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(54) **Title:** ENGINEERING CLOSTRIDIA NEUROTOXINS WITH ELEVATED CATALYTIC ACTIVITY

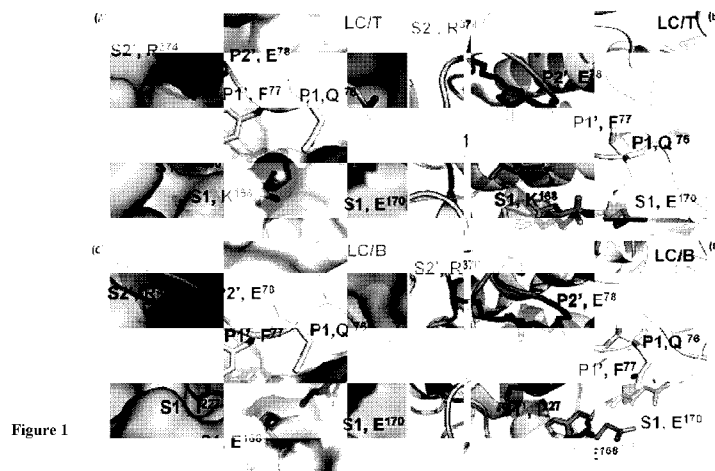


Figure 1

(57) **Abstract:** This invention provides methods of modulating activity of Botulinum Neurotoxin and Tetanus Neurotoxin. In one embodiment, the present invention provides active derivatives of the light chain of Tetanus Neurotoxin (LC/T) and Botulinum Neurotoxin (LC/B) or derivatives of the entire Tetanus Neurotoxin and Botulinum Neurotoxin. In another embodiment, the present invention provides methods to improve current therapy using Botulinum Neurotoxin. In another embodiment, the present invention provides novel methods to use Botulinum Neurotoxin or light chain of Botulinum Neurotoxin for therapeutic or cosmetic purposes. In one embodiment, the present invention provides method for reducing immunoresistance to Botulinum Neurotoxin in various applications of the toxin.

**ENGINEERING CLOSTRIDIA NEUROTOXINS WITH ELEVATED CATALYTIC
ACTIVITY**

CROSS-REFERENCE TO RELATED APPLICATION

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[0001] This application claims the benefit of U.S. Serial No. 61/868,560, filed August 21, 2013. The entire contents and disclosures of this prior application are incorporated herein by reference into this application.

10 **[0002]** Throughout this application, various references or publications are cited. Disclosures of these references or publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

FIELD OF THE INVENTION

15 **[0003]** This invention relates to modification of Clostridia Neurotoxins including Botulinum Neurotoxin and Tetanus Neurotoxin and applications of the derivatives obtained thereof.

BACKGROUND OF THE INVENTION

[0004] The Clostridia Neurotoxins (CNTs) are among the most potent protein toxins for humans
20 and are responsible for botulism, a flaccid paralysis elicited by the Botulinum Neurotoxins (BoNT), and spastic paralysis elicited by Tetanus Neurotoxin (TeNT). CNTs are 150 kDa dichain proteins with typical A-B structure-function properties, where the B (binding) domain binds to surface components on the mammalian cell and translocates the A (active) domain to an intracellular location (1). CNTs are organized into three functional domains: an N-terminal
25 catalytic domain (light chain, LC), an internal translocation domain (heavy chain, HCT), and a C-terminal receptor binding domain (heavy chain, HCR) (2). The CNTs are zinc metalloprotease that cleave SNARE (Soluble NSF Attachment REceptor) proteins which interfere with synaptic vesicle fusion to the plasma membrane and ultimately block neurotransmitter release in nerve cells (1, 3). Mammalian neuronal exocytosis is driven by the formation of protein complexes
30 between a vesicle SNARE protein called vesicle associated membrane protein-2 (VAMP2), and two plasma membrane SNARE proteins SNAP25 (Synaptosome-Associated Protein of 25 kDa) and syntaxin 1a (4). There are seven BoNT serotypes (termed A–G) that cleave specific residues on one of the three SNARE proteins: BoNT serotypes B, D, F and G, and TeNT cleave VAMP2, BoNT serotypes A and E cleave SNAP25, and BoNT serotype C cleaves SNAP25 and syntaxin
35 1a (3, 5-7).

[0005] BoNTs are most widely used in protein therapy. BoNT serotype A (BoNT/A) was approved by the United States Food And Drug Administration (FDA) to treat strabismus, blepharospasm, hemifacial spasm and also for cervical dystonia, cosmetic use, glabellar facial lines and axillary hyperhidrosis as early as 1989. The efficacy of BoNT/A in treating dystonia and other disorders related to involuntary skeletal muscle activity, and the satisfactory safety profile associated with BoNT/A have prompted empirical/off-label use of BoNT/A in a variety of ophthalmological, gastrointestinal, urological, orthopedic, dermatological, secretory, and painful disorders (8-17). On December 11, 2000, a Botulinum Neurotoxin serotype B product (MYOBLOC™) was approved by the FDA in the United States as a treatment for patients with cervical dystonia to reduce the severity of abnormal head position and neck pain associated with cervical dystonia (18, 19).

[0006] The therapeutic benefits of BoNT for conditions associated with involuntary muscle spasm and contractions, cosmetic use, or other applications are transient and repeated injections of BoNT are necessary. In some patients, BoNT could elicit neutralizing antibodies against the corresponding toxin, thus reducing the availability of BoNT. The resulted immunoresistance limits the beneficial effects of BoNT and may render the patient completely unresponsive to further treatment (9, 10, 20-26). The exact percentage of patients who may develop immunoresistance to BoNT treatment is unknown, but it is commonly believed that there are fewer patients who develop blocking antibodies when treated with Botulinum Neurotoxin serotype A (BoNT/A) than with serotype B (BoNT/B) (9, 26). This is probably due to the use of lower doses of the BoNT/A complex than the BoNT/B complex (27). The development of blocking antibodies is also more common in patients who receive treatment for cervical dystonia or spasticity, which requires larger doses and periodic administration of the toxin, while it is less common in patients who are treated for laryngeal dystonia, blepharospasm or for cosmetic use, all of which require smaller doses for treatments (9, 28, 29). Apparently, lowering the treatment dose may help to reduce the development of immunoresistance to BoNT treatment.

[0007] To date, there is no effective solution for handling the BoNT immunoresistance issue. Attempt has been made to block the epitopes on the BoNTs that are involved in the induction of neutralizing antibody. By reacting neutralizing antibodies from resistant patients to different domains of BoNT/A and BoNT/B, a series of immunogenic regions that may be involved in the induction of neutralizing antibody have been identified (39-41). It has been reported that conjugating peptides with monomethoxypolyethylene glycol (mPEG) could suppress the immune response against the peptides. Regions on the heavy chain of serotype A (HC/A) which showed strong immune response were conjugated to mPEG and the resulting derivatives were

used to pre-immunize mice before the administration of BoNT/A. It was shown that some of the mPEG-conjugated peptides could actually reduce the production of the neutralizing antibody (42), indicating that the tolerization procedure might be potentially useful for clinical applications to immunoresistant patients.

- 5 **[0008]** Since therapeutic properties of BoNTs and development of BoNTs immunoresistance are interrelated, the best way to overcome the immunoresistant problem in BoNTs is to engineer more active BoNTs that could reduce the amounts of toxins required for therapy. Rummel et al have modified a ganglioside binding motif of the heavy chain (HC) domain of BoNT/B that enhances the binding and toxicity to up to three-fold relative to the wild type toxin (43).
- 10 However, engineering of BoNT through modification(s) of its receptor binding sites may affect the selectivity of the binding event. In addition, modification of the binding site(s) may not increase the potency significantly enough to prevent the development of immunoresistance. Rather, altering BoNT activity by modification of the light chain (LC) may be a better way to achieve this goal. Hence, there is a need to modify Clostridia Neurotoxins such as Botulinum
- 15 Neurotoxins to enhance therapeutic efficacies.

SUMMARY OF THE INVENTION

- [0009]** This invention provides methods for modulating activity of Botulinum Neurotoxin and Tetanus Neurotoxin.
- 20 **[0010]** In one embodiment, the present invention provides methods for enhancing substrate recognition of the light chain of Tetanus Neurotoxin (LC/T) and Botulinum Neurotoxin (LC/B) via elevated catalytic activity.
- [0011]** In one embodiment, the present invention provides derivatives of Botulinum Neurotoxin and Tetanus Neurotoxin and their applications thereof.
- 25 **[0012]** In one embodiment, the present invention provides derivatives of the light chain of Tetanus Neurotoxin (LC/T) or derivatives of the entire Tetanus Neurotoxin with altered activity.
- [0013]** In another embodiment, the present invention provides derivatives of the light chain of Botulinum Neurotoxin (LC/B) or derivatives of the entire Botulinum Neurotoxin with altered activity.
- 30 **[0014]** In one embodiment, the LC/B derivatives or Botulinum Neurotoxin derivatives described herein can be used in various therapies, cosmetics or other applications. In another embodiment, the LC/B derivatives or Botulinum Neurotoxin derivatives described herein are used in therapy for reducing immunoresistance to Botulinum Neurotoxin.

