In Silico Study of Membrane-Inserted Synaptotagmin Conformational Changes in Complex with Botulinum Toxin B1

Fodil Azzaz[1]*, Oussama El Far[2] and Jacques Fantini[1]

[1]Aix-Marseille Univ, INSERM UA16, Marseille, France

[2] Aix-Marseille Univ, INSERM U1325, DyNaMo, Marseille, France

* Corresponding author: Fodil Azzaz, INSERM UA16

E-mail: azzaz.fodil@gmail.com

Tel: +33 651 942 957

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Abstract

Synaptotagmins 1 and 2 (SYT1 and SYT2) are essential membrane proteins in neurotransmission, aiding in synaptic vesicle fusion. The juxtamembrane domains of both SYT1 and SYT2 are recognized by botulinum neurotoxin B1 (BoNT/B1), the agent of botulism. Current experimental models describe SYT1 and SYT2 interactions with BoNT/B1 but lack membrane insertion, which may bias the observed interactions. **Objective:** To explore the interactions of membrane-inserted SYT1 and SYT2 with BoNT/B1 and assess the impact of membrane constraints on these interactions. **Methods:** We used molecular dynamics simulations to study the conformational changes of membrane-inserted SYT1 and SYT2 when bound to BoNT/B1, focusing on the influence of membrane constraints. **Results:** Our simulations predict that membrane constraints reshape SYT1 and SYT2, enabling their juxtamembrane domains to interact with the BoNT/B1 lipid-binding loop, a unique interaction not seen in Xray models but in agreement with experimental data. SYT1's conformation is particularly affected, increasing solvent exposure at its BoNT/B1 binding site. The GT1b ganglioside stabilizes this complex by reducing solvent exposure, providing mechanistic insights into its role in BoNT/B1-SYT1 interaction, as experimental data support. **Conclusion:** Our study reveals overlooked molecular mechanisms in non-membrane models, highlighting dynamics essential to BoNT/B1-SYT interactions. These findings could guide the design of BoNT/B1-targeted antidotes or drugs able to enhance binding efficacy for therapeutic purposes.

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

Introduction

Synaptotagmins are membrane proteins defined structurally by a short, disordered extracellular domain in the N-terminal position, followed by a transmembrane domain and two calcium binding cytoplasmic C2 domains (C2A and C2B) [1]. In human, there are a total of seventeen synaptotagmin isoforms [2], classified into six different classes on the basis of their biological role, shared structural properties and sequence similarity of their extracellular domain [1]. These proteins are involved in neurotransmission by activating neurotransmitter release at the synapse [3, 4]. Synaptotagmin 1 (SYT1) and 2 (SYT2) are functional protein receptors of botulinum neurotoxin B (BoNT/B), a bacterial toxin that is produced by the spore-forming anaerobic gram-positive bacillus clostridium botulinum [5, 6]. It intoxicates neurons by interacting with specific membrane receptors at the neuromuscular junction and causes the disease botulism, a flaccid paralysis, by inhibiting acetylcholine release in peripheral nervous system through the cleavage of SNARE (Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptor) proteins. BoNT/B intoxication can lead to death due to respiratory failure [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].

Like the other serotypes, BoNT/B is a large protein of 150 kDa composed of two distinct domains, the 50 kDa light chain (LC) and the 100 kDa heavy chain (HC) [14, 15]. The LC is a metalloprotease that is associated with the HC via a disulfide bond and noncovalent bonds. The HC, consisting approximately to ⅔ of the toxin mass is divided into two subdomains, the N-terminal translocation subdomain (HN) and the C-terminal subdomain which consists of two functionally distinct domains of 25 kDa each which are H_{CN} and H_{cc}. HN has the biological role to translocate the LC into the cytoplasmic compartment while Hcc is the subdomain that specifically recognizes its membrane receptors.

The structures of co-crystallized BoNT/B with SYT1 or SYT2 have been resolved by Xray diffraction, allowing to identify the interaction site of SYT1 or SYT2 on the surface of BoNT/B. The crystallographic data suggest that BoNT/B interacts with a short

protein stretch corresponding to an alpha-helix defined by residues 39 to 49 or 47 to 58 for rat SYT1 and SYT2 respectively [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].
Moreover, BoNT/B has an intriguing structure called the lipid binding loop (LBL) which is formed by residues E1245 to E1252. This structure seems to be important for the BoNT/B to bind its protein and glycolipid receptors, but we still lack a detailed molecular model to understand how the LBL interacts with these membrane receptors [30, 31].

