Membrane constraints reshape synaptotagmin recognition by botulinum neurotoxin B1

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Abstract

Synaptotagmins 1 and 2 (SYT1 and SYT2) are essential Ca^{2+} sensors in neurotransmission and the functional receptors of botulinum neurotoxin B1 (BoNT/B1). While crystallographic models have defined key contacts between SYT1/2 and BoNT/B1, these structures neglect the membrane environment, potentially overlooking critical interactions. Here, we used molecular dynamics simulations of membrane-inserted SYT1 and SYT2 in lipid rafts to uncover how membrane constraints reshape their juxtamembrane domains and enable unique contacts with the BoNT/B1's lipid-binding loop (LBL). These interactions, absent in soluble crystal structures, reconcile experimental findings from nanodisc and mutagenesis assays. Notably, in the SYT1 complex, ganglioside GT1b dynamically bridges the gap created by membrane stress, stabilizing the SYT1-BoNT/B1 interface while SYT2 remains stably engaged. Our results demonstrate that lipid rafts and intrinsically disordered protein domains act synergistically to create functional binding modes missed by crystallography or AI-based structure prediction. This study highlights the epigenetic dimension of protein structure (EDPS), where environmental constraints dictate receptor conformation, and provides mechanistic insights for designing BoNT/B1 antidotes or therapeutics that exploit lipid-assisted binding.

Keywords: Molecular dynamics simulation, lipid rafts, ganglioside, conformational changes, synaptotagmin, botulinum neurotoxin, geometric constraints

Introduction

Synaptotagmins (SYTs) are synaptic vesicle proteins that function as calcium sensors for neurotransmitter release [1-4]. Synaptotagmin 1 and 2 (SYT1/2) serve as specific protein receptors for botulinum neurotoxin B (BoNT/B), a category A agent that causes botulism by cleaving SNARE (Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptor) proteins and inhibiting neurotransmission [5-7]. BoNT/B is one of the eight serotypes classified from A to X, of which the serotypes A, B, E and F are associated with human botulism [8-11]. Each serotype is divided into subtypes with different amino acid sequences [12]. Additionally, there are “mosaic BoNTs formed by the combination of the amino acid sequences of different serotypes [13].

The 150 kDa BoNT/B toxin is composed of a light chain (LC, metalloprotease) and a heavy chain (HC) [14, 15]. The HC C-terminal domain (Hcc) mediates receptor binding. X-ray structures of BoNT/B bound to SYT1 or SYT2 fragments have defined the interaction interface within residues 39-49 of SYT1 and 47-58 of SYT2. [16, 17].

In addition to its protein receptor, BoNT/B binds to human gangliosides GT1b and GD1a through a common ganglioside-binding site which is conserved in other toxin types [18-21]. Commonly, gangliosides are packed with cholesterol and sphingolipids in lipid rafts, which are electronegatively charged platforms that attract cationic molecules such as virus proteins or amyloid proteins on the membrane surface [22-28]. Cesare Montecucco proposed a model in which the neurotoxin interacts simultaneously with its protein and glycolipid receptor [29].

However, these structural models present a static and incomplete picture, as they fundamentally lack the native membrane environment. This omission is critically relevant for understanding the function of a unique structural feature of BoNT/B: its lipid-binding loop (LBL, residues E1245 to E1252) [30, 31]. While the LBL is hypothesized to be important for receptor binding, its precise mechanistic role has remained enigmatic. This gap in understanding is highlighted by a key biochemical paradox: although the LBL is dispensable for SYT binding in soluble assays, it is absolutely essential for toxin binding in a membrane context. The current crystallographic data, which show no role for the LBL, are thus insufficient to explain its mandatory function in a physiological setting.

This discrepancy points to a broader limitation: existing models cannot capture the

geometric constraints imposed by the lipid bilayer on the conformation of synaptotagmin or the potential synergy between its protein and glycolipid receptors. Therefore, to resolve this paradox and unravel the authentic molecular mechanism of BoNT/B intoxication, there is an urgent need for a structural model that incorporates the toxin in complex with its full repertoire of membrane-embedded receptors within a realistic lipid environment.

For this purpose, we present an all-atom molecular dynamics simulation (MDS) study of membrane-inserted SYT1 or SYT2 in complex with BoNT/B1 within a lipid raft environment, revealing how membrane constraints fundamentally redefine this critical host-pathogen interaction and resolve the long-standing mystery of the LBL's essential role.