[0015] In another embodiment, the present invention provides methods to improve current therapy using Botulinum Neurotoxin. In another embodiment, the present invention provides method of using derivatives of Botulinum Neurotoxin or light chain of Botulinum Neurotoxin for various therapeutic or cosmetic applications.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0016] Figure 1 shows an illustration of the S2', S1' and S1 pockets of the light chain of Tetanus Neurotoxin (LC/T) and light chain of Botulinum Neurotoxin (LC/B), and the P sites of VAMP2. Amino acid residues at the pockets which interacts with different P sites of the VAMP2 are shown. Figure 1A shows the interacting residues of LC/T and the VAMP2, wherein the LC/T is represented as a surface model. Figure 1B shows the interacting residues of LC/T and the VAMP2, wherein the LC/T is represented as a cartoon model. Figure 1C show the interacting residues of LC/B and VAMP2, wherein the LC/B is represented as a surface model. Figure 1D show the interacting residues of LC/B and VAMP2, wherein the LC/B is represented as a cartoon model. The nomenclatures of the P site of VAMP2 and the S pockets of LC/T are as follow. The two residues that formed the scissile bond of VAMP2 were designated as P1-P1' from C- to N-terminus. Residues from P1 to the C-terminal direction were designated as P2, P3 etc., while residues from P1' to the N-terminal direction were designated as P2', P3' etc. The pockets in LC/T or LC/B that recognize the specific P sites were designated as the corresponding S pockets. The S2' pocket of LC/T and LC/B, which recognize the P2' site of VAMP2 (residue E⁷⁸), is composed of R³⁷⁴ and R³⁷⁰ respectively. The S1' pocket of LC/T and LC/B, which recognizes the P1' site of VAMP2 (residue F⁷⁷), is composed of L²³⁰ and I²²⁷ respectively. The S1 pocket of LC/T and LC/B, which recognizes the P1 site of VAMP2 (residue Q⁷⁶), is composed of K¹⁶⁸NE¹⁷⁰ and E¹⁶⁸NE¹⁷⁰ respectively.

[0017] Figure 2 shows catalytic activities of LC/T, LC/B and their derivatives on cleaving substrate VAMP2. Figure 2A shows the activities of LC/T and derivatives on cleaving VAMP2. Figure 2B shows the activities of LC/B and derivatives on cleaving VAMP2. Error bar represents standard deviation of three independent repeats for each assay.

[0018] Figure 3 shows orientation of S1' residue in LC/B, LC/T and the LC/T derivative [K¹⁶⁸E, L²³⁰I]. Figure 3A compares the orientation of S1' pocket residue in LC/B (I²²⁷), in LC/T (L²³⁰) and in LC/T [K¹⁶⁸E, L²³⁰I] (I²³⁰) as labeled. Line refers to the peptide backbone, while stick refers to residues. Figure 3B shows a relative flat surface demonstration of S1' pocket comprising I²²⁷ in LC/B (black), and I²³⁰ in LC/T [K¹⁶⁸E, L²³⁰I] (grey). Figure 3C shows the bulky surface of S1' pocket of LC/T comprising L²³⁰ with a mesh surface demonstration. Figure

3D shows the bulky surface of S1' pocket of LC/T comprising L²³⁰ with a solid surface demonstration.

[0019] Figure 4 shows the distances between residues in the S1' and S2' pockets of LC/T (Figure 4A), LC/B (Figure 4B) and LC/T [K¹⁶⁸E, L²³⁰I] (Figure 4C). The distances between the side chains of S1' residue and S2' residues were measured using the PyMol program. The distance between R³⁷⁰ and L²³⁰ in LC/T is about 7.2 Å, the distance between R³⁷⁰ and I²²⁷ in LC/B is 7.9 Å and the distance between R³⁷⁴ and I²³⁰ in LC/T [K¹⁶⁸E, L²³⁰I] is about 8.3 Å.

[0020] Figure 5 shows the cleavage of endogenous VAMP2 by recombinant LC/B, LC/T and their derivatives. Figure 5A shows the cleavage of endogenous VAMP2 by LC/T and LC/T [K¹⁶⁸E, L²³⁰I]. Figure 5B shows the cleavage of endogenous VAMP2 by LC/B and LC/B [S²⁰¹P]. Upper panel in each figure shows cleavage of VAMP2 analyzed by western blotting using anti-VAMP2 antibody and anti-actin antibody; lower panel shows quantification of the bands obtained from the western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

[0021] In order to enhance the therapeutic efficacies of BoNTs and minimize the induction of neutralizing antibody against BoNTs, it is highly desirable to engineer the BoNTs into more potent derivatives such that a lower effective therapeutic dose of BoNT can be used in various treatments and applications.

[0022] Through studying the substrate recognition and specificity of the Botulinum Neurotoxin and Tetanus Neurotoxin, the present invention has opened up a new opportunity to engineer Botulinum Neurotoxin and Tetanus Neurotoxin into novel derivatives with enhanced activity and substrate specificity.

[0023] In the present invention, comparative characterization of the substrate recognition pockets in the active sites of the light chain of Botulinum Neurotoxin (LC/B) and the light chain of Tetanus Neurotoxin (LC/T) was performed.

[0024] In one embodiment, the present invention provides methods for optimizing substrate recognition of the light chain of Botulinum Neurotoxin B (LC/B) or the light chain of Tetanus Neurotoxin (LC/T), and provides derivatives of these toxins with elevated catalytic activity.

[0025] In one embodiment, the present invention provides methods for modulating activity of Botulinum Neurotoxin and Tetanus Neurotoxin.

[0026] In one embodiment, the present invention provides derivatives of the light chain of Botulinum Neurotoxin B (LC/B) or derivatives of the entire Botulinum Neurotoxin B with elevated activity. In one embodiment, the Botulinum Neurotoxin described herein comprises a polypeptide of SEQ ID NO.: 1. In another embodiment, derivatives of Botulinum Neurotoxin

described herein comprise one or more alteration in the amino acid sequence. In one embodiment, derivatives of Botulinum Neurotoxin comprise a change of amino acid at position 201 identified according to SEQ ID NO.:1 from serine (S²⁰¹) to proline (P²⁰¹). In another embodiment, the derivative of Botulinum Neurotoxin [S²⁰¹P] comprises a sequence of SEQ ID NO.: 2.

5 [0027] In one embodiment, derivatives of Botulinum Neurotoxin comprise a change of amino acid alanine at position 263 identified according to SEQ ID NO.:1. In another embodiment, derivatives of Botulinum Neurotoxin comprise a change of amino acid isoleucine at position 264 identified according to SEQ ID NO.:1.

[0028] In one embodiment, the present invention provides derivatives of the light chain of
10 Tetanus Neurotoxin (LC/T) or derivatives of the entire Tetanus Neurotoxin. In one embodiment, the Tetanus Neurotoxin described herein comprises a polypeptide of SEQ ID NO.: 3. In another embodiment, derivatives of Tetanus Neurotoxin described herein comprise one or more alteration in the amino acid sequence. In one embodiment, derivatives of Tetanus Neurotoxin comprise a change of amino acid at position 230 as identified according to SEQ ID NO: 3 from
15 leucine (L²³⁰) to isoleucine (I²³⁰). In another embodiment, derivatives of Tetanus Neurotoxin comprise a change of amino acid at position 168 as identified according to SEQ ID NO: 3 from lysine (K¹⁶⁸) to glutamate (E¹⁶⁸). In one embodiment, derivative of Tetanus Neurotoxin [L²³⁰I] comprises a sequence of SEQ ID NO.: 4.

[0029] In another embodiment, derivatives of Tetanus Neurotoxin comprise a change from
20 Leucine-230 (L²³⁰) to Isoleucine-230 (I²³⁰) and a change from Lysine-168 (K¹⁶⁸) to Glutamate-168 (E¹⁶⁸) identified according to SEQ ID NO: 3. In one embodiment, derivative of Tetanus Neurotoxin [K¹⁶⁸E, L²³⁰I] comprises a sequence of SEQ ID NO:5.

[0030] Wildtype of the light chain of Botulinum Neurotoxin B (LC/B) (SEQ ID NO. 1):

25 MPVTINNFNYNPDIDNNNIIMMEPPFARGTGRYYKAFKITDRIWIIPERYTFGYKPEDFNK
SSGIFNRDVCEYYDPDYLNTNDKKNIFLQTMIKLFNRIKSKPLGEKLLEMIINGIPYLGDR
RVPLEEFNTNIASVTVNKLISNPGEVERKKGIFANLIIFGPGPVLNENETIDIGIQNHFAFRE
GFGGIMQMKFCPEYVSVFNNVQENKGASIFNRRGYFSDPALILMHELIHVLHGLYGIKV
DDLPIVPNEKKFFMQSTDAIQAEELYTFGGQDPSIITPSTDKSIYDKVLQNFRGIVDRLNK
30 VLVCISDPNININIKYKFKDKYKFVEDSEGKYSIDVESFDKLYKSLMFGFTETNIAENYK
IKTRASYFSDSLPPVKIKNLLDNEIYTIEEGFNISDKDMEKEYRGQNKAINKQAYEEISKE
HLAVYKIQMCKSV

[0031] Derivative of the light chain of Botulinum Neurotoxin B (LC/B) (SEQ ID NO. 2):

MPVTINNFNYNPDIDNNNIIMMEPPFARGTGRYYKAFKITDRIWIIPERYTFGYKPEDFNK
35 SSGIFNRDVCEYYDPDYLNTNDKKNIFLQTMIKLFNRIKSKPLGEKLLEMIINGIPYLGDR

RVPLEEFNTNIASVTVNKLISNPGEVERKKGIFANLIIFGPGPVLNENETIDIGIQNHFA
 SREGFGGIMQMKFCPEYVPVFNNVQENKGASIFNRRGYFSDPALILMHELIHVLHGLYGIKV
 DDLPIVPNEKKFFMQSTDAIQAEELYTFGGQDPSIITPSTDKSIYDKVLQNFRGIVDRLNK
 VLVCISDPNININIKNFKDKYKFVEDSEGKYSIDVESFDKLYKSLMFGFTETNIAENYK
 5 IKTRASYSFSDSLPPVKIKNLLDNEIYTIEEGFNISDKDMEKEYRGQNKAINKQAYEEISKE
 HLAVYKIQMCKSV

[0032] Wildtype of the light chain of Tetanus Neurotoxin (LC/T) (SEQ ID NO. 3):

MPITINNFRYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFNP
 PSSLIEGASEYYDPNYLRTDSDKDRFLQTMVKLFNRIKNNVAGEALLDKIINAIPYLGNS
 10 YSLLDKFDTNSNSVSFNLEQDPSGATTKSAMLTNLIIFGPGPVLNKNEVRGIVLRVDNK
 NYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTISLTIGKSKYFQDPALLLMHELIHVLHG
 LYGMQVSSHEIIPSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLDNDYKAI
 ANKLSQVTSCNDPNIDIDSYKQIYQQKYQFDKDSNGQYIVNEDKFQILYNSIMYGFTIEIE
 LGKKFNIKTRLSYFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMRVNTN
 15 AFRNVDGSGLVSKLI