Molecular models showing the interaction between SYT1 or SYT2 with BoNT/B1 are limited because synaptotagmin is in a water-soluble environment rather than a membrane environment. This feature could result in describing a non-accurate interaction mechanism due to the absence of the appropriate environment for SYT1 and SYT2. Therefore, it is important to develop a molecular model in which the toxin interacts simultaneously with its protein receptor and gangliosides in a membrane context. For this purpose, we present in this study a molecular dynamics simulation (MDS) consisting of membrane inserted SYT1 or SYT2 in complex with BoNT/B1 in a lipid raft environment.

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/B (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 environment imposes geometric constraints on synaptotagmin conformation

The BoNT/B1-SYT1 and BoNT/B1-SYT2 complexes were inserted in the membrane in a way that respects the topology of a lipid raft. The Root Mean Square Fluctuation (RMSF) of BoNT/B1 in complex with SYT1 and SYT2 was analyzed. Most residues displayed RMSF values below 2 Å, with an average RMSF of 1.27 Å for the BoNT/B1-SYT1 system and 1.37 Å for the BoNT/B1-SYT2 system **(Figure 1)**. These values suggest that BoNT/B1 maintains overall structural stability in both complexes, with only limited flexibility observed in specific regions.

We observed during the beginning of the simulation 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 25 ns of simulation, forces the conformation of its structure on the side of the ganglioside interaction site of BoNT/B1 (circled in light orange) 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. This preliminary observation suggests a better chemical affinity between SYT2 and BoNT/B1.

The membrane constraints allow a close contact between the LBL structure of BoNT/B1 and 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 3** 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.

The gap formed between SYT1 and BoNT/B1 due to membrane constraints is filled with a GT1b molecule

As shown in **figure 2 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**). 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 4 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 4 B, C, D, E and F**). The GT1b molecule appears to act as a bridge between BoNT/B1 and SYT1 (**figure 4 F**) that stabilizes the link between the toxin and its protein receptor by occupying the space generated by the membrane constraints.

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 5**. 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].

The major information predicted by the present simulations is that the lipid raft environment imposes constraints on SYT1 and SYT2. However, SYT1 seems to be more impacted by the lipid constraints, which is illustrated by the freed space between SYT1 and BoNT/B1 (**figure 2 A**). Yet it is predicted that this free space can be rapidly occupied by the GT1b molecule that was initially interacting with the residue W1262 of the toxin. This interesting observation could explain why the binding of BoNT/B1 to SYT1 is dependent on the presence of gangliosides both *in vitro* and in cell context [43].

Also, we observe that the juxtamembrane domain of SYT1 and SYT2 interacts with the LBL of BoNT/B1. These observations are in good agreement with the study of Stern et al. [31] where they addressed by SPR the capacity of BoNT/B to bind rat SYT2 in soluble or nanodisc environments. The results showed that BoNT/B binds with the same capability to rat SYT2 in both environments. Interestingly, when the LBL structure of BoNT/B is deleted (residues F1247 to F1250) the toxin is still capable of binding SYT2 in soluble environment with the same dissociation constant than the wild type but fails to bind SYT2 in nanodisc. This observation cannot be explained by the experimental models because, as shown in **figure 6**, when studied in a soluble environment, the LBL structure of BoNT/B is not involved in the interaction with the protein receptor while it is involved in the models predicted by our simulations since our systems consider a proper membrane environment.

Finally, we observed in the simulations that a lipid raft-associated GT1b molecule adapts its conformation to bind simultaneously the residue R1242 of BoNT/B and more importantly the residue F1250 of the LBL structure of BoNT/B1. Once again, this observation is agreement with what has been experimentally shown in previous studies [31]. BoNT/B can bind to a nanodisc containing gangliosides but if the toxin is deprived of the amino acids forming its LBL structure, BoNT/B fails to bind those gangliosides embedded in nanodiscs. Moreover, when F1250 is replaced by an arginine, BoNT/B can no longer bind gangliosides contained in nanodisc, in full agreement with our model.