Material and methods

System set-up

The initial coordinates of ganglioside and cholesterol molecules composing the lipid raft were obtained using the glycolipid modeler tool and bilayer builder available on charmm-gui [32]. The structure of BoNT/B1 (from 537 to 1291) was obtained from PDB : 2NP0 and the missing regions were built with SWISS-MODEL [33]. The luminal domain of SYT1 and SYT2 were made de-novo using HyperChem [34]. A molecular docking of the complex BoNT/B-SYT1 or 2 were obtained with HyperChem in a way that respects the geometric constraints of membrane topology. The glycosylations of SYT1and SYT2 were added using the tool PDB reader of charmm-GUI [35] [36]. An energy minimization of the complexes was performed with the Polak-Ribière conjugate gradient algorithm, with the Bio+(Charmm) force field in Hyperchem and a root-mean-square (RMS) gradient of 0.01 kcal. $Å^{-1}.mol^{-1}$. Finally, the complexes and the lipid raft were placed in a lipid bilayer that mimics the lipid composition of neural membranes (outer leaflet: 50% POPC and 50% cholesterol | inner leaflet: 30% POPS/ 20% POPE and 50% cholesterol) [37]. Then, the systems were solvated with TIP3P water and neutralized with the addition of Na^+ and Cl^- ions at a final concentration of 0.15 mol/L using the tools “Solvation” and “add ions” available on VMD [38].

Molecular dynamics simulations

The system was simulated using NAMD 2.14 for Windowsx64 [39] coupled with the all-atom Force Field Charmm36m which has been improved for the calculation of IDPs [40]. The cutoff for the calculation of non-bonded interaction was set to 12 Å. The PME calculation algorithm was used to calculate long range electrostatic interactions. The covalent bonds involving hydrogen atoms were constrained using the algorithm SHAKE. First, the whole systems were minimized for 20000 steps. The apolar parts of the lipids were melted for 1 ns at 310K in the NVT ensemble to get rid of the crystalline organization of the lipid bilayer. Then, the systems were equilibrated at constant temperature (310 K) and constant pressure (1 atm) during 15 ns with constraint on protein followed by 15 ns of equilibration with constraint on backbone only. Finally, the non-constrained runs were performed at constant temperature (310 K) and constant pressure (1 atm) for 100 ns with a time step of 2 fs. The coordinates of the system evolution were backed up every 0.1 ns corresponding to 50000 steps.

Results

Membrane constraints reshape synaptotagmin conformation and alter BoNT/B1 binding surfaces.

The BoNT/B1-SYT1 and BoNT/B1-SYT2 complexes were inserted into a lipid raft membrane to model the native neuronal environment. To assess the stability of the toxin, we calculated the Root Mean Square Fluctuation (RMSF) of BoNT/B1 in both complexes. The low average RMSF values (1.27 Å for SYT1 and 1.37 Å for SYT2) indicate that BoNT/B1 maintains its overall structural integrity, with most residues displaying a minimal flexibility (< 2 Å) **(Figure 1)**.

Shortly after the beginning of the simulation, we observed that SYT1 and SYT2 undergo conformational changes due to the constraints imposed by the membrane. As shown in **figure 2 upper panel**, SYT1 undergoes stress which, after 10 ns of simulation, forces the conformation of its structure on the side of the ganglioside interaction site of BoNT/B1 (yellow arrow) to move closer to the membrane surface. This movement cancels out intermolecular interactions between the two partners, forming a gap (highlighted by a red arrow). As shown in **figure 2 lower panel**, the

same phenomenon is observed for SYT2 but interestingly, no gap is formed between both partners. We quantified this loss of contact by calculating the solvent-accessible surface area (SASA) of the synaptotatmin-binding interface on BoNT/B1. The average SASA for the SYT1 complex was significantly larger than that for the SYT2 complex (1944 $Å^2$ versus 1796 $Å^2$, **figure 3 A and B**). These preliminary observations suggests a stronger chemical affinity between SYT2 and BoNT/B1 under membrane constraints.

Membrane constraints enable the lipid-binding loop (LBL) of BoNT/B1 to establish direct contacts with synaptotagmin.

Due to the forces that constrained the structure of SYT1 and SYT2 to move closer to the membrane surface, contact points were created between the LBL structure of BoNT/B1 and the synaptotagmins. As shown in **figure 4** residues subject to contact are I1248 of the LBL of BoNT/B1 and the residues M47 of SYT1 and P55 of SYT2. Distances between the lateral chain of residues I1248-M47 and I1248-P55 were plotted. The plots reveal that the distances are generally below 5 Å throughout the simulation, indicating that the residues are often in contact together via van der Waals interactions.

GT1b ganglioside bridges BoNT/B1 and SYT1, stabilizing the membrane-induced complex.