[0033] Derivative of the light chain of Tetanus Neurotoxin (LC/T) (SEQ ID NO. 4):

MPITINNFRYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFNP
 PSSLIEGASEYYDPNYLRTDSDKDRFLQTMVKLFNRIKNNVAGEALLDKIINAIPYLGNS
 YSLLDKFDTNSNSVSFNLEQDPSGATTKSAMLTNLIIFGPGPVLNKNEVRGIVLRVDNK
 20 NYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTISLTIGKSKYFQDPALILMHELIHVLHGL
 YGMQVSSHEIIPSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLDNDYKAIA
 NKLSQVTSCNDPNIDIDSYKQIYQQKYQFDKDSNGQYIVNEDKFQILYNSIMYGFTIEIEL
 GKKFNIKTRLSYFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMRVNTNA
 FRNVDGSGLVSKLI

[0034] Derivative of the light chain of Tetanus Neurotoxin (LC/T) (SEQ ID NO. 5):

MPITINNFRYSDPVNNDTIIMMEPPYCKGLDIYYKAFKITDRIWIVPERYEFGTKPEDFNP
 PSSLIEGASEYYDPNYLRTDSDKDRFLQTMVKLFNRIKNNVAGEALLDKIINAIPYLGNS
 YSLLDKFDTNSNSVSFNLEQDPSGATTKSAMLTNLIIFGPGPVLNENEVRGIVLRVDNK
 NYFPCRDGFGSIMQMAFCPEYVPTFDNVIENTISLTIGKSKYFQDPALILMHELIHVLHGL
 30 YGMQVSSHEIIPSKQEIYMQHTYPISAEELFTFGGQDANLISIDIKNDLYEKTLDNDYKAIA
 NKLSQVTSCNDPNIDIDSYKQIYQQKYQFDKDSNGQYIVNEDKFQILYNSIMYGFTIEIEL
 GKKFNIKTRLSYFSMNHDPVKIPNLLDDTIYNDTEGFNIESKDLKSEYKGQNMRVNTNA
 FRNVDGSGLVSKLI

[0035] In one embodiment, the present invention provides pharmaceutical composition
 35 comprising derivatives of Botulinum Neurotoxin or Tetanus Neurotoxin. In one embodiment, the

Botulinum Neurotoxin, Tetanus Neurotoxin and the derivatives of Botulinum Neurotoxin and Tetanus Neurotoxin described herein are fused or coupled with one or more supplementary polypeptides, wherein the supplementary polypeptide is from Botulinum Neurotoxin, Tetanus Neurotoxin or other organisms. In one embodiment, the supplementary polypeptides fused or coupled with the present toxins are native or recombinant polypeptides. In another embodiment, the supplementary polypeptide fused or coupled with the present toxins are artificial polypeptides.

[0036] In one embodiment, the DNA of the light chain (LC) and heavy chain (HC) of Botulinum Neurotoxin or Tetanus Neurotoxin or their derivatives described herein are cloned into the same expression vector, thereby expressing a fusion protein comprising the light chain and heavy chain. In one embodiment, the light chain and heavy chain are originated from the same serotype of the toxin. In another embodiment, the light chain and heavy chain are originated from different serotypes of the toxin.

[0037] In one embodiment, the Botulinum Neurotoxin derivatives described herein are used in a pharmaceutical, clinical, or cosmetic procedure. In one embodiment, the Botulinum Neurotoxin derivatives described herein are used to treat or improve diseases or conditions in various therapies or other applications. In one embodiment, the Botulinum Neurotoxin derivatives described herein can be used in a novel therapy with a lower chance of developing immunoresistance to Botulinum Neurotoxin.

[0038] In one embodiment, the Botulinum Neurotoxin derivatives described herein are used to treat or improve strabismus, blepharospasm, hemifacial spasm, cervical dystonia, spasticity, glabellar facial lines, axillary hyperhidrosis, lower urinary tract disorders, gastrointestinal tract disorders, spasmodic dysphonia, temporomandibular disorder, sialorrhea, chronic diabetic neuropathy, wound healing, vaginism, musculoskeletal pain, disorders related to involuntary skeletal muscle activity such as involuntary muscle spasm and contractions, or any other applicable diseases or conditions.

[0039] In one embodiment, the present invention provides methods for improving or treating diseases or conditions by administering to the subject an effective amount of the Botulinum Neurotoxin derivatives described herein. In another embodiment, the present invention provides methods for improving current therapy using Botulinum Neurotoxin.

[0040] In one embodiment, the present invention provides methods of using Botulinum Neurotoxin with enhanced therapeutic efficacy or pharmaceutical properties. In another embodiment, the present invention provides methods for reducing immunoresistance to Botulinum Neurotoxin in various therapies and applications.

[0041] In one embodiment, the Tetanus Neurotoxin derivatives described herein can be used as a molecular marker or an effective tool to study the mechanisms of exocytosis in central neuron.

[0042] The present invention provides a modified light chain polypeptide of Botulinum Neurotoxin, wherein the Botulinum Neurotoxin has the amino acid sequence of SEQ ID NO.:1, and the modified light chain comprises one or more mutations at positions as identified according to SEQ ID NO.: 1.

[0043] In one embodiment, the modified light chain polypeptide of Botulinum Neurotoxin described herein, wherein the mutations occur at amino acid residues selected from the group consisting of Serine²⁰¹, Alanine²⁶³ and Isoleucine²⁶⁴.

[0044] In one embodiment, the modified light chain polypeptide of Botulinum Neurotoxin described herein comprises an amino acid sequence of SEQ ID NO.: 2.

[0045] The present invention provides a composition comprising the modified light chain polypeptide of Botulinum Neurotoxin described herein, wherein the polypeptide is fused with or coupled with one or more second polypeptide, and wherein the second polypeptide is a polypeptide from Botulinum Neurotoxin or other organisms.

[0046] In one embodiment, the second polypeptide is native or artificial.

[0047] The present invention provides a composition comprising the modified light chain polypeptide of Botulinum Neurotoxin described herein and a pharmaceutical acceptable carrier.

[0048] The present invention provides a method for improving or treating diseases or conditions in a subject, comprising a step of administering to the subject an effective amount of the modified light chain polypeptide of Botulinum Neurotoxin described herein.

[0049] In one embodiment, the diseases or conditions are selected from the group consisting of strabismus, blepharospasm, hemifacial spasm, cervical dystonia, spasticity, glabellar facial lines, axillary hyperhidrosis, involuntary muscle spasm, lower urinary tract disorders, gastrointestinal tract disorders, spasmodic dysphonia, temporomandibular disorder, sialorrhea, chronic diabetic neuropathy, wound healing, vaginism, musculoskeletal pain and involuntary muscle contractions.

[0050] In one embodiment, the method for improving or treating diseases or conditions described herein, wherein reduced immunoresistance to Botulinum Neurotoxin in a subject is induced in the subject. In another embodiment, said reduced immunoresistance to Botulinum Neurotoxin is induced in the subject as compared to treatment with wild-type Botulinum Neurotoxin.

[0051] The present invention provides a modified light chain polypeptide of Tetanus Neurotoxin, wherein the Tetanus Neurotoxin has the amino acid sequence of SEQ ID NO.: 3, and the

modified light chain comprises one or more mutations at positions as identified according to SEQ ID NO.: 3.

[0052] In one embodiment, the modified light chain polypeptide of Tetanus Neurotoxin described herein, wherein the mutations occur at amino acid residues selected from the group consisting of Lysine¹⁶⁸ and Leucine²³⁰.

[0053] In one embodiment, the modified light chain polypeptide of Tetanus Neurotoxin described herein comprises an amino acid sequence of SEQ ID NO.: 4 or 5.

[0054] The present invention provides a composition comprising the modified light chain polypeptide of Tetanus Neurotoxin described herein, wherein the polypeptide is fused with or coupled with one or more second polypeptide, and wherein the second polypeptide is a polypeptide from Tetanus Neurotoxin or other organisms.

[0055] In one embodiment, the second polypeptide is native or artificial.

[0056] In one embodiment, the composition comprising the modified light chain polypeptide of Tetanus Neurotoxin described herein is used as a molecular marker to study exocytosis in the neuron.

[0057] The present invention provides a composition comprising the modified light chain polypeptide of Tetanus Neurotoxin described herein and a pharmaceutical acceptable carrier.

[0058] The present invention provides a method for improving or treating diseases or conditions in a subject, comprising a step of administering to the subject an effective amount of the modified light chain polypeptide of Tetanus Neurotoxin described herein.

[0059] This invention will be better understood by reference to the examples which follow. However, one skilled in the art will readily appreciate that the examples provided are merely for illustrative purposes and are not meant to limit the scope of the invention which is defined by the claims following thereafter.

[0060] Throughout this application, it is to be noted that the transitional term “comprising”, which is synonymous with “including”, “containing” or “characterized by”, is inclusive or open-ended, and does not exclude additional, un-recited elements or method steps.

EXAMPLE 1

Modification of Botulinum Neurotoxin and Tetanus Neurotoxin

Plasmid Construction For Protein Expression

[0061] Plasmids for the expression of LC/T (1-436) (accession no. of the Tetanus Neurotoxin: X06214.1), LC/B (1-440) (accession no. of the Botulinum Neurotoxin: AB084152.1) and VAMP2 (1-97) and subsequent protein expression and purification were performed as previously described (30-32). Site directed mutagenesis of pLC/T, pLC/B and pVAMP2 were performed

using QuickChange (Stratagene) protocols as previously described (30, 31). Plasmids were sequenced to confirm the mutation and that additional mutations were not present within the ORFs. Mutated proteins were produced and purified as described (30-33).