In conclusion, our molecular dynamics simulation predictions are in line with previous experiments studies. The models extracted from our simulation explain at the atomic resolution these experimental observations in a realistic environment that mimics a lipid raft environment. 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

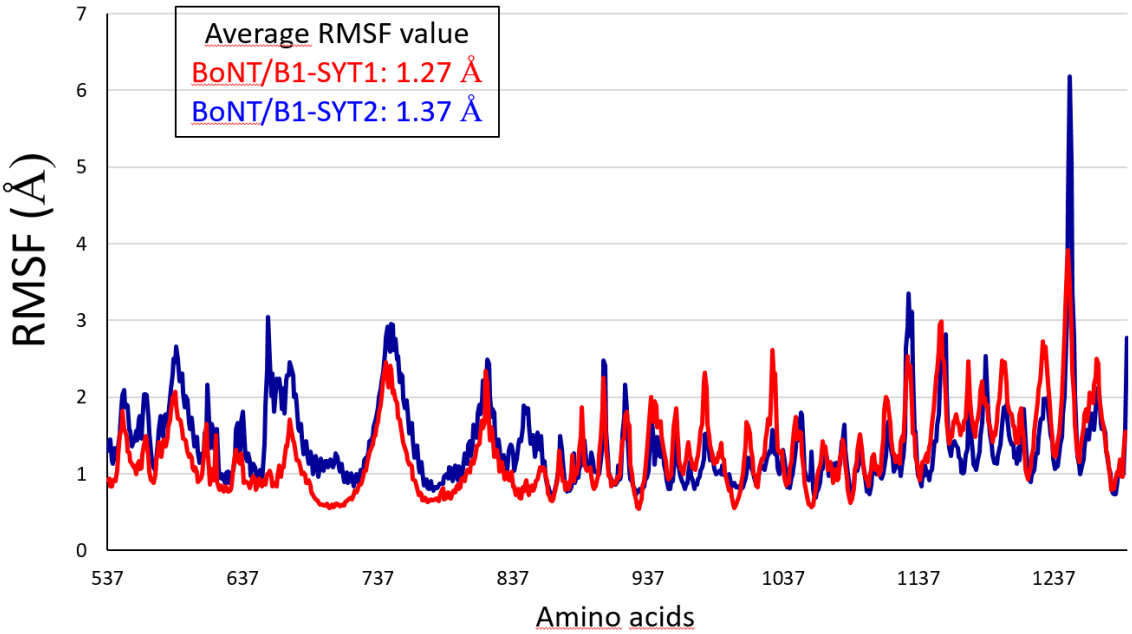

Figure 1: Root Means Square Fluctuation (RMSF) value of BoNT/B1 in complex with SYT1 (red lines) or SYT2 (blue lines). Is framed on the graph the average RMSF value for both conditions.