As shown in **figure 2 A and figure 3 A**, a gap is formed between SYT1 and BoNT/B1 due to the membrane constraints while such a gap is not formed between SYT2 and BoNT/B1 (**figure 2 B, figure 3 B**). In response to this gap, the simulation predicts that the GT1b molecule that was initially mainly interacting with the aromatic residue W1262 of BoNT/B1 (**figure 5 A**), adapts its conformation to end up interacting with the cationic residues K1187 and K1188 of BoNT/B1 and the aromatic residue H52 of SYT1 (**figure 5 B, C, D, E and F**). The GT1b molecule appears to act as a bridge between BoNT/B1 and SYT1 (**figure 5 F**) that stabilizes the link between the toxin and its protein receptor by occupying the space generated by the membrane constraints. This ganglioside bridging illustrates a synergy between protein and glycolipid receptors that cannot be captured without membrane context.

A lipid raft-associated GT1b molecule interacts with the LBL of BoNT/B

In addition to SYT1 and SYT2, the simulation suggests that the LBL structure of

BoNT/B1 also interacts with a lipid raft-associated GT1b molecule. The GT1b molecule interacts with this structure mainly via residues R1242 and F1250. Snapshots showing the kinetics of the interaction mechanism between the ganglioside and the lipid-binding loop are shown in **figure 6**. These snapshots show that, initially and after 10 ns of simulation, the sugar moiety of the ganglioside is in a closed conformation able to interact only with the aromatic residue F1250. After 35 ns, the polar part of the ganglioside opens, allowing simultaneous interaction with residues R1242 and F1250, as shown in the snapshot obtained after 68 ns of simulation.

Discussion

In this manuscript, MDS was used to explore the evolution of the conformational landscape of BoNT/B and the luminal domain of SYT1 and SYT2 in a lipid raft membrane context. The current molecular data of BoNTs in interaction with their membrane receptor, obtained after co-crystallization, have significantly improved our knowledge by identifying the different pockets which accommodate the protein receptor and the sugar moiety of gangliosides. However, the limitations of these models is that they do not take into consideration the membrane topology as well as the constraints that can cause such environment on the conformation of synaptotgamin. Moreover, a former study suggested a direct link between gangliosides and synaptotagmins and demonstrated that gangliosides specifically interact with the juxtamembrane domain of synaptotagmins, thanks to the recognition of a ganglioside binding site (GBS) motif, a critical feature that is absent in crystal models [41, 42].

A key prediction of our study is that the lipid raft environment imposes distinct geometric constraints on SYT1 and SYT2. Notably, SYT1 is more severely impacted, resulting in a discernible gap at its interface with BoNT/B1 (**figure 2 A**). We propose that this membrane-induced destabilization of the SYT1 complex is compensated by the dynamic recruitment of a GT1b molecule, which moves from its initial interaction with W1262 to bridge the freed space, engaging both BoNT/B1 (K1187 + K1188) and SYT1 (H52) (**Figure 5**). This provides a compelling mechanistic explanation for the long-standing observation that BoNT/B1 binding to SYT1 is strictly ganglioside-dependent [43].

Furthermore, our simulations reveal a novel and functionally critical interaction: the

direct contact between the juxtamembrane domains of both SYT1 and SYT2 with the BoNT/B1 lipid-binding loop (LBL) (**Figure 4**). This finding resolves a significant paradox in the field. As demonstrated by Stern et al., the LBL is dispensable for SYT2 binding in soluble assays but becomes essential in a membrane environment [31]. Our model accounts for this discrepancy directly; in the context of membrane constraints, the LBL serves as a crucial docking site for synaptotagmin, a role that is unnecessary and thus invisible in soluble structures (**Figure 7**).

A principal strength of our molecular dynamics approach is its capacity to resolve atomic-level dynamics that are presently beyond the reach of direct experimental observation. While techniques like cryo-electron microscopy provide indispensable static snapshots, they cannot capture the real-time, lipid-dependent conformational rearrangements that are central to the BoNT/B1 recognition mechanism. The dynamic formation of the GT1b bridge, the membrane-induced contact with the LBL, and the differential stability of the SYT1 and SYT2 complexes are paradigm examples of such transient yet functionally critical events. Thus, our simulations provide a vital functional complement to structural databases, offering a mechanistic narrative for biochemical observations that have until now lacked a structural rationale.

