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Botulinum neurotoxins C, E and F bind gangliosides via a conserved binding site prior to stimulation-dependent uptake with botulinum neurotoxin F utilising the three isoforms of SV2 as second receptor

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Abstract

The high toxicity of clostridial neurotoxins primarily results from their specific binding and uptake into neurons. At motor neurons, the seven botulinum neurotoxin serotypes A–G (BoNT/A–G) inhibit acetylcholine release, leading to flaccid paralysis, while tetanus neurotoxin blocks neurotransmitter release in inhibitory neurons, resulting in spastic paralysis. Uptake of BoNT/A, B, E and G requires a dual interaction with gangliosides and the synaptic vesicle (SV) proteins synaptotagmin or SV2, whereas little is known about the entry mechanisms of the remaining serotypes. Here, we demonstrate that BoNT/F as wells depends on the presence of gangliosides, by employing phrenic nerve hemidiaphragm preparations derived from mice expressing GM3, GM2, GM1 and GD1a or only GM3. Subsequent site-directed mutagenesis based on homology models identified the ganglioside binding site at a conserved location in BoNT/E and F. Using the mice phrenic nerve hemidiaphragm assay as a physiological model system, cross-competition of full-length neurotoxin binding by recombinant binding fragments, plus accelerated neurotoxin uptake upon increased electrical stimulation, indicate that BoNT/F employs SV2 as protein receptor, whereas BoNT/C and D utilise different SV receptor structures. The co-precipitation of SV2A, B and C from Triton-solubilised SVs by BoNT/F underlines this conclusion.

Keywords: botulinum neurotoxin, ganglioside binding site, ganglioside dependence, protein receptor, stimulated neurotoxin uptake, synaptic vesicle glycoprotein 2, tetanus neurotoxin.

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The family of clostridial neurotoxins (CNTs) consists of tetanus neurotoxin (TeNT) and the seven botulinum neurotoxin serotypes (BoNT/A–G), which cause the diseases tetanus and botulism, respectively. With a median lethal dose < 1 ng/kg body weight they represent the most toxic agents known (Gill 1982), with their mode of action presumably related to their specific binding to unmyelinated areas of nerve terminals (Dolly *et al.* 1984). At motor neurons, BoNTs inhibit acetylcholine release, leading to flaccid paralysis, while TeNT is transported retrogradely to inhibitory neurons and blocks release of glycine or GABA, which results in spastic paralysis.

The crystal structures of the 150 kDa large BoNT/A, B and E holotoxins (Lacy *et al.* 1998; Swaminathan and Eswaramoorthy 2000; Kumaran *et al.* 2009) revealed that all

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Abbreviations used: BoNT, botulinum neurotoxin; BoNT/A–G, BoNT serotypes A–G; CNT, clostridial neurotoxin; GD3S, GD3-synthetase; GST, glutathione-*S*-transferase; HC, 100 kDa heavy chain; H_C, 50 kDa C-terminal half of H_C; H_{CC}, 25 kDa C-terminal half of H_C; H_{CN}, 25 kDa N-terminal half of H_C; HNE buffer, HEPES–NaCl–EDTA buffer; KO, knockout; LC, 50 kDa light chain; MPN, mouse phrenic nerve; NAcGal, *N*-acetylgalactosamine; NAcGalT, *N*-acetylgalactosamine-transferase; NAcNeu, *N*-acetylneuraminic acid or sialic acid; NMRI, Naval Medical Research Institute; scBoNT, single chain BoNT; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SV, synaptic vesicle; SV2, synaptic vesicle glycoprotein 2; Syt-X, isoform X of synaptotagmin; TeNT, tetanus neurotoxin; wt, wild-type.