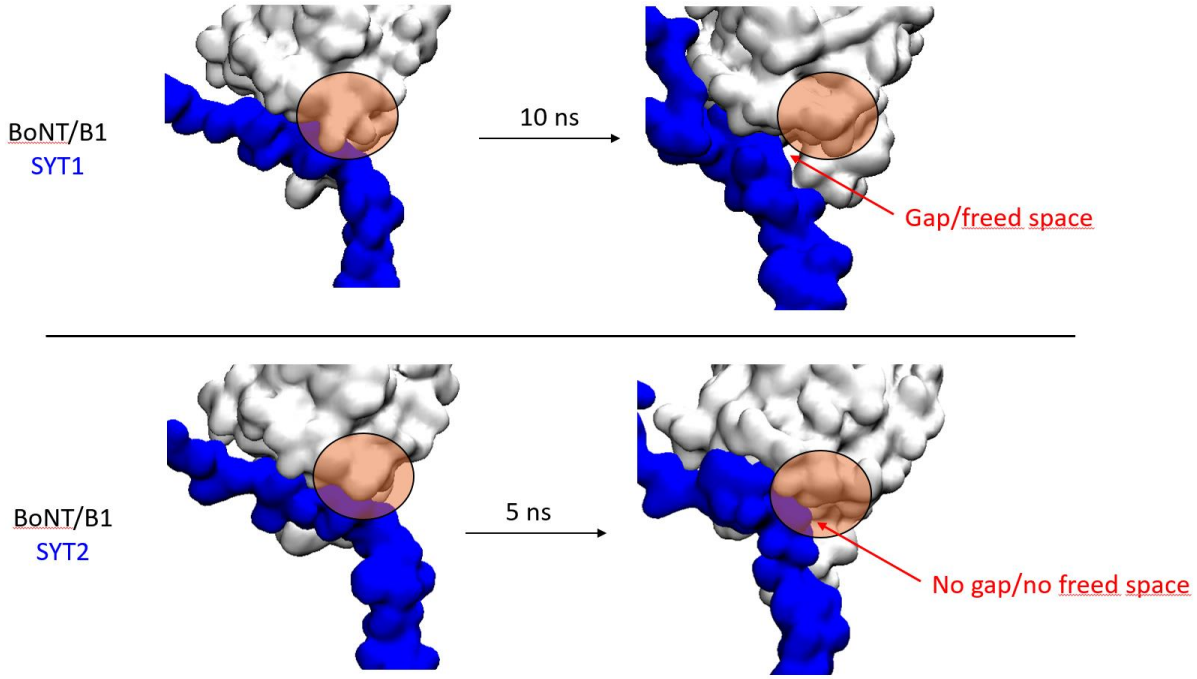

Figure 2: The membrane environment places stress on the synaptotagmin structure. This reduces the contact surface between synaptotagmin and BoNT/B1 on the GBS side (circled in light orange). Synaptotagmin is shown as a blue surface while BoNT/B1 is shown as a white surface.