The functional importance of the LBL is further underscored by its role in ganglioside recognition. We observed a lipid raft-associated GT1b molecule engaging the LBL through residues R1242 and F1250 (**Figure 6**). This is in full agreement with experimental data showing that LBL deletion or a F1250R point mutation abolishes BoNT/B binding to gangliosides in nanodiscs [31], validating our model's predictive power

In conclusion, our work moves beyond static models to demonstrate that the membrane is an active participant in forming the BoNT/B1 receptor complex. From a fundamental point of view, our study reinforces the concept according to which the structure of an initially disordered domain of the juxtamembrane part of synaptotagmin can be controlled by different ligands that can act in synergy, particularly when these ligands are part of a lipid raft. This particularity of intrinsically disordered proteins (IDPs) is an unexpected refinement of biology which is totally ignored by an artificial intelligence such as AlphaFold which only considers the amino acid sequence to predict 3D structures of proteins. This algorithm lacks the consideration of IDP ligands and more broadly all the environmental parameters that we have grouped together in the concept of Epigenetic Dimension of Protein Structure (EDPS). This new theory of

protein structure has already made it possible to highlight the role of raft lipids (gangliosides and cholesterol) in the propagation of transmembrane conformational waves initiated by the binding of a ligand to its receptor. Taking into account this conformational flexibility inherent to the intrinsic disorder of critical receptor domains will make it possible in the future to develop innovative therapeutic approaches that are less “frozen in stone” than those suggested by classic models [44, 45].

Figure legend

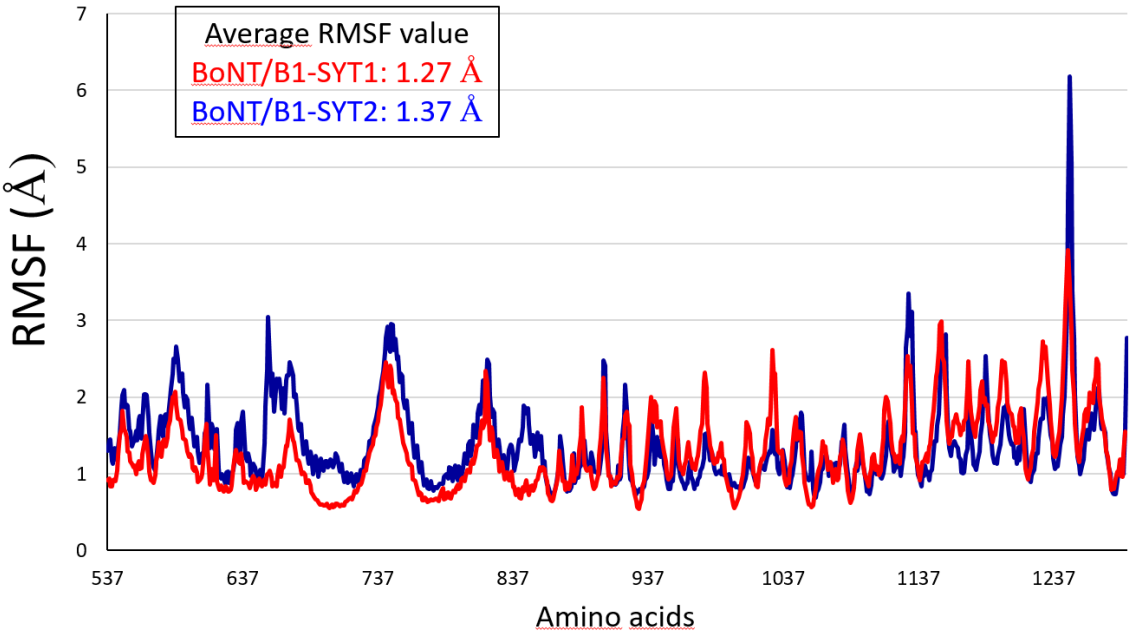

Figure 1: BoNT/B1 exhibits minimal flexibility in complex with SYTs. Root Means Square Fluctuation (RMSF) value of BoNT/B1 in complex with SYT1 (red lines) or SYT2 (blue lines). Is framed on the graph the average RMSF value for both conditions.

