Structure of dual receptor binding to botulinum neurotoxin B

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Botulinum neurotoxins are highly toxic, and bind two receptors to achieve their high affinity and specificity for neurons. Here we present the first structure of a botulinum neurotoxin bound to both its receptors. We determine the 2.3-Å structure of a ternary complex of botulinum neurotoxin type B bound to both its protein receptor synaptotagmin II and its ganglioside receptor GD1a. We show that there is no direct contact between the two receptors, and that the binding affinity towards synaptotagmin II is not influenced by the presence of GD1a. The interactions of botulinum neurotoxin type B with the sialic acid 5 moiety of GD1a are important for the ganglioside selectivity. The structure demonstrates that the protein receptor and the ganglioside receptor occupy nearby but separate binding sites, thus providing two independent anchoring points.
Botulinum neurotoxins (BoNTs) are the most toxic substances known, with a lethal dose (LD₅₀) value of ~1 ng kg⁻¹ (ref. 1). Seven distinct serotypes of BoNTs (A–G) exist⁵. Because of their extreme toxicity, they are one of the top potential bioterrorism agents. However, members of the BoNTs (BoNT/A and B) are also extensively used therapeutically to treat numerous medical conditions, such as cervical dystonia, cerebral palsy, strabismus, hemifacial spasm and myofascial pain, as well as for cosmetic purposes⁶–⁹.

These toxins are composed of three domains: the light chain (LC), the translocation domain (H₉) and the binding domain (H₈). The LC (~50 kDa), is a Zn-protease responsible for the cleavage of the SNARE (soluble NSF attachment protein receptor) proteins targeted by the toxin. SNARE proteins are central in vesicle exocytosis; cleavage of SNARE proteins by BoNTs inhibits acetylcholine release at the neuromuscular junctions⁷. H₈ (~50 kDa) is responsible for transport of the LC over the endosomal membrane. H₉ (~50 kDa) binds to receptors on nerve terminals²,⁸,⁹. H₈ is further divided into H₉N and H₉C, with H₉N being highly conserved and H₉C being diverse in sequence. The receptors bound by H₉C and the final protein being targeted by the LC varies between serotypes. In general, BoNTs achieve their high affinity and specificity for neurons by binding two receptors: gangliosides and one of the two synaptic vesicle proteins, synaptotagmin (Syt) or synaptic vesicle protein 2 (SV2). BoNT/A, D and E use SV2 as their protein receptor¹⁰–¹³, BoNT/F might also utilize SV2¹⁴,¹⁵, although this has not been functionally confirmed¹⁶. BoNT/B and BoNT/G as well as a BoNT subtype, type DC, bind Syt, both the Syt-I and Syt-II isoforms, as their protein receptors¹⁷–²⁴. Gangliosides bind in a ganglioside-binding site (GBS), conserved in all serotypes except C and D, consisting of a SXWY motif¹⁴,¹⁵,²⁵–³⁰. BoNT/C and D have in the same site an analogous GBS³¹–³³. Independent of which receptor the BoNT bind, the end result is similar; cleavage of SNARE proteins by the LC leading to muscle paralysis⁸.

Structures showing BoNT binding to either its protein receptor or gangliosides alone have been reported, including BoNT/B in complex with its protein receptor, synaptotagmin II (Syt-II)¹⁹,²², BoNT/A with ganglioside GT1b²⁸ and BoNT/F with ganglioside GD1a³⁴. Structures are also available for BoNT/B-HC, BoNT/C and D bound with Gal4. The remaining two subunits of GD1a, Glc1 and Gal2, is furthermore forming crucial aromatic stacking interactions I₁₂₄₀, H₁₂₄₁, S₁₂₆₀ and E₁₁₉₀. GalNAc₃ and Sia₆ form one hydrogen bond to N₁₂₇₃, N₁₁₀₅, G₁₂₇₇ and Y₁₂₆₃, Gal₄ to hydrogen bond to N₁₂₇₃ and N₁₁₀₅. Sia₅ forms hydrogen bonds to N₁₂₇₃, H₁₂₄₁ and S₁₂₆₀ and Sia₆ form a hydrogen bond to N₁₂₇₃. The asymmetric unit contained two BoNT/B molecules, which were virtually identical (root-mean squared deviation of 0.4 Å).

