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The N-terminal half of the receptor domain of botulinum neurotoxin A binds to microdomains of the plasma membrane

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ABSTRACT

Botulinum neurotoxin type A (BoNT/A) is largely employed in human therapy because of its specific inhibition of peripheral cholinergic nerve terminals. BoNT/A binds to them rapidly and with high specificity via its receptor binding domain termed HC. Recent evidence indicate that BoNT/A interacts specifically with polysialogangliosides and with a luminal loop of the synaptic vesicle protein SV2 via the C-terminal half of HC.

Here we show that the N-terminal half of HC binds to sphingomyelin-enriched membrane microdomains and that it has a defined interaction with phosphatidylinositol phosphates (PIP). We have identified a PIP binding site in this half of HC and we show how this interaction could predispose BoNT/A for membrane insertion, which is the step subsequent to binding, in the four-steps route leading BoNT/A inside nerve terminals.

Anaerobic and sporigenic bacteria of the genus *Clostridium* produce very potent neurotoxins capable of binding specifically to peripheral nerve terminals, termed botulinum neurotoxins (seven known serotypes from BoNT/A to BoNT/G). These neurotoxins cause a flaccid paralysis with autonomic symptoms termed botulism [1]. If injected directly in the brain they act as well on neurons of the central nervous system [2]. However, a key aspect of their toxicity and pharmacological properties is the neurospecific binding to peripheral cholinergic terminals [3].

BoNTs consist of three distinct 50 kDa domains, termed L, HN and HC [4,5]. This structure is functional to the four-steps of their cellular mode of intoxication [6,7] (a) binding carried out by the HC which consists of two sub-domains of equal size (25 kDa), termed Hc-N and Hc-C; Hc-N is highly conserved among the different serotypes whilst Hc-C is not [6] (b) endocytosis inside synaptic vesicles (SV), (3) membrane translocation of L, mediated by the intermediate domain termed HN, from the acidic vesicle lumen into the cytosol; where the (4) step takes place: the L domain is a metalloprotease that cleaves specifically and at single points any of the three SNARE proteins. These proteins form a SNARE complex which arranges in a rosette supercomplex to mediate neuroexocytosis [7,8].

Recently, major advances in the identification of the receptors and mode of binding of BoNTs have been made, particularly for types A, B and G and E. A polysialoganglioside binding site has been mapped in Hc-C [9,10], and is close to the binding site for the luminal domain of the SV protein synaptotagmin in BoNT/B and /G [11,12]. BoNT/A and BoNT/E were shown to bind another SV protein termed SV2 [13–15]. These findings clearly indicate that the BoNT use SV to enter nerve terminals, as tetanus neurotoxin does in the central nervous system [16]. Another important recent advancement was the set up of a reliable assay of the membrane crossing mediated by HN which has revealed the partial unfolding of the L chain during translocation inside HN [17,18].

Less defined are the molecular events taking place between BoNT binding and the subsequent insertion into the lipid bilayer. Even for the more defined case, that of BoNT/B, there are different views about how the toxin is located with respect to the membrane surface after polysialoganglioside and synaptotagmin binding [11,12,19].

Here, we show that the 25 kDa N-terminal domain of the HC receptor binding domain of BoNT/A (Hc-N/A) binds to microdo-

Abbreviations used: BoNT, botulinum neurotoxin; EGFP, enhanced green fluorescent protein; H, heavy chain; HN, N-terminal portion of heavy chain; HC, C-terminal portion of heavy chain; Hc-N, N-terminal portion of HC; Hc-C, C-terminal portion of HC; mCherry, monomeric red fluorescent protein; L, light chain; TG, triglyceride; DAG, diacylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; CL, cardiolipin; PI, phosphatidylinositol; PI(3)P, PI(4)P, PI(5)P, PI monophosphates; PI(3,4)P, PI(3,5)P, PI(4,5)P, biphosphates; PI(3,4,5)P, PI triphosphate; S, sulfatide (3-sulfogalactosylceramide); SM, sphingomyelin; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor.