5 Linear Velocity And Kinetic Constant Determinations For VAMP2 Cleavage By LC/B And LC/T

[0062] Linear velocity reactions (10 μ l) were performed as previously described (30, 31, 33). VAMP2 proteins (5 μ M) were incubated with varying concentrations of LC/T, LC/B or derivatives in 10 mM Tris-HCl (pH 7.6) with 20 mM NaCl at 37°C for 10 min. Reactions were
10 stopped by adding SDS-PAGE buffer, and VAMP2 and cleavage product were resolved by SDS-PAGE. The amount of VAMP2 cleaved was determined by densitometry. K_m and k_{cat} were determined using the same assay where VAMP2 concentrations were adjusted to 1-300 μ M to achieve ~10% cleavage by LC/T and derivatives. Data were fit into the Michaelis-Menten equation (reaction velocity versus substrate concentration) and kinetic constants were derived
15 using the GraphPad Program (San Diego, CA). At least five independent assays were performed to determine the kinetic constants for each protein.

Compensatory Assay

[0063] Effect of compensatory mutations within LC/T on the cleavage of VAMP2 and VAMP2
20 mutant [F⁷⁷D] was determined as previously described with modification (31). Briefly, 5 μ M VAMP2 or VAMP2 mutant were incubated with LC/T or derivatives. Uncleaved and cleaved VAMP2 were then resolved by SDS-PAGE and quantified. The amount of wild type LC/T or derivatives in the reaction were plotted verses % cleavage of VAMP2. The amount of LC required to cleave 50% of VAMP2 or VAMP2 mutant was then calculated (EC_{50}).

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LC Crystallization And Structure Determination

[0064] LC/T derivative [K¹⁶⁸E, L²³⁰I] was crystallized by hanging drop vapor diffusion method. LC/T [K¹⁶⁸E, L²³⁰I] was stored in a buffer of 10 mM Tris, 20 mM NaCl (pH 7.9) at a concentration of 7.5 mg/ml. Each of the hanging drop contained 1 μ l protein solution and 1 μ l
30 mother liquor (250 mM Mg(NO₃)₂ and 15% PEG 3350). Crystals were grown at 16°C for 4-5 days until maturation. For data collection, crystals were harvested and cryoprotected in the mother liquor supplemented with 20% glycerol. Data was collected at 100K on Rigaku MicroMaxTM-007HF x-ray machine and processed using iMOSFLM (34). The crystals were found to have a space group of monoclinic group C222 with a cell parameter of $a = 105.38 \text{ \AA}$, $b = 176.83 \text{ \AA}$, $c = 57.36 \text{ \AA}$ and were diffracted to 2.6 $\text{\AA}$. There was one molecule per asymmetric
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unit. The structure of LC/T [$K^{168}E$, $L^{230}I$] was solved by molecular replacement using the PHASER module in the CCP4i suite of programs with LC/T (PDB ID: 1Z7H) as the homology model (35). Subsequent structural refinement was conducted using REFMAC module in CCP4 (36). Manual structure rebuilding was done using WINCOOT (37). The final structure has been deposited to the protein data bank (PDB) with the PDB ID: 4J1L.

Cleavage of Endogenous VAMP of Neuro2A Cell By Recombinant LC/T, LC/B And Their Derivatives

[0065] Neuro-2A cells were cultured in minimum essential medium supplemented with 10% newborn calf serum, 1.4% sodium bicarbonate, and 0.5% penicillin-streptomycin at 37 °C in 5% CO₂. Confluent cells were harvested and lysed by passing through a 25 gauge needle for 20~30 times on ice. Nuclei and unbroken cells were spun down by centrifuging the lysed product for 5 min at 2500 rpm and the supernatant was collected for assay. After incubating the cell lysate with different amount of LCs at 37°C for 10 min in a reaction volume of 10 µl, the reaction was stopped by adding equal amount of SDS-PAGE sample buffer and boiling the reaction mixture at 100°C for 10 min. Cleavage of VAMP was analyzed by western blotting using anti-VAMP2 and anti-actin antibody.

Results

[0066] BoNT/B and TeNT were found to cleave substrate VAMP2 at the same scissile bond, while their efficiencies of substrate hydrolysis were different, with LC/B being more active than LC/T by about 20-folds. Earlier characterization of VAMP2 recognition by LC/B and LC/T showed that the active site of the two LCs displayed a similar arrangement for VAMP2 recognition. The major difference between the two systems was found to be at the P2'-S2' substrate recognition site (33, 38). Mutation at the P2' site of VAMP2 (E^{78} to alanine, [$E^{78}A$]) reduced LC/B cleavage by ~8-fold but reduced LC/T cleavage by ~240-fold, suggesting different role of VAMP2 E^{78} on LC/B and LC/T substrate cleavage (33).

[0067] Further biochemical characterization of the S2' pocket of LC/B and LC/T showed that the S2' pocket of LC/B and LC/T is formed by R^{370} and R^{374} respectively (Figure 1). Compensatory mutational assay suggested that R^{370} of LC/B directly interacts with E^{78} of VAMP2, while the interaction of R^{374} of LC/T with E^{78} of VAMP was not evidenced (38). These data prompted current study to examine the different mechanism of substrate recognition at the P2' site of VAMP (E^{78}) by LC/B and LC/T.

Optimization of LC/T and LC/B S1' Pockets Enhanced Their Catalytic Activities

[0068] Figure 1 illustrates the active site of LC/B and LC/T for interacting with the substrate VAMP2. Comparison of the structures of LC/B and LC/T revealed that the S2' pocket of the two LCs are similar and comprise an arginine residue (R³⁷⁰ in LC/B and R³⁷⁴ in LC/T), while their S1' pockets were different. The S1' pocket of LC/B is composed of F⁹⁵, V²⁰⁰, S²⁰¹, L²²⁶ and I²²⁷, whereas the S1' pocket of LC/T is composed of F¹⁹⁹, V²⁰⁴, P²⁰⁵, L²²⁹, L²³⁰ and L²³¹. Mutation of these S1' pocket residues to alanine showed no effect on LC/B substrate hydrolysis except for I²²⁷A, which showed ~80-fold reduction of k_{cat} , but not K_m (38). For LC/T, mutation of the S1' pocket residues did not affect substrate hydrolysis except mutations LC/T [L²³⁰A] or LC/T [P²⁰⁵A] that reduced k_{cat} by ~30-fold without affecting K_m (38). Within these S1' pocket residues, P²⁰⁵ of LC/T is found to be structurally aligned well with S²⁰¹ of LC/B while L²³⁰ of LC/T is aligned well with I²²⁷ of LC/B (Figure 1). The different composition of S1' pocket of LC/B and LC/T may contribute not only to the different recognition of P1', but also the P2' site of VAMP, which may further affect the different catalytic activity of LC/B and LC/T.

[0069] Mutations were then performed to swap the residues between the S1' pocket of LC/B and LC/T. Mutation of LC/T [P²⁰⁵S] showed a ~80-fold reduction in k_{cat} , while retained the same K_m . Mutation of LC/T [L²³⁰I] showed a ~20-fold elevated k_{cat} and similar K_m (Table 1, Figure 2a). These data suggested that proline and isoleucine are the optimal residues to form the S1' pocket of LC/T for interacting with the P1' site residue F⁷⁷ of VAMP.

[0070] To check this hypothesis, residues swapping was then performed in the S1' pocket of LC/B. Mutation of LC/B [I²²⁷L] showed a ~590-fold reduction in k_{cat} , but no change in K_m . Mutation of LC/B [S²⁰¹P] showed a ~10-fold increase in k_{cat} and no change in the K_m (Table 1, Figure 2b). These data further confirmed that proline and isoleucine constitute the most optimal S1' pocket for LC/B and LC/T, and indicate that the current substrate recognition pockets of LC/B and LC/T are not optimal. Optimization of these pockets could improve the catalytic activity of the toxins. To verify that changing the S1' pocket of LC/B and LC/T did not alter their substrate specificity, the above four mutants' cleavage activities on VAMP2 F⁷⁷D were examined. F⁷⁷ of VAMP2 is an important residue for the scissile action by both LC/B and LC/T and mutation of this residue likely affects the cleavage activity of the LC/B and LC/T. Since none of the above mutants of LC/B and LC/T could cleave F⁷⁷D, suggesting that the substrate specificity for VAMP2 of these LC/B and LC/T mutants remained the same regardless of the mutations (Figures 2A-B).

TABLE 1

Kinetic constants of LC/B, LC/T and their derivatives on cleaving VAMP2

LCs	LC Pockets	LC derivative	K_m (μM)	k_{cat} (S^{-1})	k_{cat}/K_m ($\text{S}^{-1} \cdot \mu\text{M}^{-1}$)
LC/B		Wt-LC/B	1.7 $\pm$ 0.3	1.1	6.5 $\times 10^{-1}$
	S1'swapping	S ²⁰¹ P	1.6 $\pm$ 0.2	11.5	7.2
	S1' swapping	I ²²⁷ L	1.6 $\pm$ 0.4	1.8 $\times 10^{-3}$	1.1 $\times 10^{-3}$
LC/T		Wt-LC/T	4.1 $\pm$ 0.2	1.6 $\times 10^{-1}$	3.9 $\times 10^{-2}$
	S1' swapping	P ²⁰⁵ S	4.4 $\pm$ 0.4	2.1 $\times 10^{-3}$	4.7 $\times 10^{-4}$
	S1' swapping	L ²³⁰ I	4.2 $\pm$ 0.2	4.1	9.7 $\times 10^{-1}$
	S1, S1' swapping	K ¹⁶⁸ E, L ²³⁰ I	4.0 $\pm$ 0.4	15.9	4.0
	S3, S1, S1'swapping	K ¹⁶⁸ E, L ²³⁰ I, R ¹⁸⁸ M	ND	ND	ND

ND: Not Determined, as the enzyme is too inactive to determine the kinetic constants.