CNTs are composed of four functionally independent domains that perform individual tasks in the multistep intoxication process. The 100 kDa heavy chain (HC) contains three of the four domains. The carboxyl-terminal 25 kDa H_{CC} domain of H_C mediates the neurospecific binding and uptake as first step (Brunger and Rummel 2009). The role of the amino-terminally neighbouring 25 kDa H_{CN} domain in the intoxication mechanism is still unclear, although it is known that BoNT/A H_{CN} weakly interacts with phosphatidylinositol phosphates (Muraro *et al.* 2009). Both domains form the 50 kDa H_C-fragment, which was proteolytically isolated from full-length BoNT or autonomously expressed in *Escherichia coli* (*E. coli*) and displays intact neurospecific binding (Lalli *et al.* 1999). Thereafter, the amino-terminal half of HC, the 50 kDa translocation domain H_N forms a channel and delivers the partially unfolded, disulphide bond linked 50 kDa light chain (LC) into the cytosol (Koriazova and Montal 2003; Fischer and Montal 2007). Within the cytosol, the liberated LC (catalytic domain) hydrolyses one member of the three neuronal soluble *N*-ethylmaleinimid-sensitive factor attachment protein receptor proteins, thereby blocking exocytosis (Schiavo *et al.* 2000).

It has been demonstrated that the H_{CC} domains of BoNT/B and G carry conserved interaction sites for one polysialo ganglioside, as well as for their protein receptor – the vesicular molecule synaptotagmin (Syt) (Swaminathan and Eswaramoorthy 2000; Rummel *et al.* 2004b, 2007; Chai *et al.* 2006; Jin *et al.* 2006; Kohda *et al.* 2007). Binding to both receptors allows their neurospecific uptake confirming the double receptor mechanism (Montecucco 1986). Consistent with this finding, a conserved ganglioside binding site, containing the E(D)…H…S(G)XWY…G(S) motif, which interacts with the terminal *N*-acetylgalactosamine–galactose (NAcGalβ3-1Galβ) moiety of GT1b and GD1a (nomenclature for gangliosides is in accordance with Svennerholm 1994), could also be described for BoNT/A (Rummel *et al.* 2004b; Stenmark *et al.* 2008), BoNT/C (Tsukamoto *et al.* 2008) and TeNT (Fotinou *et al.* 2001; Rummel *et al.* 2003). Until recently, corresponding ganglioside binding sites in BoNT/E and F were still to be characterised. However, an amino acid sequence alignment of all H_{CC} domains does not indicate the existence of such a conserved motif in BoNT/D (Rummel *et al.* 2004b) and its binding to gangliosides is currently ambiguous. In addition, at the homologous location of the Syt binding site in BoNT/B and G, the TeNT H_{CC} domain contains a second ganglioside binding site, interacting either with the disialic acid branch of GD1b and GT1b (Fig. 1a) (Emsley *et al.* 2000; Fotinou *et al.* 2001; Rummel *et al.* 2003; Jayaraman *et al.* 2005; Chen *et al.* 2008) or eventually with a glycosylated protein receptor (Herreros *et al.* 2000).

Widely distributed gangliosides represent low affinity neuronal acceptors responsible for accumulating CNTs on neuronal surfaces. This interaction has been studied extensively using *in vitro* assays for all serotypes (reviewed in Halpern and Neale 1995; Yowler and Schengrund 2004). Their physiological role has been demonstrated for all the CNTs barring BoNT/F, using mice or mouse hippocampal neurons displaying different expression patterns of polysialo gangliosides, as a result of specific gene knockouts (KOs) (Kitamura *et al.* 1999, 2005; Bullens *et al.* 2002; Tsukamoto *et al.* 2005; Dong *et al.* 2007, 2008; Rummel *et al.* 2007).