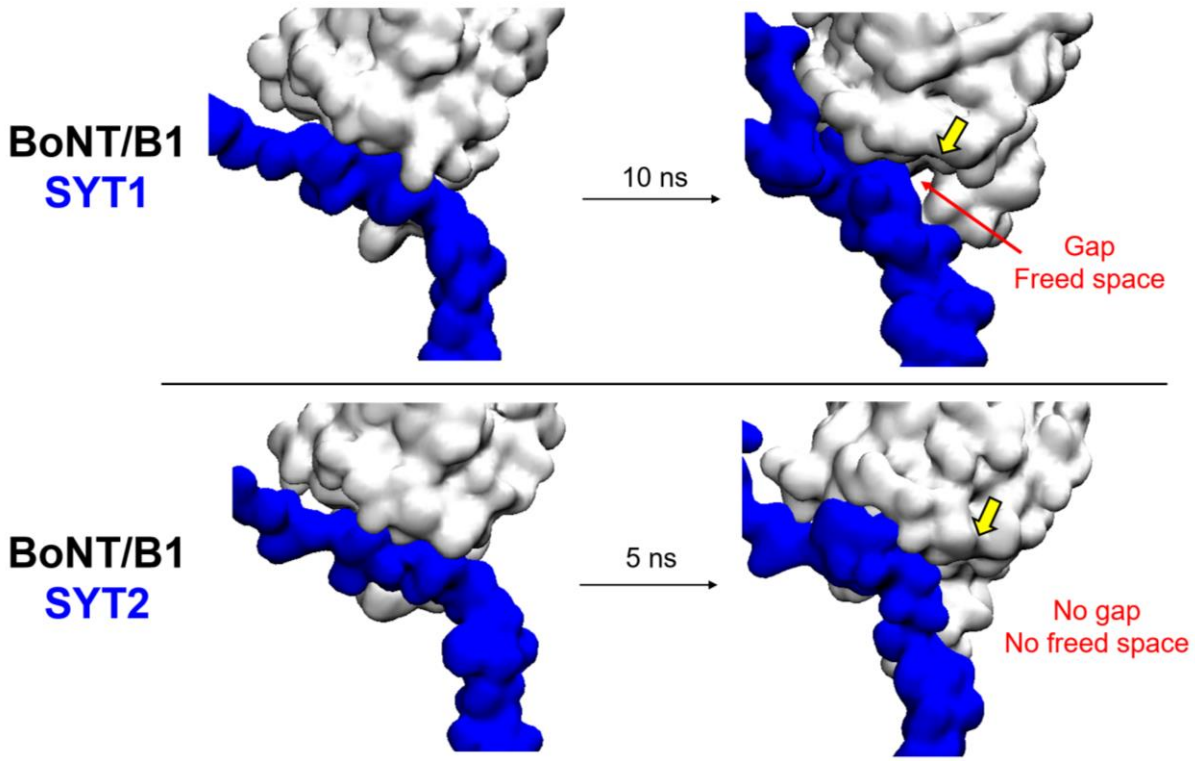

Figure 2: Membrane constraints reshape the conformation of SYT1 and 2. The membrane environment places stress on the synaptotagmin structure. This reduces the contact surface between synaptotagmin and BoNT/B1 (highlighted by a yellow arrow). Synaptotagmin is shown as a blue surface while BoNT/B1 is shown as a white surface.