Here, we solve the structure of the first ternary complex of a BoNT in complex with both its protein receptor and ganglioside. We determine the 2.3 Å structure of BoNT/B bound to Syt-II and the ganglioside GD1a. We show that the binding of GD1a does not influence the toxins affinity for Syt-II. Our results show that the two receptor-binding sites are independent of each other.

**Results**

**Overall structure of the complex.** In a similar approach as previously reported, BoNT/B-HC was constructed and purified as a fusion construct together with Syt-II ²². This recombinant protein was then pre-incubated with GD1a, a major brain ganglioside known to bind BoNT/B³⁶, devoid of the ceramide part. Subsequent co-crystallization experiments yielded crystals that diffracted to 2.3 Å (Table 1). The structure was solved using molecular replacement. Residues 862–1291 of BoNT/B-HC were visible in the electron density, with the exception of two flexible loops between residues 1,151–1,159 and 1,245–1,252. Well-defined electron density became visible during refinement, accounting for both the fused Syt-II toxin-binding region (residues 45–59), and the entire GD1a ganglioside sugar (Fig. 1a). The remainder of the fused Syt-II was disordered and not visible in the electron density. The asymmetric unit contained two BoNT/B molecules, which were virtually identical (root-mean squared deviation of 0.4 Å).

Ganglioside binding to BoNT/B. This is the first structure of BoNT/B in complex with a complex ganglioside. The GBS on BoNT/B has been previously mapped, mainly through mutagenesis studies, and contains a conserved SXWY motif²⁶. The ternary complex structure revealed that GD1a indeed binds to this established GBS (Figs 1 and 2). Four subunits of GD1a (sialic acid (Sia₅), galactose (Gal₄), N-acetylgalactosamine (GalNAc₃) and Sia₆) make contacts to the GBS of BoNT/B (Fig. 1b). Sia₅ forms hydrogen bonds to N₁₂₇₃, N₁₁₀₅, G₁₂₇₇ and Y₁₂₆₃, Gal₄ to I₁₂₄₀, H₁₂₄₁, S₁₂₆₀ and E₁₁₉₀. GalNAc₃ and Sia₆ form one hydrogen bond each, to E₁₁₉₀ and W₁₂₆₂, respectively. W₁₂₆₂ is furthermore forming crucial aromatic stacking interactions with Gal₄. The remaining two subunits of GD1a, Glc₁ and Gal₂, do not interact with the GBS, but are still visible in the electron density due to the crystal packing to the second monomer in the asymmetric unit (Fig. 3). These crystal contacts are not biologically relevant; BoNT/B has previously been shown to

| Table 1 | Data collection and refinement statistics |
|-----------------|-----------------|
| **Data collection** | |
| Space group | P2₁ |
| Cell dimensions | |
| a, b, c (Å) | 47.2, 158.2, 75.2 |
| α, β, γ (°) | 90.0, 108.4, 90.0 |
| Resolution (Å) | 44.7–2.3 (2.42–2.3) |
| Rₘᵢₙₓ (%), I/σ(I) | 14.1 (79.3), 10.9 (2.3) |
| Completeness (%) | 99.7 (98.5) |
| Redundancy | 4.3 |
| **Refinement** | |
| Resolution | 44.7–2.3 |
| No. of unique reflections | 50,612 |
| Rₘᵢₙₓ / Rₜₒₒₑₑₓ | 15.5/18.9 |
| **No. of atoms** | |
| Protein | 7161 |
| Syt-II ligand | 284 |
| GD1a ligand | 117 |
| Water | 264 |
| **B-factors** | |
| Protein | 25.0 |
| Syt-II ligand | 31.2 |
| GD1a ligand | 26.9 |
| Water | 21.1 |
| **R.m.s. deviations** | |
| Bond lengths (Å) | 0.008 |
| Bond angles (°) | 1.28 |

Abbreviation: R.m.s., root-mean squared. The data was collected from one crystal. Values in parenthesis are for the highest resolution shell.