Several studies demonstrated an accelerated uptake of TeNT (Schmitt *et al.* 1981; Simpson 1985), BoNT/A (Black and Dolly 1986) and BoNT/E (Lawrence *et al.* 2007) into the phrenic nerve (which is prepared together with the adjacent diaphragm) upon electrical stimulation. As a consequence, increased nerve stimulation resulted in an earlier onset of muscle paralysis upon application of BoNT/A (Hughes and Whaler 1962; Simpson 1980) and TeNT (Schmitt *et al.* 1981). Other studies used high K^+ concentrations to stimulate neurons, leading to an accelerated uptake of BoNT/A, B, E and G into spinal cord and hippocampal preparations (Keller *et al.* 2004; Dong *et al.* 2007, 2008). As neuronal stimulation causes increased rates of exo- and endocytosis of synaptic vesicles (SVs), the amino-terminal intra-vesicular domains of the SV membrane proteins Syt-I and Syt-II were identified as the protein receptors for BoNT/B (Nishiki *et al.* 1993, 1994, 1996a,b; Kozaki *et al.* 1998; Dong *et al.* 2003). Co-crystallisation of Syt-II with BoNT/B refined the binding segment to an α-helical 17-mer peptide adjacent to the transmembrane domain (Chai *et al.* 2006; Jin *et al.* 2006). BoNT/G interacts with identical segments of Syt-I and Syt-II, but none of the remaining CNTs bind to either Syt-I or Syt-II (Rummel *et al.* 2004a). Thereafter, the SV glycoprotein 2 (SV2) was identified as a protein receptor for BoNT/A. SV2 is a highly glycosylated protein, possessing 12 transmembrane domains, which exists in three isoforms SV2A, B and C. Interestingly, *in vitro* BoNT/A displays highest affinity for the unglycosylated, 125 amino acid luminal domain 4 of isoform SV2C, followed by SV2A and SV2B (Dong *et al.* 2006; Mahrhold *et al.* 2006). Recently, it was demonstrated that a singular *N*-glycosylation of the luminal domain 4 of SV2A and SV2B enables the binding and uptake of BoNT/E into hippocampal neurons (Dong *et al.* 2008). The protein receptors of the remaining BoNT serotypes have still to be identified.

In this report, employing mice phrenic nerve (MPN) hemidiaphragm preparations derived from mice only expressing either GM3 or the a-series gangliosides GM3, GM2, GM1 and GD1a, we demonstrate that the neurotoxicity of BoNT/A, C, E and F depends on complex polysialo gangliosides (GM3, GM2, GM1, GD1a, GD1b, GT1b). Based on homology models, key residues within a conserved ganglioside binding site were identified in BoNT/C, E and F. Corresponding mutations led to a drastic decrease in binding to gangliosides and synaptosomal membranes, as well as in neurotoxicity concomitant with reduced uptake of the respective toxins.

Furthermore, the number of protein receptors for the CNT was narrowed down by inhibiting the uptake of the eight CNTs through the systematic addition of identified non-toxic H_C-fragments to the MPN assay system. BoNT/A H_C, as well as BoNT/F H_C, was able to inhibit the activity of BoNT/E and BoNT/F, indicating an involvement of SV2 as a protein receptor for BoNT/F. Furthermore, we show that uptake of BoNT/F by the MPN is stimulation dependent as expected for a vesicle receptor. Moreover, the BoNT/F H_C-fragment co-precipitates with all three SV2 isoforms from detergent-solubilised vesicles, whereas no interaction with other vesicle proteins (such as Syt) was detectable. This conclusively demonstrates that SV2 acts as co-receptor for BoNT/F. Moreover, the stimulation-dependent uptake of BoNT/C and BoNT/D suggests the involvement of SV structures during their neuronal entry step, although a molecular interaction of BoNT/C with any of the SV2 or Syt isoforms could not be detected.

Materials and methods

Isolated gangliosides were purchased from Merck Biosciences GmbH (Darmstadt, Germany). Statistical analysis was performed using GraphPad prism 4.03 software (GraphPad Software Inc., San Diego, CA, USA).

Generation of homology models

The amino acid sequences of BoNT/E H_C (residues 845–1252) and BoNT/F H_C (residues 866–1278) were aligned to their closest relatives – BoNT/A (3BTA.pdb) and TeNT (1A8D.pdb) – using the SWISS-MODEL Protein Modeling Server (Schwede *et al.* 2003). The geometry of the resulting structural models was refined using Discovery Studio (Accelrys Inc., San Diego, CA, USA). The stereochemical quality of the models was assessed by structural analysis, using both the protein health report function found in Discovery Studio, as well as by superimposition of the C-alpha backbone of the recently published crystal structures of BoNT/E (3ffz.pdb) and BoNT/F H_C (3fuq.pdb) using Discovery Studio.