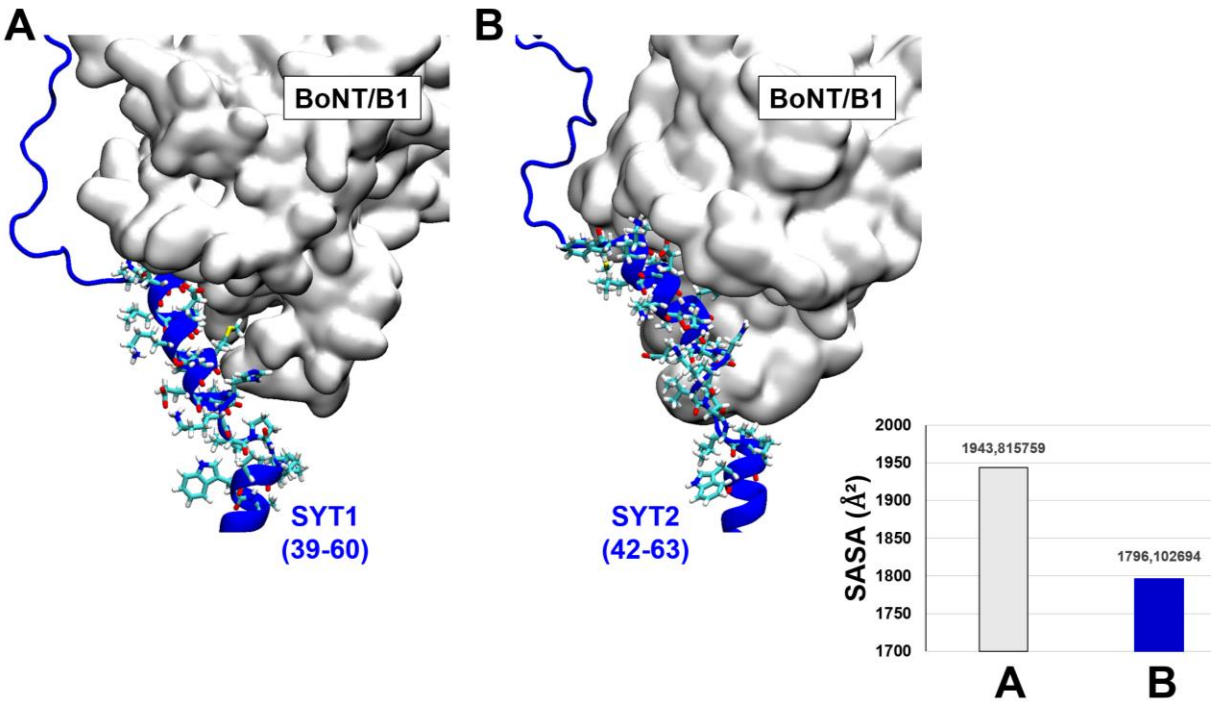

Figure 3: The differential impact of membrane constraints on SYT1 versus SYT2 leads to a larger solvent-exposed surface for SYT1. Surface representation of BoNT/B1 (white) in complex with SYT1 the juxtamembrane domain of SYT1 depicted in blue and the residues 39-60 are depicted as sticks colored by atom names (A). Same as (A) but for BoNT/B1-SYT2 complex **(B)**. Bar graph showing the average solvent-accessible surface area (SASA) of SYT1 (residues 39-60, white bar) and SYT2 (residues 42-63, blue bar) over time. The observed increase in SASA for the SYT1 complex is consistent with a sustained loss of contact compared to the SYT2 complex. Numerical values represent the mean SASA for each system.