This is the first structure of BoNT/B bound to a complex ganglioside. The GBS on BoNT/B has been previously mapped, mainly through mutagenesis studies, and contains a conserved SXWY motif²⁶. The ternary complex structure revealed that GD1a indeed binds to this established GBS (Figs 1 and 2). Four subunits of GD1a (sialic acid (Sia₅), galactose (Gal₄), N-acetylgalactosamine (GalNAc₃) and Sia₆) make contacts to the GBS of BoNT/B (Fig. 1b). Sia₅ forms hydrogen bonds to N₁₂₇₃, N₁₁₀₅, G₁₂₇₇ and Y₁₂₆₃, Gal₄ to I₁₂₄₀, H₁₂₄₁, S₁₂₆₀ and E₁₁₉₀. GalNAc₃ and Sia₆ form one hydrogen bond each, to E₁₁₉₀ and W₁₂₆₂, respectively. W₁₂₆₂ is furthermore forming crucial aromatic stacking interactions with Gal₄. The remaining two subunits of GD1a, Glc₁ and Gal₂, do not interact with the GBS, but are still visible in the electron density due to the crystal packing to the second monomer in the asymmetric unit (Fig. 3). These crystal contacts are not biologically relevant; BoNT/B has previously been shown to
have a single GBS. Key residues in the GBS mapped by previous mutagenesis studies, are highlighted with a star in the figure (Fig. 1b).

We next compared the structure of BoNT/B with the previously reported structures of BoNT/A and BoNT/F (Fig. 2). We found that there are significant differences in the position of most sugar moieties (Fig. 2a–d). However, the position of Gal4 is fairly conserved in all three structures. Interestingly, GD1a has shifted one unit when compared with the previously determined structure of BoNT/B with bound sialyllactose (Fig. 2e,f). This shift could indicate plasticity in the binding site, with different sugars being able to occupy the binding sites in different binding registers. Alternatively, the use of a partial mimic of the ganglioside or soaking could have influenced the position.

**BoNT/B ternary complex.** Importantly, the structure of BoNT/B in the ternary complex, does not undergo any significant conformational changes as compared with apo BoNT/B and the BoNT/B–Syt-II complex with an HCC root-mean squared deviation of 0.4 and 0.3 Å, respectively. Binding of the receptors induces only minor adjustments of the positions of side chains in the binding sites. For instance, W1262 moves slightly in the direction of Gal4. Gal2 and Glc1 are located between the membrane and the toxin, and are likely flexible. However, even allowing these moieties to be flexible, sterical and distance restraints likely prevent the ganglioside to reach the protein receptor in BoNT/B for a direct interaction. The closest distance between GD1a and Syt-II is 15 Å. E1190 interacts with GD1a and E1191 interacts with Syt-II, this is the closest connection between the binding sites. This result further establishes that the high binding affinity mainly is the product of two independent anchoring points, with no direct interactions between the binding sites or the receptors (Figs 1 and 4). Furthermore, the toxins binding to the receptors impose sterical restraints on the interaction with the membrane (Fig. 4).

GD1a does not influence the affinity for Syt-II. To further confirm the lack of allosteric effects between the dual receptors, we measured the binding affinity of rat Syt-II peptide (residues

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**Figure 1 | Dual receptor binding to BoNT/B.** (a) Overall view of BoNT/B (grey) with GD1a (green) and mouse Syt-II (orange) bound. Lower panel shows a close-up view on the GD1a- and Syt-II-binding sites, with the GD1a moieties and the Syt-II residues, interacting with BoNT/B, shown as sticks. Phenylalanine 54 is indicated, this position is a leucine in human Syt-II that has a drastically lower affinity to BoNT/B than mouse Syt-II. (b) Schematic overview of possible hydrogen bonds between GD1a and BoNT/B (red dashed lines) and an internal hydrogen bond (blue dashed line). The hydrogen bond distance is shown for each bond (Å). Red stars show residues that previously, via mutagenesis, have been shown to be important for ganglioside binding. In GD1a, a ceramide is present at the R position, the GD1a oligosaccharide used here has a hydrogen in this position. (c) Stereo view of the bound GD1a, with its electron density (2Fo-Fc map contoured at 1.5σ) in blue, and the possible hydrogen bonds to BoNT/B residues as dashed lines.
40–60) to BoNT/B-H₂C, without and with a pre-incubation with GD1a oligosaccharide. Isothermal titration calorimetry (ITC) gave a $K_D$ of $0.14 \pm 0.05 \mu M$ and $0.18 \pm 0.06 \mu M$, respectively (Fig. 5). The presence of GD1a did thus not significantly affect the binding affinity between Syt-II and BoNT/B, further indicating that there is no allosteric contribution in the binding of Syt-II and GD1a.