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mains of the plasma membrane which are enriched in sphingomyelin, and in particular to phosphatidylinosytol-phosphate. On this basis, with the help of molecular modelling, we suggest that the Hc-N sub-domain is highly conserved because it serves the essential and common function of binding to selected microdomains of the synaptic vesicle membrane thus orienting the HN domain for the subsequent insertion into the membrane.

Materials and methods

Materials. Escherichia coli XL1-Blue was from Stratagen; *E. coli* BL21 pLysS and anti His-tag antibody were from Novagen. pEGFP-N1 was from Clonetech. pRSETa, Alexa Fluor-555 goat anti-rabbit IgG, Alexa 488 goat anti-rabbit IgG antibody and SimplyBlue Safestain were from Invitrogen. Restriction enzymes were from New England Biolabs. HiTrap Chelating HP, ECL Advanced were from GE Healthcare Life Science. DMEM, MEM-EBSS were from Gibco; FBS was from Euroclone; Poly-L-lysine, retinoic acid, gentamicin and optiprep were from Sigma. BSA, Complete Protease Inhibitor Cocktail and Expand High Fidelity PCR System were from Roche, Indianapolis, IN. Anti-GFP (Ab6556) was from Abcam; anti-flotillin was from BD Biosciences and anti-SNAP-25 clone SM181 from Sternberger. Membrane Lipid Strips and PIP array were from Echelon Biosciences.

Plasmids. The Hc-N/A was cloned by PCR from pGEX-4T-3-Kin-HA-HCA (kind gift of Dr. G. Schiavo, Cancer Research UK). Forward and reverse primer sequences were 5′-AAACTCGAGAGTACAGATAT ACCTTTTCAGCTT and 5′-AAAAAGCTTTGAATTTGATTGATTATCATAT AAATCTTT, respectively. The DNA fragments obtained were digested with XhoI and HindIII, introduced at overhangs of the used oligonucleotides, and inserted into the vector pRSETa . The EGFP sequence was cloned from pEGFP-N1 and mCherry was cloned from pRSETb-mCherry [20]. Forward and reverse primer sequences were 5′ AAAGGATCCATGGTGAGCAAGGGCGAG 5′-AAACTCGAGCT TGTACAGCTCGTCCATGCC, for both EGFP and mCherry sequences. The amplified sequences were digested with BamHI and XhoI and Hc-N/A sequence was cloned upstream in the pRSETa vector to obtain pRSETa-EGFP-Hc-N/A or pRSETa-mCherry-Hc-N/A. DNA inserts sequencing were sequenced by CRIBI (Padova). His-Venus-NT-Lysenin (kindly provided by Prof. T. Kobayashi) was described elsewhere [21].

Recombinant protein expression and purification. BL21 (DE3) pLysS *E. coli* strain transformed either with pRSETa-EGFP-Hc-N/A or pRSETa-mCherry-Hc-N/A were grown in LB medium until the OD_{600} (optical density at 600 nm) reached 0.6. Cultures were induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 4 h at 30 °C. Cells were pellet, frozen in liquid nitrogen and keep at −80 °C. Cells were then lysed by two passages of pre-cooled French Press. The lysate was centrifuged (17,000g, 20 min), and the supernatant was loaded on a HiTrap Chelating HP on a ÄKTA-prime system (GE Healthcare) and eluted with a linear gradient from 0 to 500 mM Imidazole. Protein purity was assessed by staining with SimplyBlue Safestain of a 12% SDS–PAGE gel. Protein identity was confirmed by Western blotting using an anti-His-Tag antibody.

Cell culture. Primary rat spinal *cord* motor neurons (SCMNs) were isolated from Sprague–Dawley rat embryos (embryonic day 14) and cultured following previously described protocols [22]. All experiments were performed using SCMNs differentiated for 5–8 d.