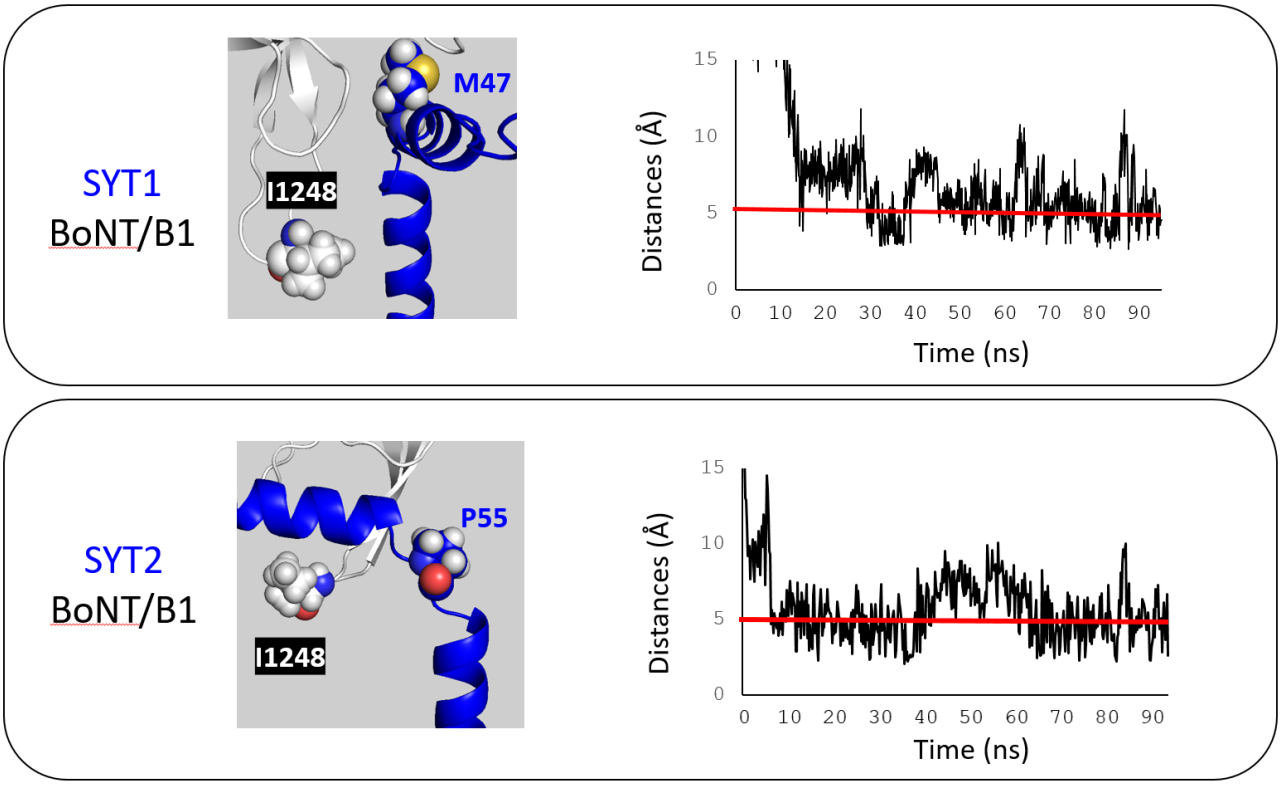

Figure 3: Snapshots showing residues involved in the interaction between the LBL structure of BoNT/B1 and the SYT1 **(upper panel)** or SYT2 **(lower panel)**. Line graphs on the right side plot the evolution of the minimal distance between the lateral chain of residues I1248-M47 (BoNT-SYT1) and I1248-P55 (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.

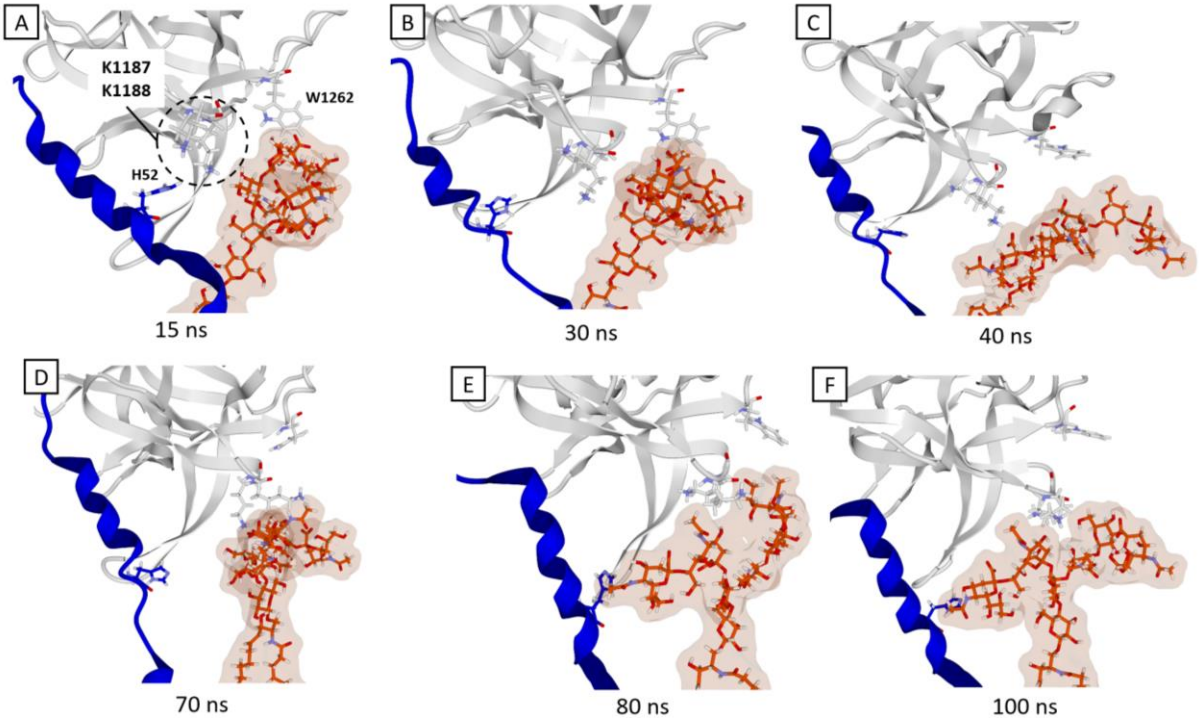

Figure 4: Snapshots taken at 15, 30, 40, 70, 80, 90 ns showing the panel of conformations adopted by the gangliosides and its insertion in the SBP (**A, B, C, D, E and F**). The ganglioside is in orange, h-SYT1 in blue and the toxin in white.