Discussion

The dual receptor concept for BoNT family toxins was proposed 27 years ago. The ternary structure, of BoNT/B bound to both Syt-II and GD1a concurrently, presented here, now proves the dual receptor model at the structural level. The structure demonstrates that the binding sites are separate and there are only minor changes in the structure upon binding of the receptors. There is no allosteric contribution in the binding of Syt-II and GD1a to BoNT/B. Due to the high sequence similarity between the recognition domains of Syt-I and Syt-II, it is likely that these receptors bind the toxin in a very similar way.

This is the first structure reported of BoNT/B bound to a complex ganglioside. Previous experiments have shown that GT1b and GD1a support BoNT/B binding, but that GD1b and GM1 bind substantially weaker. The structure presented here reveals the structural basis for this selectivity that arises from the interactions of Sia5. The Sia5 moiety is strongly bound to BoNT/B with key interactions for the overall binding of the ganglioside. Both GT1b and GD1a have a Sia5 moiety, whereas GD1b and GM1 do not, explaining the differences in affinity. Furthermore, K1265 and R1269, close to W1262, are well...
The detailed description of dual receptor binding to BoNT/B can be used to develop toxin countermeasures targeting both sites simultaneously, thus benefitting from the high affinity achieved by binding multiple sites, as naturally occurring in the BoNTs.

Methods

Constructs and peptides. The cDNA encoding BoNT/B-HC (residues 857–1291, GenBank: AB232927) was synthesized by GeneArt (Regensburg, Germany), with codon optimized for Escherichia coli expression. The cDNA encoding mouse Syt-II (residues 8–61) by overlapping PCR, and subcloned into pET-28a vector at Ndel/XhoI site. The following additional residues from pET28 vectors were also expressed in the fusion protein: N-terminus before BoNT/B-HC: MGSSHHHHHHSSGLVPRGSH; C-terminus after Syt-II: VLEHHHHHH. Peptide corresponding to the toxin-binding site in rat Syt-II (residues 40–60) was synthesized (>95% purity) by JPT Peptide Technologies GmbH (Germany).

Protein expression and purification. Double His-6 tagged BoNT/B-HC – Syt-II was expressed in E. coli (BL21 strain). The cells were grown in terrific broth medium containing 50 μg ml⁻¹ kanamycin, and grown in 21 flasks at 37°C, pH 7.0, with addition of air to mix the medium and maintain an excess of oxygen, to an OD₆₀₀ of ~1.5. The temperature was then lowered to 20°C, and expression induced by the addition of IPTG with a final concentration of 1 mM. After overnight induction, the cells were harvested and frozen in ~ – 80°C. For protein purification, the pelleted cells were thawed, resuspended to an OD₆₀₀ of ~100 in 50 mM KPi, pH 7.5, 300 mM NaCl and 10% glycerol. Lysation was performed by passing the cell suspension two times through an Emulsiflex-C3 (Avestin, Germany) at 20,000 psi. Unlysed cells and cell debris were spun down via ultracentrifugation at 4°C, 267,000g for 60 min. The supernatant was collected, imidazole, pH 7.8, was added to a final concentration of 12 mM, and incubated with 0.5 ml Ni-NTA per 10 ml supernatant at 4°C for 60 min, rotating. The material was then packed in a disposable 10 ml column (BioRad), washed 20 column volumes with wash buffer (50 mM HEPES, pH 7.8, 300 mM NaCl and 45 mM imidazole, pH 7.8). The protein was then eluted using wash buffer supplemented with 500 mM imidazole, pH 7.8. Purification was then finished by running the protein over a Superdex 200 10/300GL size exclusion column, pre-equilibrated with 20 mM HEPES, pH 7.0 and 150 mM NaCl. The peak fractions were pooled and concentrated to 5.0 mg ml⁻¹, using a 30-kDa molecular weight cutoff spin concentrator (Vivaspin). Glycerol was then added to a final concentration of 10%, and the protein was subsequently flash frozen in liquid nitrogen and stored at ~ –80°C. Expression and purification of BoNT/B-HC for ITC experiments was performed in the same way as BoNT/B-HC – Syt-II, with the exception that BoNT/B-HC was not frozen, and the final buffer in the size exclusion chromatography was 20 mM KPi, pH 7.0, 150 mM NaCl.