NSC-34 is an immortalized motor neuron cell line, (kindly provided by Dr. A. Poletti, University of Milan, Italy). These cells were routinely maintained in DMEM with sodium pyruvate, supplemented with 10% FBS, 100 UI/mL of gentamicin and grown at 37 °C in 5% CO_2 in 25 cm^2 flasks, changing medium every 72 h. Cells were induced to differentiate in DMEM 5% FBS containing 10 mM retinoic acid. For live-imaging and immunofluorescence, cells were seeded on glass-bottomed Petri dishes (Matek) previously coated with poly-L-lysine. For biochemical analysis and detergent-resistant microdomains isolation, cells were seeded on 60 mm Petri dishes. NSC-34 cells were used at the 5th day of differentiation. HeLa cells were cultivated in MEM-EBSS supplemented with 10% FBS, Caco-2 cells in MEM-EBSS, 15% FBS and 1% non-essential amino acids.

Cell imaging. SCMNs or differentiated NSC-34 were incubated with 200 nM or 50 nM EGFP-Hc-N/A, respectively, in E4 buffer supplemented with 0.5% BSA for 30 min at 37 °C. HeLA and Caco-2 were incubated 200 nM EGFP-Hc-N/A in MEM-EBSS medium supplemented with 0.5% BSA 30 min at 37 °C. mCherry-Hc-N/A was used 250 nM on NSC-34 cells and 1 μM on HeLA or Caco-2 cell. His-Venus-NT-Lysenin was used 100 nM on NSC-34, HeLA and Caco-2 cells. After washing, cells were observed under the fluorescence microscope. NSC-34 cells were fixed with 4% PFA followed by quenching with NH_4Cl, whereas HeLa and Caco-2 cells with cold acetone. The samples were then washed with PBS containing 2% BSA and 0.2% gelatin for NSC-34 and SCMNs, and PBS 2% BSA for HeLA and Caco-2 cells. Plasma membrane-localized EGFP-Hc-N/A was stained with a rabbit antibody specific for the GFP used at 1:10000 dilution, followed by an Alexa Fluor-555 goat anti-rabbit IgG antibody (Invitrogen). For cytosolic staining, the previously surface-stained cells were permeabilized with 0.1% Triton X-100 for 3 min at room temperature. After a second blocking step, labelling of the cytoplasmic chimeric protein was carried out with the same rabbit antibody and an Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen) was used as secondary antibody.

Fluorescence images were acquired with a Leica AD MIRE3 inverted microscope, equipped with a Leica DC500 CCD camera, ×63 oil immersion objective and ×1.5 zoom (NA 1.4). Stacks were acquired along the *z*-axis with 0.27 μM spacing. Data were collected using the Leica FW4000 software and analyzed with Leica Deblur and WCIF ImageJ; stacks were de-convoluted and a single 3D cell image was obtained.

Analysis of membrane fractions by ultracentrifugation. Five-days differentiated NSC-34 cells were grown on 6 cm culture dishes, rinsed with E4 buffer and then lysed on ice with 375 μl of pre-cooled buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4, 0.1% Triton X-100 (TXNE), and Complete Protease Inhibitor Cocktail. Cells were homogenized by passing for three times the ice-cold lysates through a 22G needle. Extracts were adjusted to 35% OptiPrep, placed in a SW40Ti ultracentrifuge tube (Beckman, Palo Alto, CA), and over-layed with 8.75 ml of 30% OptiPrep in TXNE and then with 1 ml of TXNE. After centrifugation (4 h, 200,000g, 4 °C), 10 fractions were collected from the top, and analyzed by Western blotting using antibodies specific for GFP antibody, for flotillin-1 and for SNAP-25.

Biochemical assays and blottings. EGFP-Hc-N/A bound to NSC-34 cells was estimated on 24-wells plates seeded with 6000 cells/well and incubated with the chimeric protein under the conditions described above. Cells were mechanically resuspended and dissolved in a lysis buffer (Promega). These samples and the gradient fractions were solubilised in Laemmli SDS buffer, boiled and loaded onto 10% or 12% polyacrylamide gels. After electrophoresis, the samples were transferred on nitrocellulose sheets, probed with a rabbit anti-GFP antibody 1:10000, and stained with a goat anti-rabbit IgG coupled to horseradish peroxidase and developed with ECL Plus.