Optimization of LC/T S1' Pocket Regained Optimal Recognition of P2' Site

- 5 [0071] Since the S1' and S2' pockets are in close proximity, the different composition of S1' pocket of LC/B and LC/T may be correlated to the different properties observed in the S2' pockets of LC/B and LC/T. Although both S2' pockets of LC/B and LC/T comprise an arginine residue, previous study showed that R³⁷⁰ of LC/B directly interacts with E⁷⁸ of VAMP, whereas R³⁷⁴ of LC/T does not directly interact with E⁷⁸ of VAMP (38). In addition, mutating E⁷⁸ to alanine (E78A) at the P2' site of VAMP was found to have less impact on cleavage by LC/B as compared to the impact on cleavage by LC/T (38).

- 10 [0072] In the present study, LC/T [L²³⁰I], which was engineered for an optimal S1' pocket, was tested for its activity on cleaving VAMP2 E⁷⁸R. As shown in Table 2, the amount of LC/T required to cleave 50% of VAMP2 was about 120 nM (EC₅₀). LC/T showed no cleavage on VAMP2 E⁷⁸R even at a concentration as high as 36,000 nM. LC/T [L²³⁰I] cleaved VAMP2 about ~24 fold more efficient than Wt-LC/T, while LC/T [L²³⁰I] cleaved VAMP E⁷⁸R at a similar efficiency as Wt-LC/T, but is 360-fold more efficient than LC/T on cleaving VAMP E⁷⁸R (Table 2). These data suggested that optimization of S1' pocket of LC/T enhanced the tolerance towards the P2' site mutation from E⁷⁸ to R⁷⁸. The less optimal LC/T S1' pocket may also explain why mutation of E⁷⁸ at the P2' site would have a greater effect on LC/T substrate cleavage than on LC/B substrate cleavage. Previous study also showed that the S2' pocket residue of LC/B, R³⁷⁰, interacts and forms salt bridge with E⁷⁸ at the P2' site of VAMP2, while the S2' pocket residue of LC/T, R³⁷⁴, does not (38). To test whether different S2'-P2' interaction between LC/B and LC/T was due to the less optimal composition of the S1' pocket, the

efficiency of LC/T [$L^{230}I$, $R^{374}E$] on cleaving VAMP2 $E^{78}R$ was examined. LC/T [$L^{230}I$, $R^{374}E$] showed no cleavage on VAMP2, while LC/T [$L^{230}I$, $R^{374}E$] was able to cleave VAMP2 at an efficiency higher than LC/T (Table 2). These data suggested that the less optimal organization of S1' pocket in LC/T is less favorable for direct interaction between R^{374} of LC/T and E^{78} of VAMP2.

TABLE 2

Results of Compensatory Mutational Assay On Cleaving VAMP2 and VAMP2 $E^{78}R$ By LC/T

	VAMP2		VAMP2 ($E^{78}R$)	
	EC ₅₀	Ratio of activity (WT: derivative)	EC ₅₀	Ratio of activity (WT: derivative)
LC/T	120 nM		>36000 nM	
LC/T($L^{230}I$)	5 nM	24	100 nM	>360
LC/T($K^{168}E$, $L^{230}I$)	1.2 nM	100	24 nM	>1440
LC/T($L^{230}I$, $R^{370}E$)	>36000 nM	<1/300	50 nM	>720

EC₅₀ refers to the concentration of LC proteins resulting in 50% cleavage of VAMP2 or VAMP2 $E^{78}R$.

Optimization of S1' And S1 Substrate Recognition Pockets Further Enhanced LC/T Activity

[0073] Previous study identified two residues that contributed to elevated activity of LC/T (38). Mutation of S1 pocket residue, LC/T [$K^{168}E$], and S3 pocket residue, LC/T [$R^{188}M$], were found to increase the catalytic activity of LC/T (38). These mutations were incorporated into LC/T together with the [$L^{230}I$] mutation to test their combined effect on LC/T activity. Double-mutation mutants, LC/T ($K^{168}E$, $L^{230}I$) was found to be ~100-fold more active than Wt-LC/T on cleaving VAMP2, as indicated by the elevated k_{cat} value, but not K_m value (Table 1, Figure 2a). The triple-mutation mutant, LC/T [$K^{168}E$, $L^{230}I$, $R^{188}M$] showed no activity on cleaving VAMP2. One possible explanation for the abolished activity is the combination of three mutations at the active site of LC/T may impair the correct conformation of LC/T (Table 1).

Orientation of Side Chain of L^{230} of LC/T Affects Its Optimal Recognition of Both P1' and P2' Sites

[0074] Both isoleucine and leucine are hydrophobic residue that are likely to interact with F^{77} of VAMP2 through hydrophobic interaction. However, in the case of LC/B and LC/T, the isoleucine in the S1' pocket of LC/B and LC/T was found to be more significant in the

interaction. It could be due to different orientation of the isoleucine residue in the pocket that favors interaction with F⁷⁷ of VAMP2. Structural analysis of the S1' pockets of LC/B and LC/T revealed that I²²⁷ in LC/B resides at a flatter position than L²³⁰ in LC/T. The bulky leucine in LC/T may push the interacting residue F⁷⁷ of VAMP2 outward, thereby restraining the interaction between R³⁷⁴ and E⁷⁸ of VAMP2. The flatter position of isoleucine in LC/B may provide an optimal position for fitting F⁷⁷ and E⁷⁸ of VAMP2 to the active site of LC/B, hence favors the interactions of I²²⁷-F⁷⁷ and R³⁷⁰-E⁷⁸.

[0075] Crystal structure of LC/T [K¹⁶⁸E, L²³⁰I] was determined and compared with Wt-LC/T. Figure 3A illustrates the F_o-F_c electron density of L²³⁰I mutations in the LC/T (K¹⁶⁸E, L²³⁰I) structure. LC/T [K¹⁶⁸E, L²³⁰I] can perfectly aligned to Wt LC/T with a Root Mean Square Deviation (RMSD) of 0.150 (370 to 370 atoms with 421 atoms aligned), suggesting that the overall conformation of LC/T [K¹⁶⁸E, L²³⁰I] is comparable to that of Wt-LC/T. LC/T [K¹⁶⁸E, L²³⁰I] also aligned well with Wt LC/B with a RMSD of 0.750 (341 to 341 atoms with 421 atoms aligned). The analysis showed that mutation of leucine to isoleucine flattens the S1' pocket to a similar level as in LC/B (Figure 3). Most importantly, distance between the side chains of L²³⁰ and R³⁷⁴ of LC/T was estimated to be 7.2 Å (Figure 4A) and the distance between the side chains of I²²⁷ and R³⁷⁰ was 7.9Å in LC/B (Figure 4B). When L²³⁰ was mutated to isoleucine, [L²³⁰I], the distance between the side chain of I²³⁰ and R³⁷⁴ of LC/T was 8.3Å (Figure 4C), which is larger than the distance 7.2 Å between L²³⁰ and R³⁷⁴ of Wt-LC/T (Figure 4A). These data further supported that distance between the S1' and S2' pockets in LC/B and LC/T determines their efficiency of recognizing the P1' and P2' sites of VAMP2. The wider space between the S1' and S2' pockets favors accommodating the P1' site residue F⁷⁷ and the P2' site residue E⁷⁸ of VAMP2 into these two pockets.

TABLE 3

Crystallographic Statistics of LC/T [K¹⁶⁸E, L²³⁰I]

	LC/T [K ¹⁶⁸ E, L ²³⁰ I]
Data collection	
Space group	C222
Unit cell parameters (Å)	
<i>a</i>	105.38
<i>b</i>	176.83
<i>c</i>	57.36
Resolution range (Å)	52.69-2.60 (2.74-2.60)
No. of total reflections	113923
No. of unique reflections	16451

I/ σ	13.9 (3.9)
Completeness (%)	97.7 (97.4)
R_{merge} (%)	9.5(18.1)
<i>Structure refinement</i>	
Resolution ($\text{\AA}$)	48.12-2.60
$R_{\text{cryst}}/R_{\text{free}}$ (%)	23.76/26.64
r.s.m.d. bonds ($\text{\AA}$)/angles ($^{\circ}$)	0.015/1.838
No. of reflections	15636
Working set	14815
Test set	821
No. of atoms	3317
Protein atoms	3179
Water molecules	138
Average B -factor ($\text{\AA}^2$)	
Main chain	34.52
Side chain	34.36
Water molecules	32.88

Activities of LC/B, LC/T And Their Derivatives On Cleaving Endogenous VAMP2

[0076] To check the activities of LC/T derivatives on cleaving natural substrate VAMP2 in neuronal cells, various LC/T derivatives were incubated with Neuro2A lysate. As illustrated in Figure 5A, LC/T [K^{168}E , L^{230}I] was more active than Wt-LC/T on cleaving endogenous VAMP2 in Neuro 2A cells. Similarly, as presented in Figure 5B, LC/B [S^{201}P] also appeared to be more active than Wt-LC/B on cleaving endogenous VAMP2.

[0077] As seen in the histograms in Figures 5A and 5B, LC/T and LC/T [K^{168}E , L^{230}I] showed a much lower activity on cleaving endogenous VAMP2 substrate than LC/B and LC/B [S^{201}P], even though the activities of LC/T [K^{168}E , L^{230}I] was slightly higher than Wt-LC/B on cleaving recombinant VAMP2. These observations maybe accounted for by the more complicated mechanism of VAMP2 recognition in neurons, wherein VAMP2 exists as a SNARE complex in association with SNAP25 and Syntaxin 1a. Hence, other factors other than accessibility of VAMP2 to LC/B and LC/T may also affect the efficiency of the toxins on cleaving native VAMP2. Previous study on LC/A found that in addition to the substrate recognition region, LC/A also interacts with SNAP25 at an additional site outside the SNARE complex to gain initial access to SNAP25 (45). Further research will be needed to figure out the different accessibility of LC/B and LC/T to VAMP2 in the SNARE complex.

[0078] In summary, these data suggest that LC/B is more active on cleaving natural substrate VAMP2 than LC/T, and imply that LC/B may be a more desirable candidate than LC/T to be used in human therapy.