Plasmid constructions

Plasmids encoding the H_C-fragments (pH_CAS, pH_CBS, pH_CCS, pH_CDS, pH_CES, pH_CFS pH_CGS and pH_CTS) and the full-length BoNT/A, BoNT/B, BoNT/D, BoNT/G and TeNT (pBoNTAS, pBoNTBS, pBoNTDS, pBoNTGS, pBoNTGS-Thro and pTeNTS) have been described previously, as has the plasmid encoding the glutathione-*S*-transferase (GST)-Syt-II 1–61 fusion protein (Rummel *et al.* 2003, 2004a,b; Bade *et al.* 2004). The plasmid pBoNTCS-Thro encoding full-length BoNT/C fused carboxyl-terminally to a Streptag and containing an *E. coli* protease sensitive peptide between LC and HC (-Thro like pBoNTGS-Thro; Rummel *et al.* 2004a) was generated by PCR, using suitable primers and purified bacteriophage DNA (acc. no. X53751) as template. The plasmid pH6BoNTES-Thro encoding full-length BoNT/E fused amino-terminally to a His_6tag, carboxyl-terminally to a Streptag and containing an *E. coli* protease sensitive peptide between LC and HC (-Thro) was generated by PCR, using suitable primers and total bacterial DNA (strain NCTC 11219, acc. no. X62683) as template. The plasmid pH6BoNTFS encoding full-length BoNT/F fused amino-terminally to a His_6tag and carboxyl-terminally to a Streptag was generated by PCR, using suitable primers and total bacterial DNA (strain NCTC 10281, acc. no. X81714) as template. Mutations in the H_C-fragments of BoNT/C, E and F were generated using the Genetailor method (Invitrogen GmbH, Karlsruhe, Germany) using suitable primers and pH_CCS, pH_CES and pH_CFS as template DNA. Mutated expression plasmids for full-length BoNT/C, E and F were generated by swapping DNA fragments between pBoNTCS-Thro, pH6BoNTES-Thro, pH6BoNTFS and mutated pH_CCS, pH_CES and pH_CFS plasmids, respectively. Plasmids encoding the H_C-fragments fused to an amino-terminal One-Streptag and a carboxyl-terminal His_6tag ($pS3H_CAH6$, $pS3H_CBH6$ $pS3H_CCH6$, $pS3H_CEH6$ and $pS3H_CFH6$) were constructed by first introducing a sequence encoding a His_6tag into the *Eco*RV site of the pPR-IBA102 vector (IBA GmbH, Göttingen, Germany) and subsequent in-frame cloning of sequences encoding the H_C-fragments between the sequences encoding for the two affinity tags. Nucleotide sequences of all mutants were verified by DNA sequencing.

Production of recombinant proteins

Recombinant full-length neurotoxins and the various H_C-fragments were purified from the *E. coli* strain M15 [pREP4] (Qiagen GmbH, Hilden, Germany), following 16 h of induction at 22°C. Proteins were purified on nickel-nitrilotriacetic acid beads (Qiagen) and/or Streptactin-Superflow (IBA GmbH), according to the manufacturers' instructions and kept in 100 mM Tris–HCl, pH 8.0. H_C-fragments intended for MPN competition measurements were dialysed against Krebs–Ringer solution (in mM: NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11, gassed with 95% O_2, 5% CO_2, pH 7.4). scTeNT was nicked into the di-chain form by digestion with beaded trypsin as described previously (Rummel *et al.* 2003). $S3H_CXH6$-fragments were purified from the *E. coli* strain BL21-DE3 (Stratagene, La Jolla, CA, USA) following 16 h of induction at 22°C. Proteins were first purified using nickel-nitrilotriacetic acid beads (Qiagen) followed by Streptactin-Superflow beads (IBA GmbH), according to the manufacturers' instructions. Highly purified $S3H_CXH6$-fragments were dialysed against HNE buffer (in mM: HEPES–KOH 25, NaCl 145 and EDTA 1, pH 7.4). GST-Syt-II 1–61, used for pull-down experiments, was purified from *E. coli* BL21 using glutathione–sepharose beads and dialysed against Tris/NaCl buffer (20 mM Tris–HCl, 150 mM NaCl and 0.5% Triton X-100, pH 7.2). All recombinant proteins were shock frozen in liquid nitrogen and kept at −70°C.