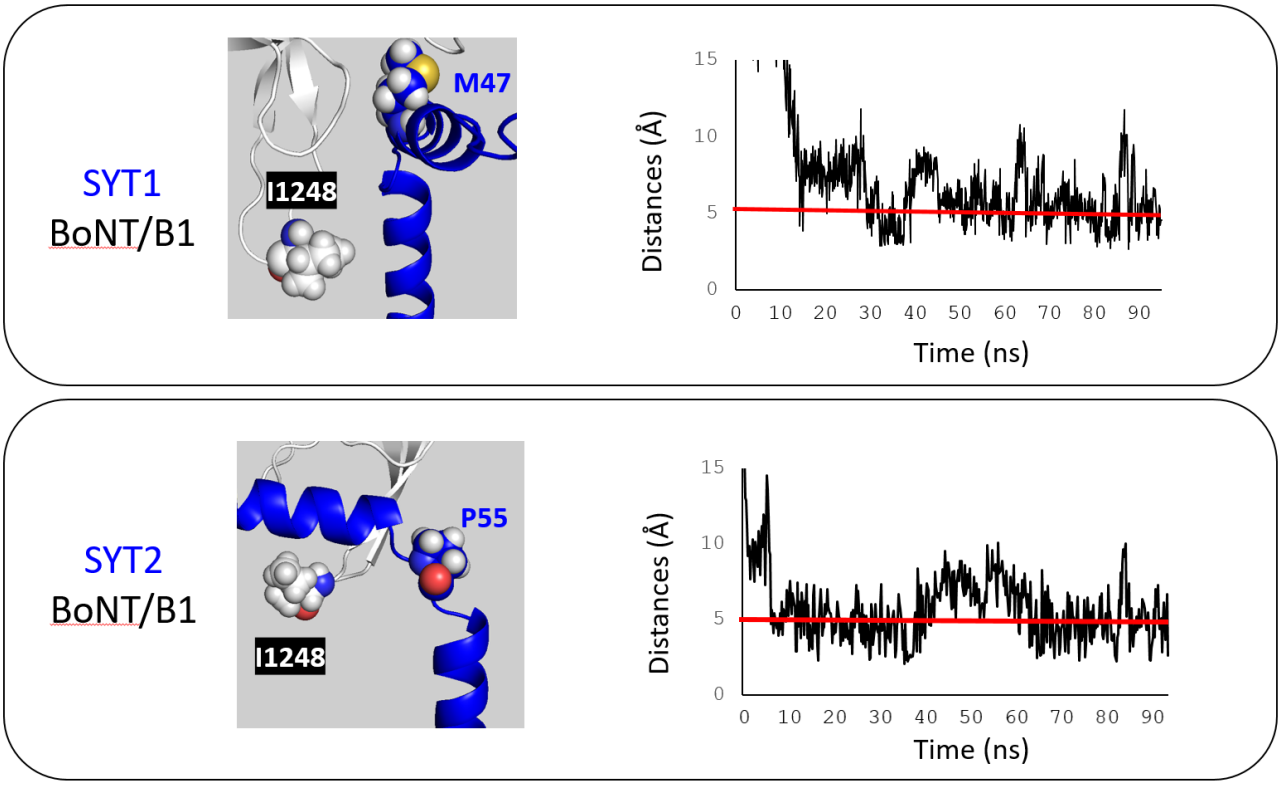

Figure 4: Membrane-driven contact between synaptotagmin and BoNT/B1 LBL. Snapshots showing residues involved in the interaction between the LBL structure of BoNT/B1 and the SYT1 **(upper panel)** or SYT2 **(lower panel)**. Line graphs on the right side plot the evolution of the minimal distance between the lateral chain of residues I1248-M47 (BoNT-SYT1) and I1248-P55 (BoNT-SYT2) were plotted over time. BoNT/B1 is represented as white cartoon, SYT1 and SYT2 are represented as blue cartoon. The residue I1248 belonging to the LBL structure of BoNT/B1 is depicted as white sphere while the residues M47 of the juxta membrane domain of SYT1 and P55 of the juxta membrane domain of SYT2 are depicted as blue spheres.

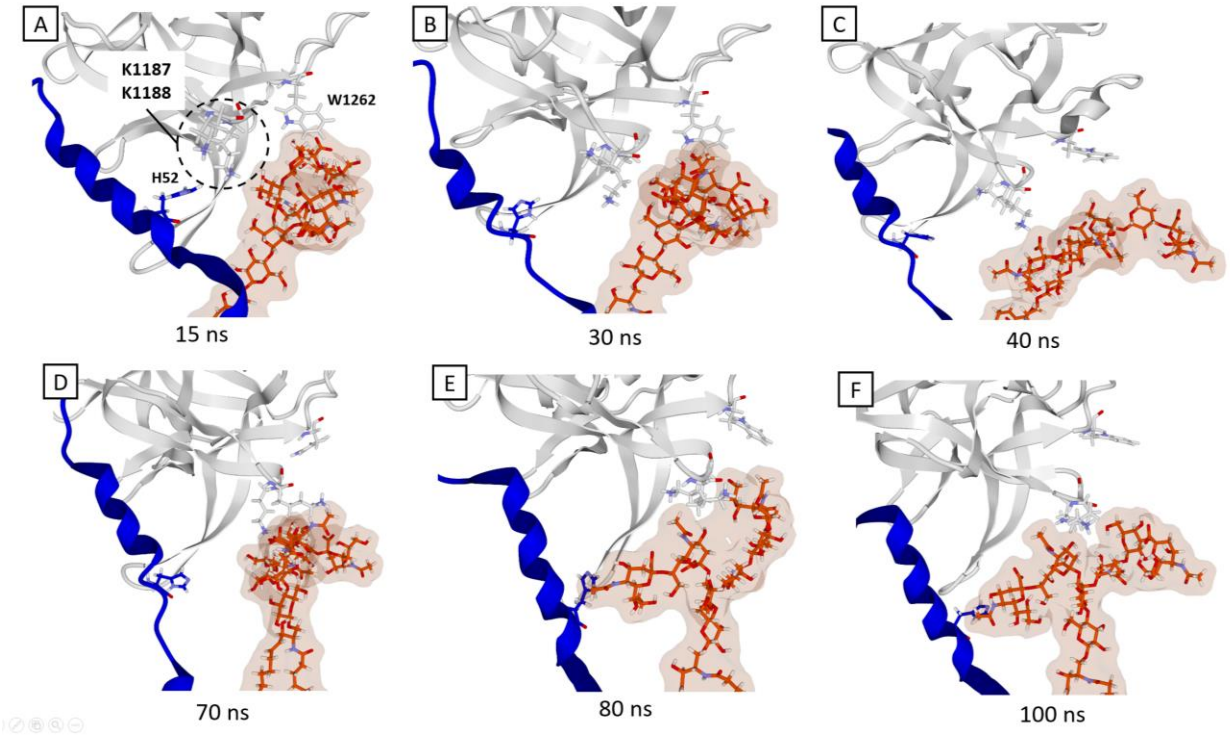

Figure 5: GT1b ganglioside bridges BoNT/B1 and SYT1. Snapshots taken at 15, 30, 40, 70, 80, 100 ns showing the panel of conformations adopted by the gangliosides and its insertion in the SBP (**A, B, C, D, E and F**). The ganglioside is in orange, h-SYT1 in blue and the toxin in white.