5 Discussion

[0079] Mutation of an active site residue of the light chain of serotype A (LC/A), [K¹⁶⁵L], resulted in a 4-fold increase in substrate hydrolysis (44). In addition, [R¹⁸⁸M], a S4 pocket mutation, was found to increase LC/T substrate hydrolysis by ~5 fold (38). The present invention provides a comparative study on LC/B and LC/T and demonstrates the possibility to engineer the light chain of CNTs for elevated activities.

[0080] The present invention found that S1 pocket mutation of LC/T from Lysine (K¹⁶⁸) to Glutamate¹⁶⁸ (E¹⁶⁸) [K¹⁶⁸E] can increase the rate of cleaving VAMP2 to a level comparable to the rate of LC/B.

15 Optimization of Substrate Recognition Pockets in LC/T

[0081] The present invention found that S1' pockets of both LC/B and LC/T are most significant to the catalytic activity of the two toxins. The side chain of Leucine²³⁰ (L²³⁰) of LC/T is more bulky in comparison to the Isoleucine²²⁷ (I²²⁷) of LC/B, which is less favorable for accommodating P1' and P2' site residues of VAMP2 and dramatically affects its catalytic activity (Figures 3-4). Mutant LC/T [L²³⁰I] was found to have a higher catalytic activity than the Wt-LC/T by ~25-folds (Table 2).

[0082] The findings herein also suggested that VAMP2 recognition by different pockets in Botulinum Neurotoxin is closely related. For instance, the less optimized S1' pocket could affect the recognition of VAMP2 by the S2' pocket. Mutant LC/T [L²³⁰I] was found to have its catalytic activity increased by ~25-folds while the double mutant LC/T [K¹⁶⁸E, L²³⁰I] has its catalytic activity increased by ~100-folds (Table 2).

Optimization of S1' and S1 Substrate Recognition Pockets in LC/B

[0083] In the case of LC/B, the native residue Isoleucine²²⁷ (I²²⁷) is favorable for cleaving VAMP2. However, the native Serine²⁰¹ (S²⁰¹) in the S1 pocket of LC/B seemed to be less optimal than the corresponding proline residue (P²⁰⁵) in LC/T. The present study found that mutation of LC/B [S²⁰¹P] could increase LC/B activity by more than 10-folds (Table 1).

Comparison of LC/B and LC/T Activity on Recombinant And Native VAMP2

[0084] The present study showed that LC/T [$L^{230}I$] and LC/T [$K^{168}E$, $L^{230}I$] have a higher activity on cleaving recombinant VAMP2 than the wildtype toxin (Table 2). The present study also showed that LC/T [$K^{168}E$, $L^{230}I$] attained a higher activity on cleaving native VAMP2 in Neuro2A cell than the wildtype toxin (Figure 5A). The highly active LC/T derivatives described in the present invention can be used as more effective tools to study the mechanisms of exocytosis in central neuron.

[0085] The present study showed that LC/B [$S^{201}P$] has a higher activity on cleaving recombinant VAMP2 than the wildtype toxin by more than 10-fold (Table 1) and on cleaving native VAMP2 in Neuro2A cell (Figure 5B). The LC/B derivative described herein can be used to replace LC/B protein currently used in various therapeutic, cosmetic or other applications, or be developed into novel therapy that may minimize immunoresistance to BoNT.

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What is claimed is:

1. A modified light chain polypeptide of Botulinum Neurotoxin, wherein the Botulinum Neurotoxin has the amino acid sequence of SEQ ID NO.:1, and the modified light chain
5 comprises one or more mutations at positions as identified according to SEQ ID NO.: 1.
2. The polypeptide of claim 1, wherein the mutations occur at amino acid residues selected from the group consisting of Serine²⁰¹, Alanine²⁶³ and Isoleucine²⁶⁴.
- 10 3. The polypeptide of claim 1, wherein the modified light chain polypeptide comprises an amino acid sequence of SEQ ID NO.: 2.
4. A composition comprising the polypeptide of claim 1, wherein the polypeptide is fused with or coupled with one or more second polypeptide, and wherein the second polypeptide is a
15 polypeptide from Botulinum Neurotoxin or other organisms.
5. The composition of claim 4, wherein the second polypeptide is native or artificial.
6. A composition comprising the polypeptide of claim 1 and a pharmaceutical acceptable
20 carrier.
7. A method for improving or treating diseases or conditions in a subject, comprising a step of administering to the subject an effective amount of the polypeptide of claim 1.
- 25 8. The method of claim 7, wherein the diseases or conditions are selected from the group consisting of strabismus, blepharospasm, hemifacial spasm, cervical dystonia, spasticity, glabellar facial lines, axillary hyperhidrosis, involuntary muscle spasm, lower urinary tract disorders, gastrointestinal tract disorders, spasmodic dysphonia, temporomandibular disorder, sialorrhea, chronic diabetic neuropathy, wound healing, vaginism, musculoskeletal
30 pain and involuntary muscle contractions.
9. The method of claim 7, wherein reduced immunoresponse to Botulinum Neurotoxin is induced in the subject as compared to treatment with wild-type Botulinum Neurotoxin.

10. A modified light chain polypeptide of Tetanus Neurotoxin, wherein the Tetanus Neurotoxin has the amino acid sequence of SEQ ID NO.: 3, and the modified light chain comprises one or more mutations at positions as identified according to SEQ ID NO.: 3.

5 11. The polypeptide of claim 10, wherein the mutations occur at amino acid residues selected from the group consisting of Lysine¹⁶⁸ and Leucine²³⁰.

12. The polypeptide of claim 10, wherein the modified light chain polypeptide comprises an amino acid sequence of SEQ ID NO.: 4 or 5.

10

13. A composition comprising the polypeptide of claim 10, wherein the polypeptide is fused with or coupled with one or more second polypeptide, and wherein the second polypeptide is a polypeptide from Tetanus Neurotoxin or other organisms.

15 14. The composition of claim 13, wherein the second polypeptide is native or artificial.

15. The composition of claim 13, wherein the composition is used as a molecular marker to study exocytosis in the neuron.

20 16. A composition comprising the polypeptide of claim 10 and a pharmaceutical acceptable carrier.

17. A method for improving or treating diseases or conditions in a subject, comprising a step of administering to the subject an effective amount of the polypeptide of claim 10.

