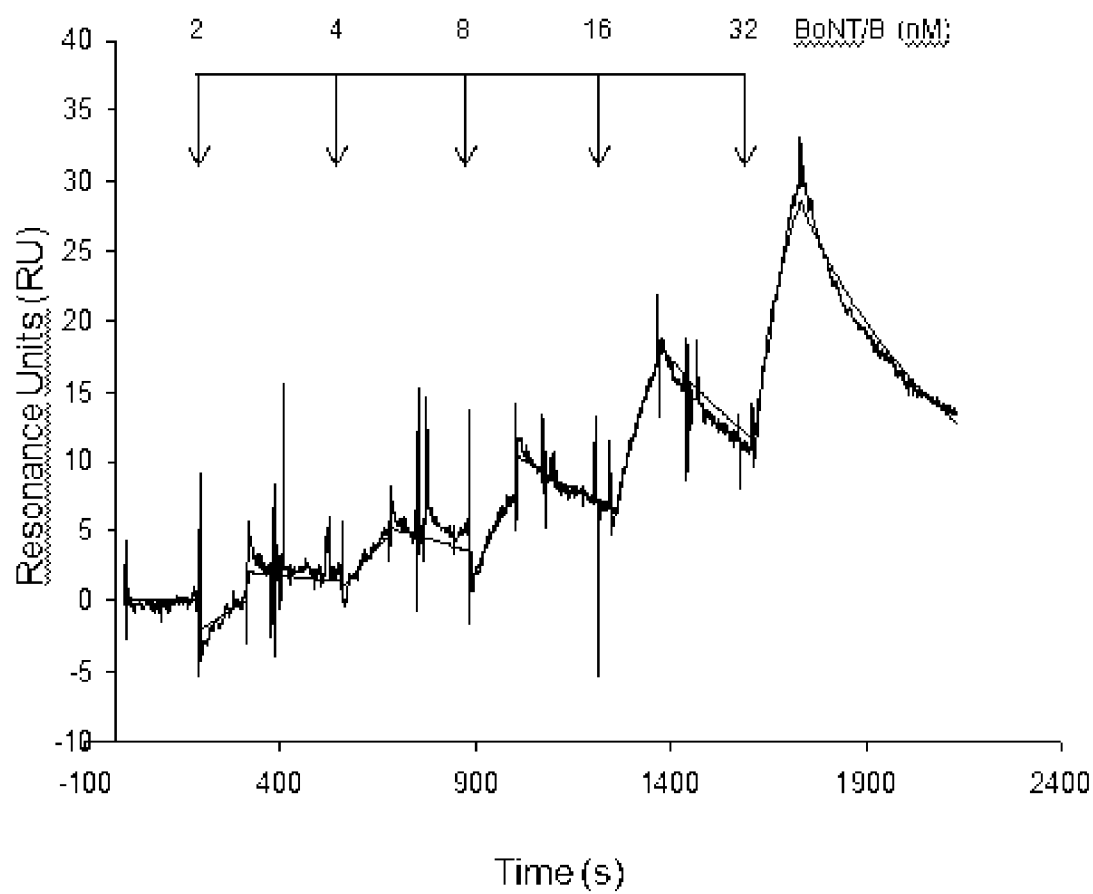
FIGURE 4B

**FIGURE 5**

**FIGURE 6**

**FIGURE 7**

**FIGURE 8**

**FIGURE 9**

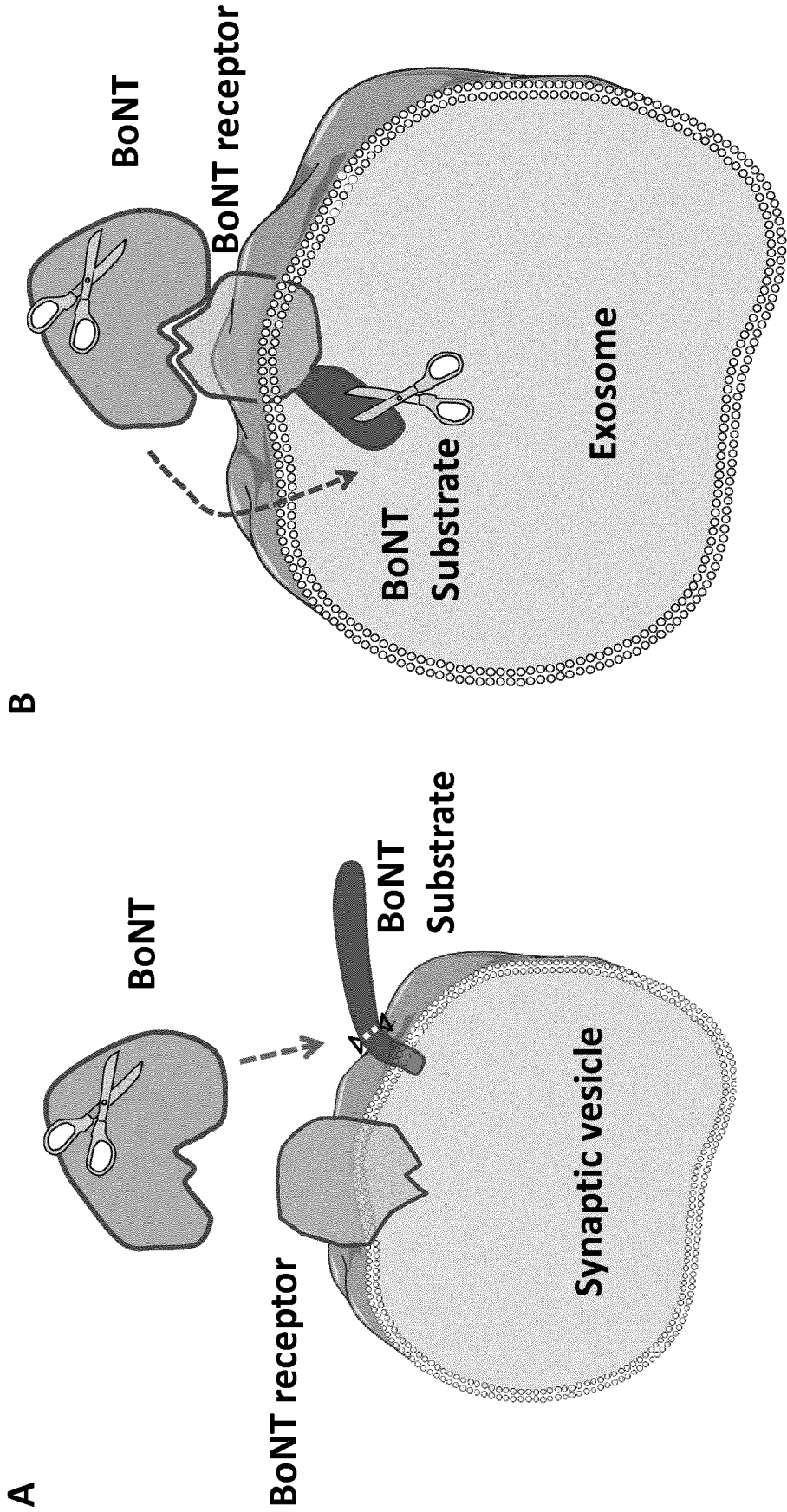


FIGURE 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/061107

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/53 G01N33/566 G01N33/569 G01N33/50
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/083370 A2 (UNIV AIX MARSEILLE II [FR]; CENTRE NAT RECH SCIENT [FR]; INST NAT SANT) 30 September 2004 (2004-09-30) abstract, p. 7, lines 9 - 23, p. 16, line 9 - p. 17, line 20, p. 27, lines 12 - 24 and Fig 6, Examples 1, 6 -----	1-20
X	FERRACCI GERALDINE ET AL: "Synaptic vesicle chips to assay botulinum neurotoxins", BIOCHEMICAL JOURNAL, vol. 391, no. Part 3, November 2005 (2005-11), pages 659-666, XP002742993, the whole document ----- -/-	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

29 June 2016

Date of mailing of the international search report

06/07/2016

Name and mailing address of the ISA/

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Authorized officer

Hoesel, Heidi

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/061107