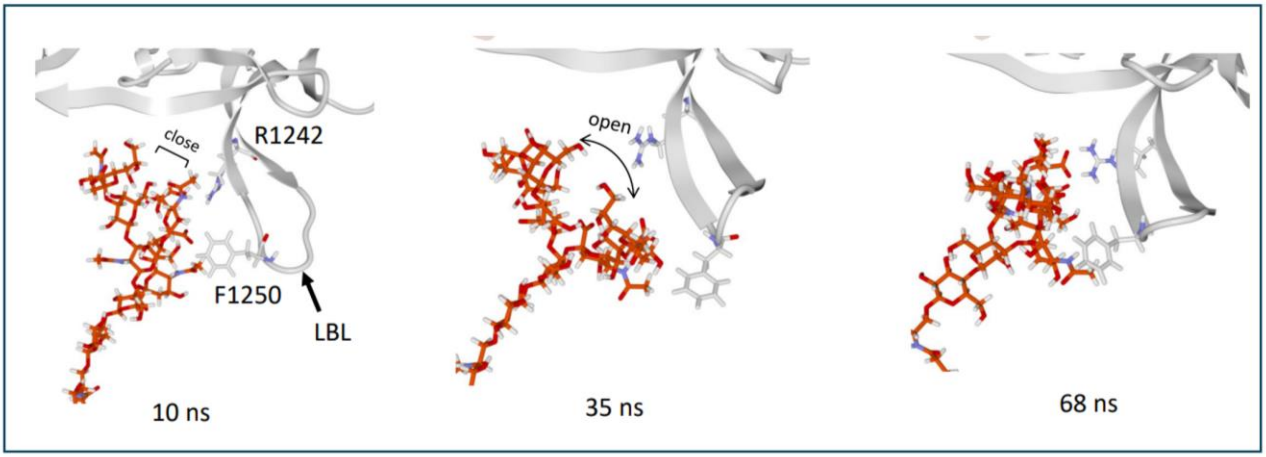

Figure 6: Snapshots of the interaction interface between the ganglioside and the lipid-binding loop of BoNT/B. Kinetics of the interaction mechanism of a GT1b molecule with the lipid-binding loop of BoNT/B1 at times 10, 35 and 68 ns. The ganglioside is shown as orange sticks, BoNT/B1 is shown as a white cartoon and residues R1242 and F1250 of BoNT/B1 are represented as white sticks.

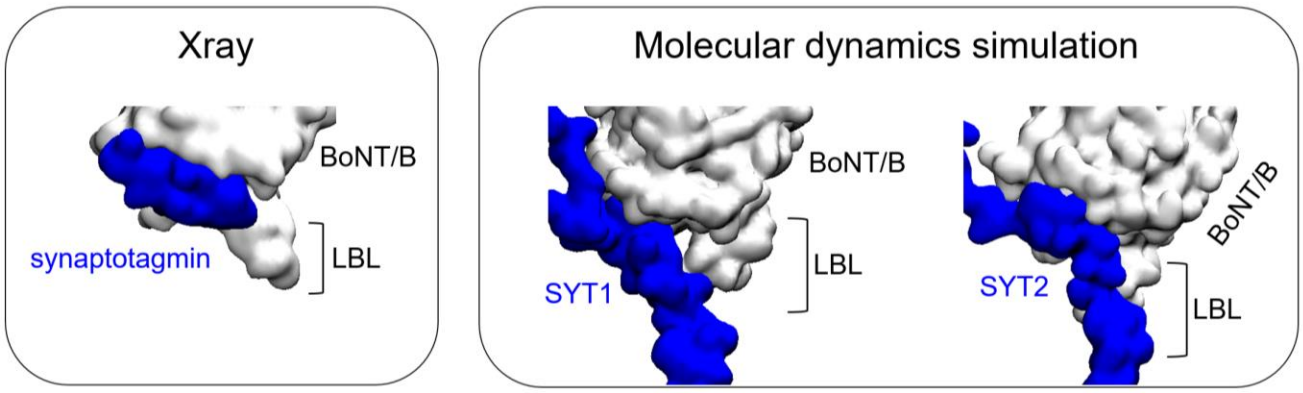

Figure 7: Snapshot showing the model of synaptotagmin in complex with BoNT/B1 in a liquid environment. These crystallographic data are obtained in the PDB (entry 6G5G) **(left panel).** Snapshots showing the model of SYT1 or SYT2 in complex with BoNT/B1 in a membrane environment **(right panel)**. BoNT/B is represented as white surface while the synaptotagmin is represented as blue surface.

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