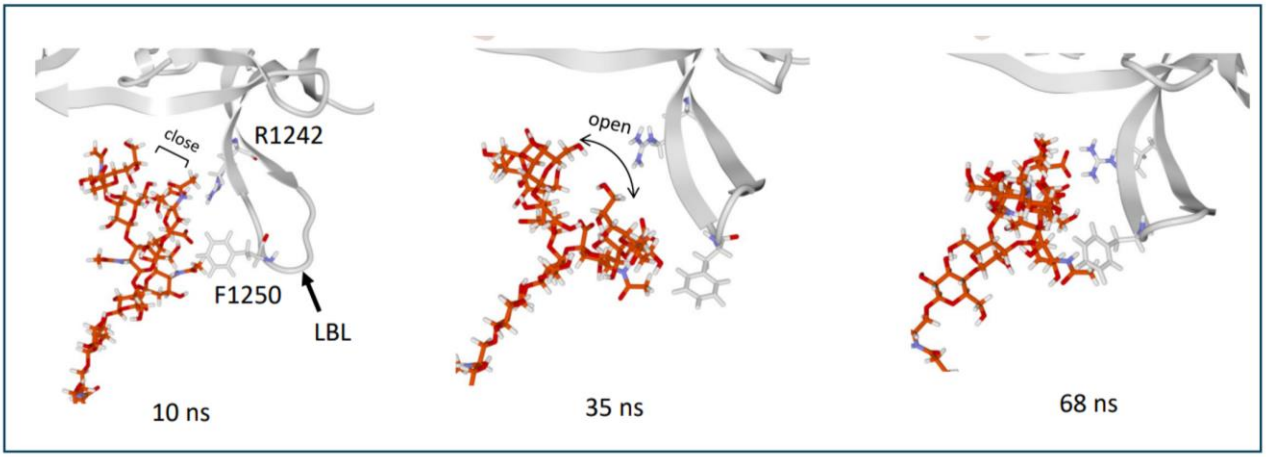

Figure 5: Snapshots of the interaction interface between the ganglioside and the lipid-binding loop of BoNT/B. Kinetics of the interaction mechanism of a GT1b molecule with the lipid-binding loop of BoNT/B1 at times 10, 35 and 68 ns. The ganglioside is shown as orange sticks, BoNT/B1 is shown as a white cartoon and residues R1242 and F1250 of BoNT/B1 are represented as white sticks.

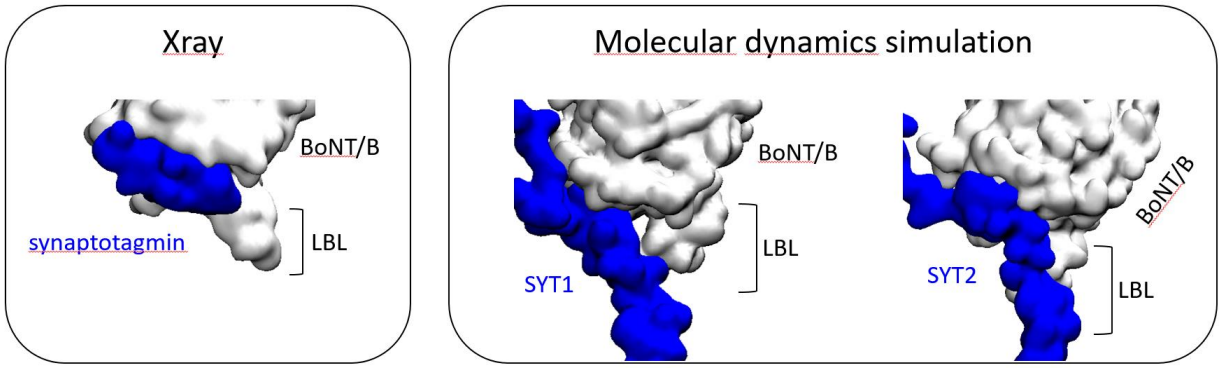

Figure 6: Snapshot showing the model of synaptotagmin in complex with BoNT/B1 in a liquid environment. These crystallographic data are obtained in the PDB (entry 6G5G) **(left panel).** Snapshots showing the model of SYT1 or SYT2 in complex with BoNT/B1 in a membrane environment **(right panel)**. BoNT/B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

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