Membrane Lipid PIP arrays were incubated, according to manufacture instructions (Echelon Bioscience), with 0.015 μg/ml of EGFP-Hc-N/A, and revealed with a rabbit anti-GFP antibody, goat anti-rabbit IgG horseradish peroxidase followed by ECL Plus.

Results and discussion

Binding of a fluorescent chimera of the N-terminal half of the binding domain of botulinum neurotoxin type A to cells

Recent studies have led to the conclusion that the poorly conserved 25 kDa C-terminal half of the HC binding domain of BoNTs is the major responsible for their neurospecific binding and internalization inside SV. This leaves open the question of the role the 25 kDa N-terminal half of HC (Hc-N), which is highly conserved among BoNTs. To study its membrane interaction, we expressed and purified a chimera consisting of Hc-N of BoNT/A linked to an enhanced green fluorescent protein (EGFP), abbreviated here as EGFP-Hc-N/A (Fig. 1S). This chimera binds to the plasma membrane of the neuronally differentiated NSC-34 cells (Fig. 1A and B) and to spinal cord motoneurons (Fig. 2SA and B); in both cases, binding is distributed all along the cell surface in discrete spots. Surprisingly, EGFP-Hc-N/A not only binds to neurons, but also to the plasma membrane of epithelial cells with the same discrete and spotty binding (Fig. 1C and D and 2S C and D). EGFP alone does not bind to these cells (not shown). This cell surface distribution of EGFP-Hc-N/A was never followed by internalization, as indicated by (a) its morphological distribution, (b) no fluorescence quenching due to exposure of the EGFP moiety to acid endosomal lumen, and (c) coincidence of the EGFP-Hc-N/A stainings obtained with an anti-GFP antibody with or without membrane permeabilization (not shown). These data suggest that Hc-N/A binds to cells with no internalization, as expected from the lack the translocation domain HN.

This general cell binding activity of EGFP-Hc-N suggested that the process may be mediated by discrete plasma membrane microdomains, common to neuronal and non-neuronal cells.

The N-terminal half of the binding domain of botulinum neurotoxin type A binds to sphingomyelin containing plasma membrane microdomains

The plasma membrane of many cells presents microdomains enriched in sphingomyelin (SM), which can be specifically identified with fluorescent lysenin, a sphingomyelin binding protein of bacterial origin [21]. Figs. 2 (and 3S) shows that the EGFP-Hc-N/A and lysenin largely co-localize in neuronal and epithelial cells, indicating that the microdomains to which the Hc-N/A binds are enriched in SM. SM-enriched microdomains can be isolated upon careful cell solubilisation with Triton X-100 and ultracentrifugation. Fig. 3A shows that, indeed, a large part of EGFP-Hc-N/A is found in an insoluble fraction together with flotillin, which is a marker of detergent-insoluble microdomains.

Hc-N/A interacts with phosphatidylinositol phosphate

To identify possible specific interactions of Hc-N/A with defined lipids, we used the well established dot-blot binding assay with nitrocellulose paper stripes dotted with various lipids. As shown in Fig. 3B, there is a defined interaction with negatively charged phospholipids PA and PS; however, Hc-N/A display a much stronger interaction with PI(4)P. The lack of interaction with SM is not in contradiction with the findings of Fig. 2 because that assay only showed colocalization with sphingomyelin-enriched membrane microdomains. To scrutinize the interaction with the PIPs, a specific assay was carried out (Fig. 3C). This result indicates that Hc-N/A is capable of interacting with PIPs, particularly with the 5 isomer. The 3 and 4 isomers are recognized as well but less strongly. In agreement with these data, the ligand also binds to $PI(3,5)P_2$. EGFP alone shows no lipid binding (not shown).