25

Figure 1

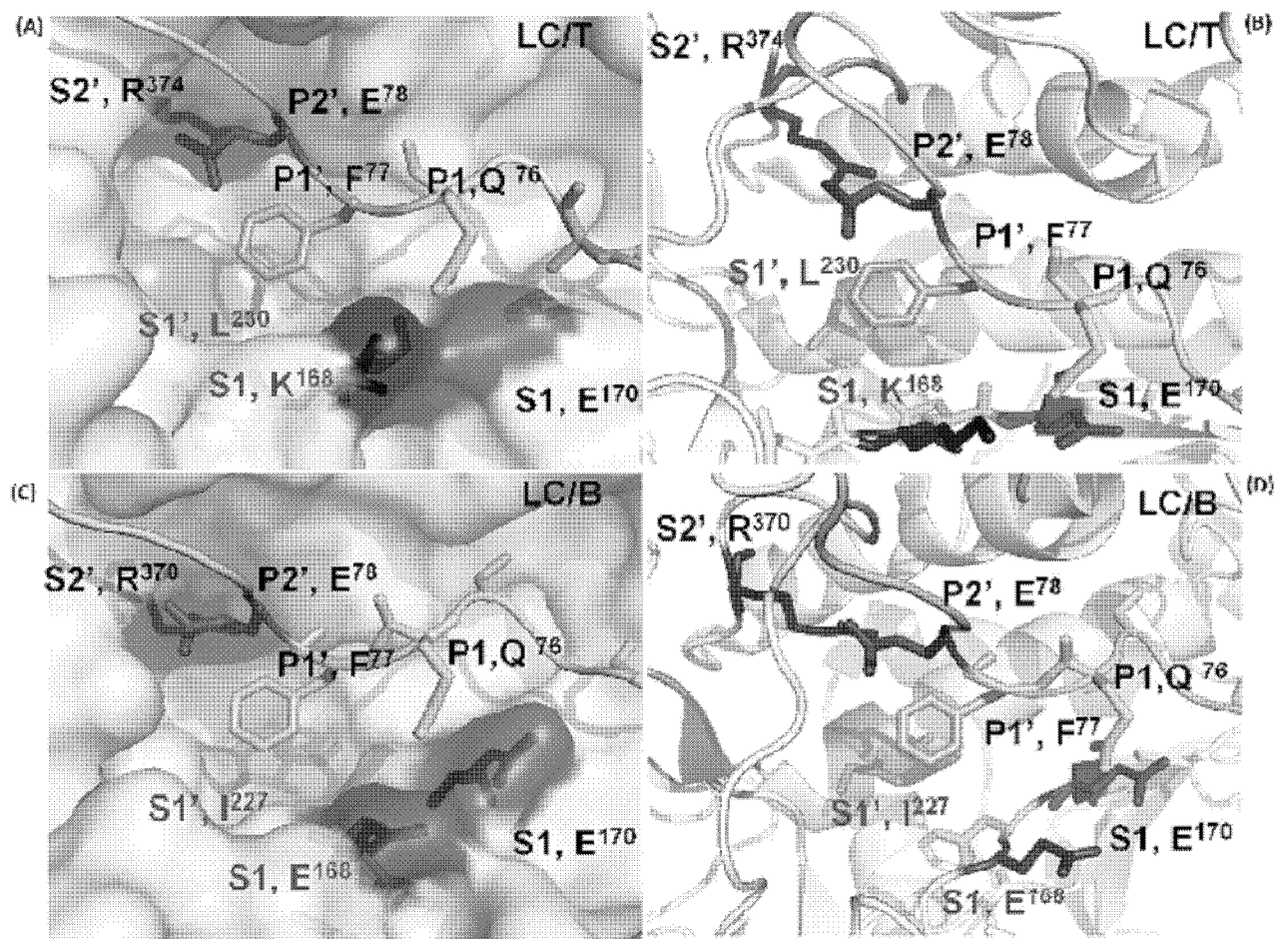


Figure 2

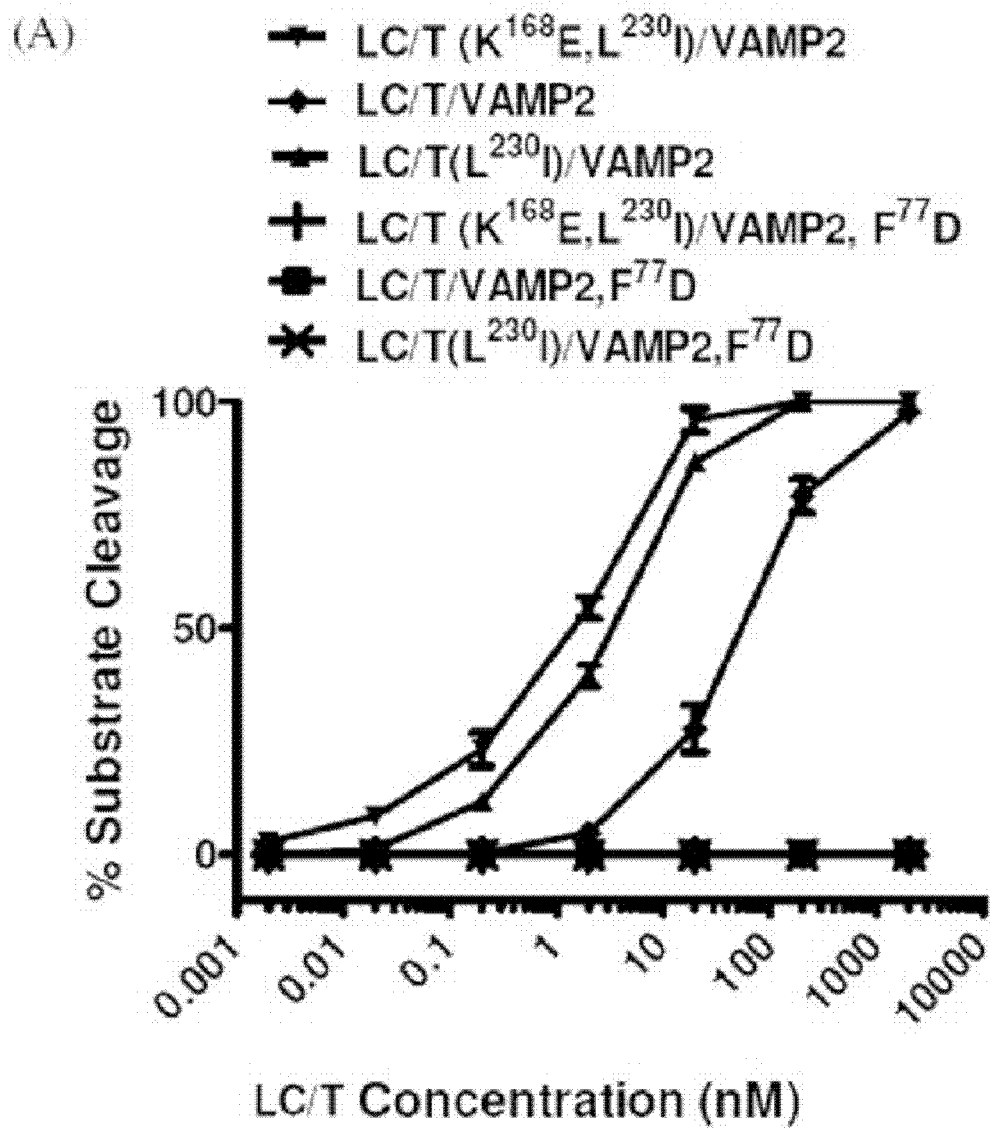


Figure 2 (Continued)