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FERRACCI G ET AL: "Real time analysis of intact organelles using surface plasmon resonance", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 334, no. 2, 15 November 2004 (2004-11-15), pages 367-375, XP027187502, ISSN: 0003-2697 [retrieved on 2004-10-19] abstract, p. 369, col. 1, 3rd par., p. 370, col. 1, 2nd par. -----	1-15
A	US 2011/268750 A1 (MAMOUN ROBERT ZAINE EI ABIDDINE [FR] ET AL) 3 November 2011 (2011-11-03) par. 0024 - 0028 -----	1-20
A	CAPEK P ET AL: "Sensing the Deadliest Toxin: Technologies for Botulinum Neurotoxin Detection", TOXINS, MOLECULAR DIVERSITY PRESERVATION INTERNATIONAL (MDPI) AG, CH, vol. 2, no. 1, 7 December 2010 (2010-12-07), pages 24-53, XP002578078, ISSN: 2072-6651, DOI: 10.3390/TOXINS2010024 [retrieved on 2010-01-07] items 11-13, 17, -----	18,19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/061107

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004083370	A2	30-09-2004	EP 1604206 A2 14-12-2005
			FR 2852393 A1 17-09-2004
			WO 2004083370 A2 30-09-2004

US 2011268750	A1	03-11-2011	CA 2718868 A1 24-09-2009
			EP 2268816 A1 05-01-2011
			FR 2928926 A1 25-09-2009
			JP 5727361 B2 03-06-2015
			JP 2011517402 A 09-06-2011
			US 2011268750 A1 03-11-2011
			WO 2009115561 A1 24-09-2009