Phosphoinositides (PIPs) are important mediators of cellular response to growth factors and in vesicular trafficking [23], also at synapse [24]. PIPs are enriched in the cytosolic leaflet of the plasma membrane, but it is possible that they are present on the cell surface in association with SM-containing microdomains. In fact, PIPs were found to localize in SM-enriched domain in the presence of cholesterol [25]. Thus, Hc-N/A, and the intact BoNT/A protein, could interact with SM-enriched membrane microdomains which contain PIPs. It is noteworthy that, using the same approach, it was recently reported that BoNT/D and Hc/D bind to PE [26].

Molecular modelling of Hc-N/A membrane binding

Taken together these results clearly indicate that Hc-N has defined membrane binding properties which were previously overlooked, most likely because all the attention was focussed on the specific binding of Hc-C to polysialoganglioside and to SV proteins. It was previously suggested that different molecules present in microdomains of the presynaptic membrane could contribute to the overall binding of BoNT and that even low affinity interactions could be relevant to toxin binding within the context of a two-dimensional solvent [27]. The present results implicate PIPs as a specific BoNT binding lipid molecule. It is as well possible that the PIP binding plays a role in the correct positioning of the toxin for the subsequent low pH-driven membrane insertion of HN [17]. On this basis, we inspected the sequences and crystallographic structures of proteins that bind PIP and compared them

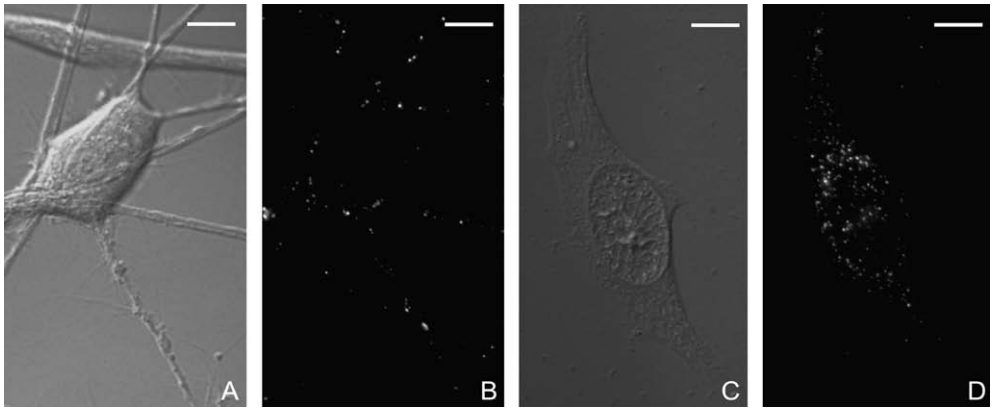

Fig. 1. Binding of EGFP-Hc-N/A to NSC-34 and HeLa cell lines. Live imaging of differentiated NSC-34 (A,B) and fixed HeLa (C,D) cells. Several images have been acquired along the zeta axes and then grouped. Scale bars: 10 μm.