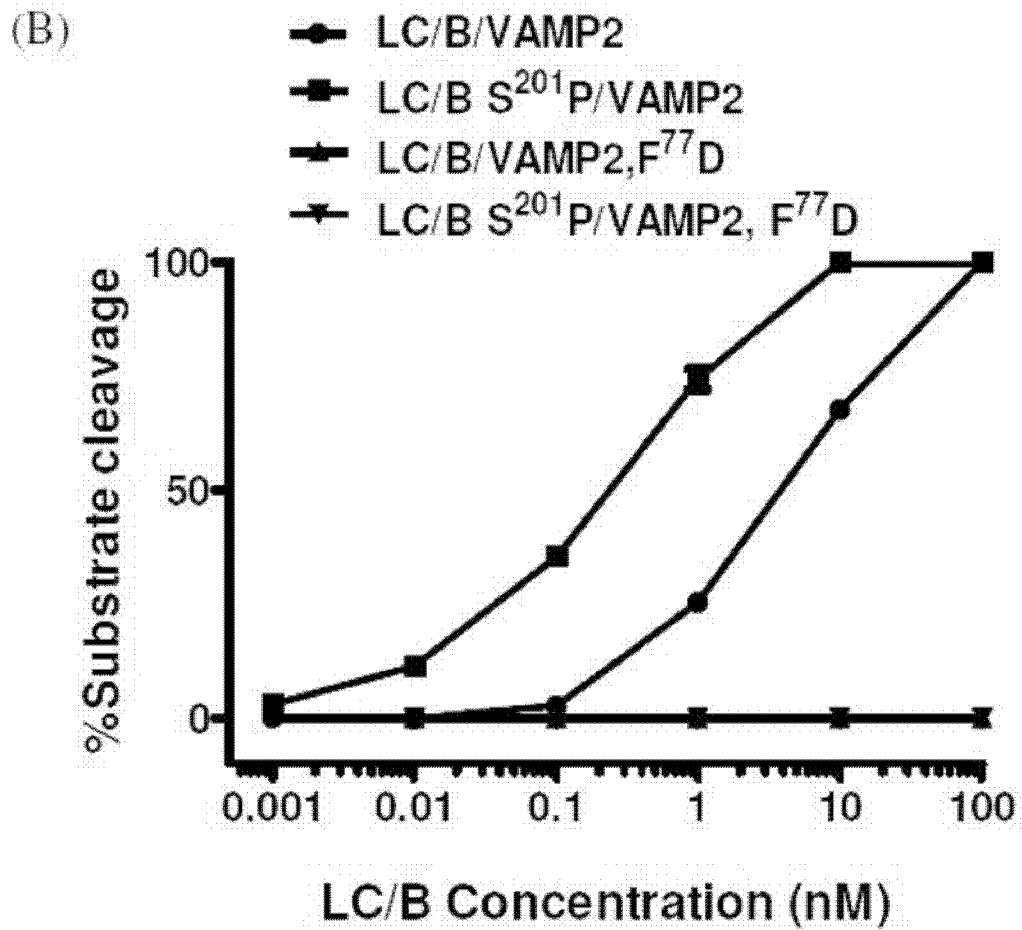


Figure 3

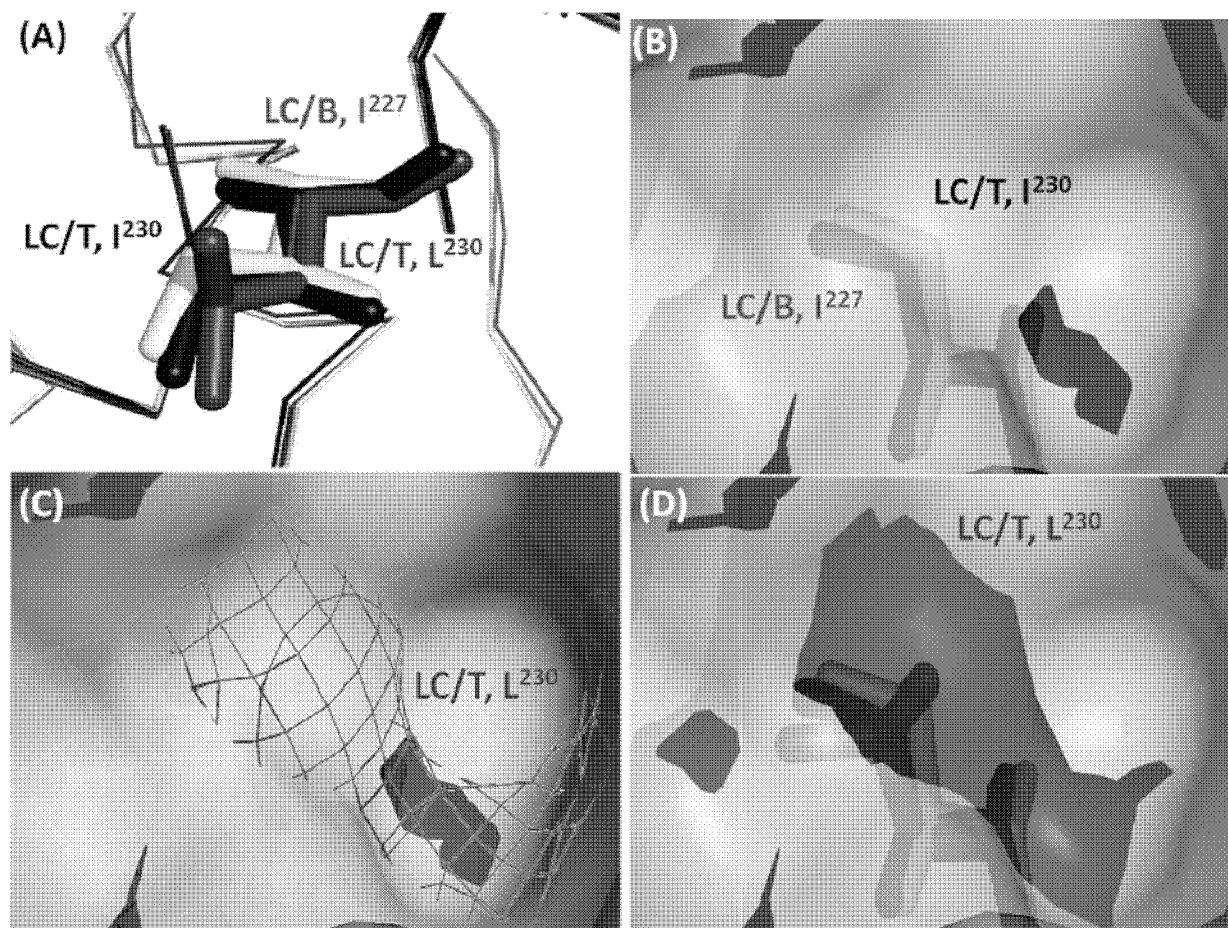


Figure 4

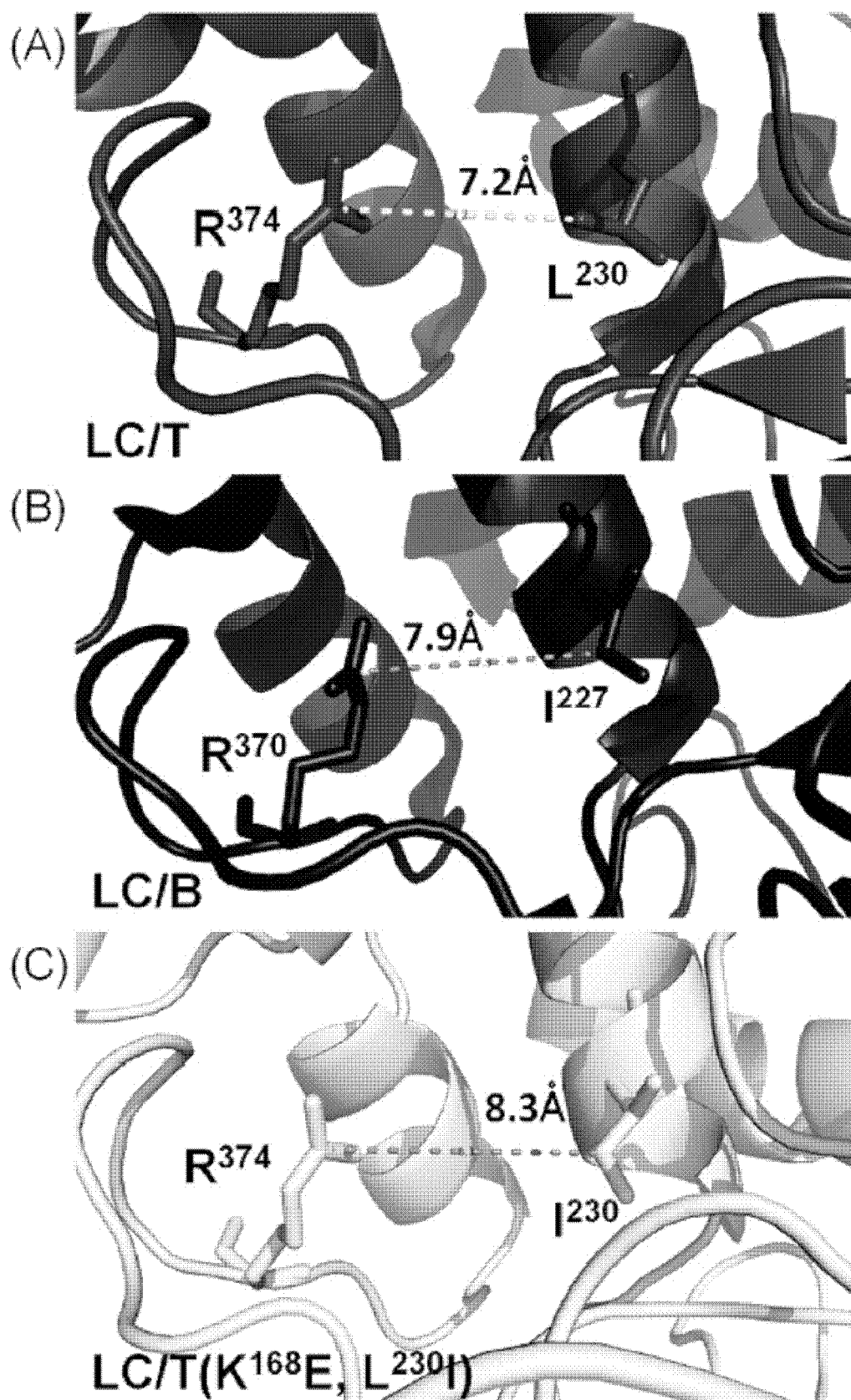
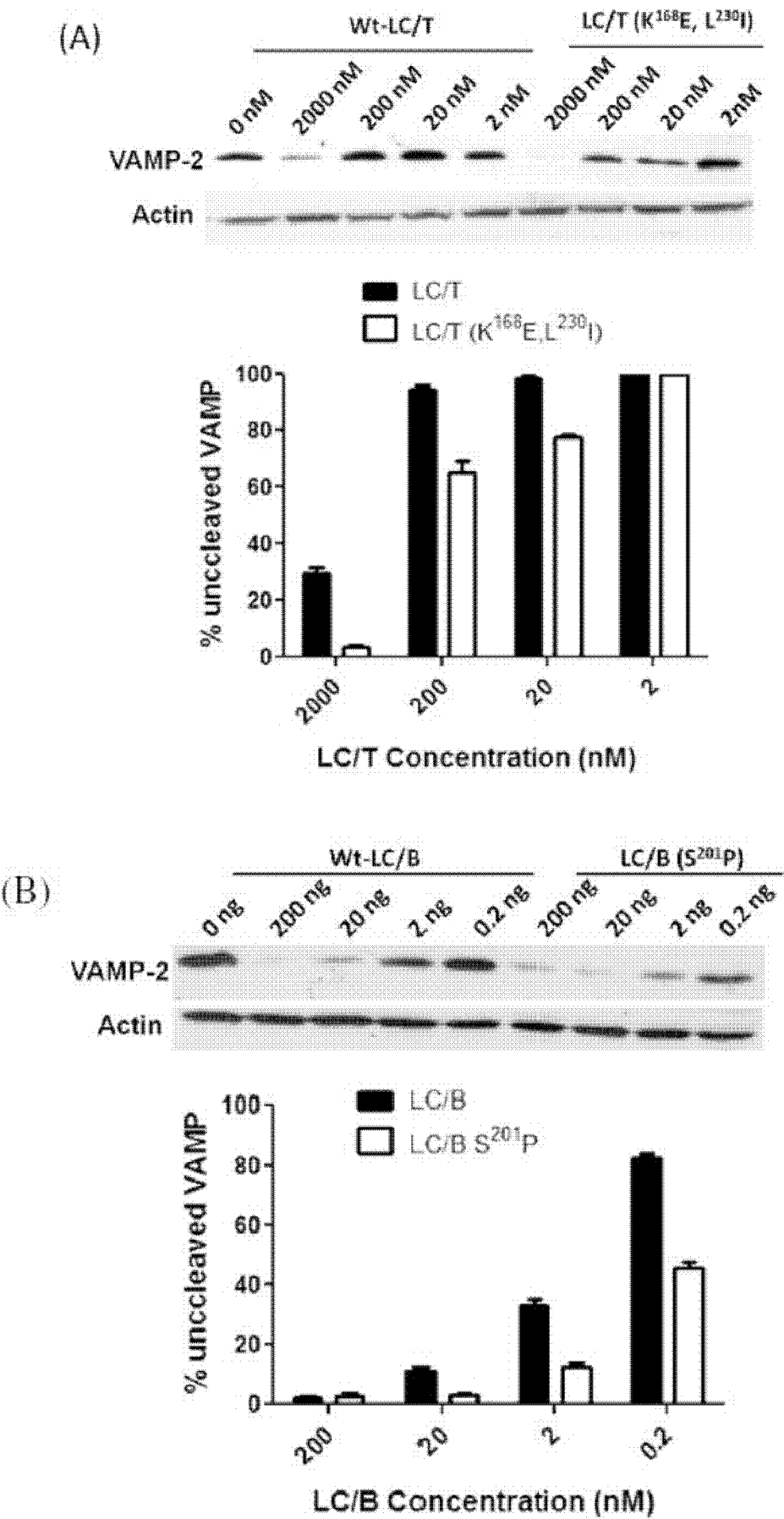


Figure 5



INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2014/084725

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/195(2006.01)i; A61K 38/16(2006.01)i

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)

C07K 14-; A61K 38-

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)

WPI, EPODOC, CNKI, CNPAT, PubMed, ISI Web of Knowledge, Google, NCBI GenBank, EBI-EMBL: Botulinum Neurotoxin, Tetanus Neurotoxin, light chain polypeptide, mutation.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SONG, Fuyang et al. "Construction, Expression and Analysis of the Activity of Mutation of Recombinant Light Chain of Botulinum Neurotoxin Serotype A (BoNT/A LC)" 《Life Science Research》, Vol. 15, No. 3, 30 June 2011 (2011-06-30), see abstract	1, 4-9
X	NEIL F. Fairweather et al. "Production of biologically active light chain of tetanus toxin in Escherichia coli Evidence for the importance of the C-terminal 16 amino acids for full biological activity" 《FEBS》, Vol. 323, No. 3, 03 June 1993 (1993-06-03), see figure 1	10, 13-17
A	NEIL F. Fairweather et al. "Botulinum and tetanus neurotoxins: structure, function and therapeutic utility" 《TRENDS in Biochemical Sciences》, Vol. 27, No. 11, 26 September 2002 (2002-09-26), see the whole document	1-17

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

(2 November 2014)

Date of mailing of the international search report

19 November 2014

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2014/084725

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **7-9, 17**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] The subject matter of claims 7-9, 17 relates to methods of treatment for diseases, and therefore does not warrant an international search according to the criteria set out in Rule 39.1(iv). However, the search has been carried out and based on the use of the composition or subject for manufacturing of a medicament.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).