Crystallization and structure determination. Purified protein was thawed, GD1a oligosaccharide (Elicityl, France) was added to a final concentration of 2.5 mM, and was subsequently crystallized using the vapour diffusion technique. Diffraction quality crystals grew from a solution containing 0.2 M MgCl₂, 0.1 M HEPES pH 7.0–7.2 and 20–24% PEG 6000. Crystals appeared between 8 and 12 days. For cryo protection, they were washed in mother liquor supplemented with 40% PEG 6000, and subsequently flash frozen in liquid nitrogen. Diffraction data was collected at 0.918 Å at beamline 14.1, BESSY, Berlin. The crystals diffracted to 3.2 Å, and were in space group P2₁. Data processing and reduction were carried out using XDS and programs from the CCP4 suite. Relevant statistics are shown in Table 1. The structure was solved via molecular replacement, using the previously solved structure of BoNT/B as a search model (PDB code: 2NM1). A few cycles of refinement in Refmac5 and Phenix.refine, interspersed with model building using Coot, were needed to build the full model and its ligands. The final structure contains residues 862–1291, with the exception of two loops, 1152–1158 and 1246–1252 that had ambiguous electron density. At the N-terminus, 6 residues of BoNT/B are missing (MNSEIL), as well as the N-terminal tag. The Syt-II-binding domain, residues 45–59, has well-defined electron density, leaving residues 1–44 of Syt-II (which acts as a linker in this construct) flexible. Furthermore, the entire GD1a molecule is visible in the electron density. The highest resolution data, to 2.3 Å, had a twin fraction of ca. 35%, as analyzed via phenix.xtriage. The structure was therefore refined using twin refinement. Another crystal form, in space group C2, diffracted to 2.6 Å and did not have twinning. The structure was also solved with this data, which was not twinned, and used for comparison with the higher resolution structure obtained from the twinned data. Final Rwork/Rfree of the unwinned data was 21.5/26.1%. No substantial differences were observed between the two structures, and as the twinned data with higher resolution gave better final electron density, we choose to use that data for the final model building.

Isothermal titration calorimetry. Binding of Syt to BoNT/B-HC was measured via ITC on a ITC200 (GE Healthcare) at 25°C and 1.000 rpm. A volume of 200 μl of
Figure 5 | ITC titrations of Syt-II to BoNT/B. Representative ITC titrations of Syt-II to BoNT/B, without (a) and with (b) pre-incubation of 75 μM GD1α. (a) $K_D = 0.14 \pm 0.05 \mu M$, (b) $K_D = 0.18 \pm 0.06 \mu M$. The error is the s.d. on at least two independent measurements. The baseline above zero in b is due to a slight buffer mismatch, and does not influence the calculated values.

BoNT/B-HC₂ (typically concentrated to 10–25 μM, in 20 mM KPi pH 7.5, 150 mM NaCl) was added to the cell. In the case of GD1α oligosaccharide addition, 0.25 mM was added to the protein and incubated for 10 min on ice before adding the protein to the cell. Binding was measured upon addition of Syt-II peptide in a stepwise manner, typically 16 injections of 2.6 μl each, at a concentration of 10–15 times the protein concentration in the cell. The first titration was 0.5 μl, and was subsequently deleted in the data analysis. The analysis was performed using the software provided by the manufacturer.

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**Author contributions**

R.P.-A.B. and L.P. performed the experiments. R.P.-A.B., M.D. and P.S. planned the experiments, performed data analysis and wrote the manuscript.

**Additional information**

Accession codes: Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession code 4KBB.

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