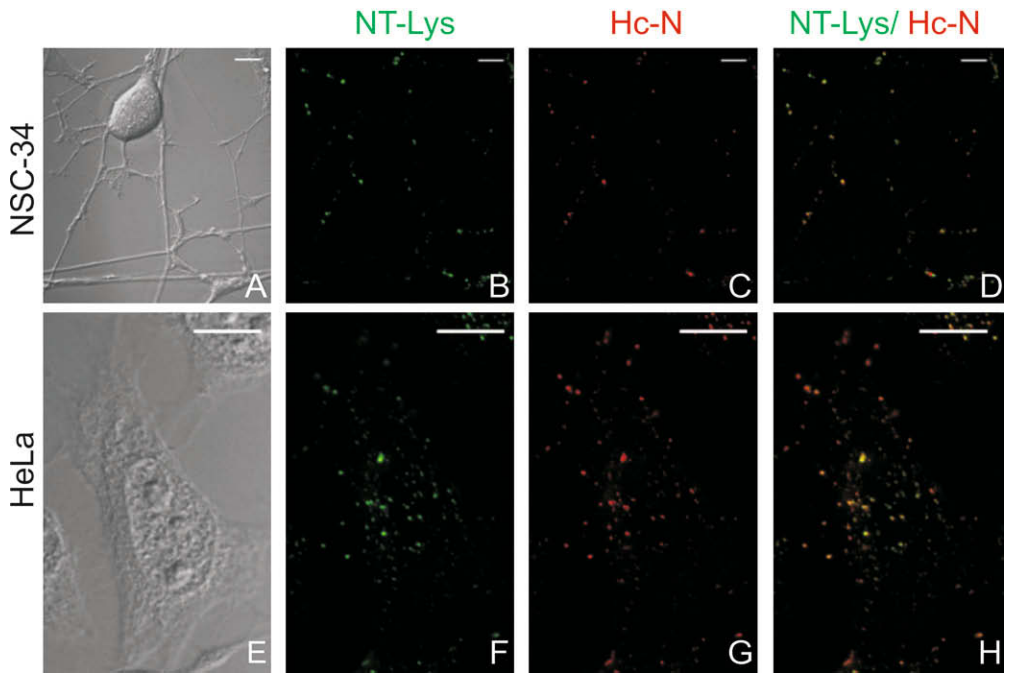

Fig. 2. EGFP-Hc-N/A co-localizes with sphingomyelin-enriched microdomains of the plasma membrane. Distribution of His-Venus-NT-Lysenin (B,F) which selectively labels sphingomyelin-enriched plasma membrane areas and of mCherry-H_C-N/A (C,G); merged images are shown in (D,H). This analysis was performed in live and differentiated NSC-34 (A–D) as well as in fixed HeLa cells (E–H). Scale bars: 10 μm.

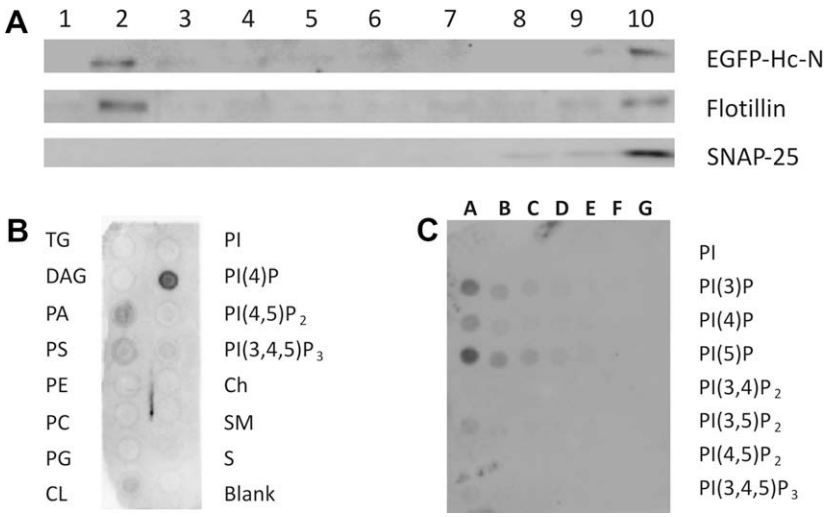

Fig. 3. EGFP-Hc-N/A localizes in detergent-resistant membrane microdomains and interacts with phosphatidylinositol phosphates. (A) EGFP-Hc-N/A is present in the insoluble fraction (lane 2) of the gradient (see Material and methods), which contains the majority of the microdomain marker flotillin, whilst SNAP-25 is found in soluble fractions (lanes 8–10). (B) Dot-blot assays performed with membrane lipid strips show that EGFP-Hc-N/A binds to phosphatidylinositol phosphate and with less affinity to the negatively charged lipids: PA, PS and CL. (C) EGFP-Hc-N/A binds preferentially to PI(5)P with small or no affinity to PI, PIP2 and PIP3; different amounts of lipids are spotted in different columns: A, 100 nmol/spot; B, 50 nmol/spot; C, 25 nmol/spot; D, 12.5 nmol/spot; E, 6.25 nmol/spot; F, 3.13 nmol/spot; G, 1.56 nmol/spot.

with those of the BoNTs. Indeed, we found a 27.5% sequence identity among the Hc-N of BoNT/A and a PI(5)P binding protein, termed the inhibitor of growth family member 2 (ING2) (Fig. 2S panel A) [28]. Visual inspection confirmed a structural similarity between HC and ING2 [29] and BoNT/A (Fig. 2S panel A and B). On this basis, we identify a putative inositol phosphate binding site in the BoNT/A region comprised between amino acid Ser-876 and Lys-929 which correspond to a β-prism. It is possible that the four positively charged residues of BoNT/A, Arg-892, Lys-896, Lys-902, Lys-910, are involved in PIP binding. Consistent with this possibility, a peptide that inhibits the binding of BoNT/A to synaptosome and a toxin neutralizing antibody [30,31] map in this portion of BoNT/A (Fig. 4S panel B). Furthermore, the side chains of several tryptophans (945, 1013, 1067, 1100, 1290) face toward this site and tryptophans are known to play frequently important roles in membrane interaction.

The 876–929 segment of BoNT/A is not highly conserved among serotypes, but it is possible that different lipids may be involved in the promotion of the membrane interaction of different neurotoxins, as indicated by the BoNT/D binding to PE [32]. We suggest that these additional interactions with the membrane surface may play the role of positioning the toxin on the membrane surface ready for membrane insertion, as it is illustrated in Fig. 4. The model suggests that the Hc-N sub-domain has the role of bringing the mem-

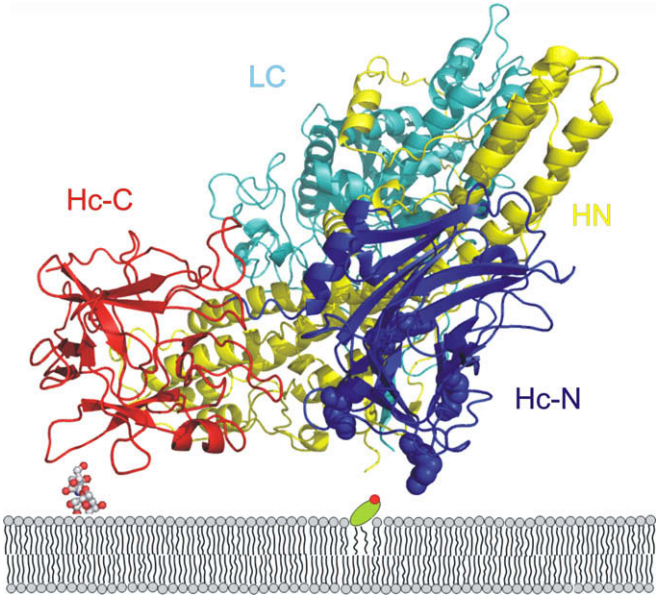

Fig. 4. A model of BoNT/A bound to plasma membrane based on the interaction of the binding domain with a polysialoganglioside and a PIP molecule. BoNT/A ribbon diagram with sub-domains labeled with Hc-C (red), Hc-N (blue), HN (yellow) and LC (light blue). Simultaneous binding of BoNT/A to the oligosaccharide moiety of a polysialoganglioside (left hand side, in grey and red) and PIP (right hand side, with inositol in green and phosphate in red) imposes a rotation of the HN and LC domains that position the HN segment 620–680 close to the membrane surface. The Hc-N positive charged residues Arg-892, Lys-896, Lys-902, Lys-910, which are suggested to play a major role in the interaction with PIP, are shown as spheres. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

brane translocating domain HN close to the membrane surface, possibly close to areas of lipid mismatching (as those present at the boundaries of membrane microdomains) to facilitate its membrane penetration. The double, ganglioside and PIP, lipid binding places the HN segment 620–680 close to the membrane surface, ready for insertion into the lipid bilayer. Further studies are clearly required to demonstrate that this is indeed the first part of the toxin which inserts into the membrane, but it is noteworthy that this segment is highly conserved. This suggestion can be tested using the available highly sensitive membrane translocation assay of BoNT [18] and other biophysical methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.037.

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