Glutathione-*S*-transferase pull-down assay

GST-Syt-II 1–61 fusion protein (0.14 nmol) was immobilised to 10 μL of glutathione–sepharose beads (GE Healthcare Europe GmbH, Freiburg, Germany) and incubated in parallel with either full-length single chain BoNT/B or G (scBoNT/B or G) and the corresponding H_C-fragments, in a total volume of 100 μL Tris/NaCl/Triton-buffer for 90 min at 4°C. Beads were then collected by centrifugation with 2000 ×*g* and washed three times each with 35 bed volumes of the same buffer. The washed pellet fractions were boiled in sodium dodecyl sulphate (SDS) sample buffer and analysed together with the corresponding supernatants using

SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining. The optical density of the GST-Syt-II 1–61 and scBoNT/X bands was determined, normalised per lane and calculated as the mol% using the corresponding molecular weight. Curves were fitted to the data points using a non-linear regression model and applying the following sigmoidal dose–response equations: y(scBoNT/B vs. H_CB) = 0.227 + (0.67)/[1 + 10^(3.318 − x)] R^2 = 0.9346; y(scBoNT/B vs. H_CG) = 0.299 + (0.684)/[1 + 10^(6.130 − x)] R^2 = 0.9311; y(scBoNT/G vs. H_CB) = 0.1684 + (0.7499)/[1 + 10^(2.734 − x)] R^2 = 0.983; y(scBoNT/G vs. H_CG) = −4.012 + (5.475)/[1 + 10^(36.6 − x) × −0.03015] R^2 = 0.9949.

Synaptosomal and ganglioside binding assays

Binding of recombinant H_C-fragments to isolated ganglioside GT1b and binding of ^{35}S-labelled *in vitro* transcribed/translated H_C-fragments to freshly prepared rat brain synaptosomes was conducted as described previously (Rummel *et al.* 2004b).

Mouse phrenic nerve hemidiaphragm assay

The MPN hemidiaphragm assay was performed as described previously (Habermann *et al.* 1980). The phrenic nerve was continuously stimulated at 5–25 mA with a frequency of 1 Hz, or, in indicated cases, 0.016 Hz (with a 0.1 ms pulse duration). Isometric contractions were transformed using a force transducer and recorded with VitroDat Online software (FMI GmbH, Seeheim, Germany). The time required to decrease the amplitude to 50% of the starting value (paralytic half-time) was determined. To determine the altered neurotoxicity of the BoNT mutants, or wild-type (wt) BoNT/A, C, E and F at ganglioside-deficient MPN hemidiaphragm preparations, a concentration–response curve, consisting of three or four data points determined in triplicate, was compiled for each of the four serotypes to which a power function could be ascribed: y(scBoNT/A wt; 1, 3.4, 10, 33.7 pM) = $150.86x^{-0.3102}$, R^2 = 0.9865; y(BoNT/C wt; 15, 50, 100, 500 pM) = $148.84x^{-0.2058}$, R^2 = 0.9842; y(BoNT/E wt; 80, 267, 800 pM) = $509.34x^{-0.3482}$, R^2 = 0.9999; y(scBoNT/F wt; 1.5, 5, 15 nM) = $3119.2x^{-0.4048}$, R^2 = 0.9931. Resulting paralytic half-times were converted to corresponding concentrations of the respective BoNT wild-type, using the equations given above, and neurotoxicity was finally expressed as the percentage of the BoNT/X wild-type neurotoxicity. For competition experiments CNTs (scBoNT/A wt, 33.7 pM organ bath concentration; scBoNT/B wt, 2 nM; BoNT/C wt, 100 pM; scBoNT/D wt, 50 pM; BoNT/E wt, 300 pM; scBoNT/F wt, 15 nM; BoNT/G wt, 20 nM; TeNT wt, 1.5 nM) were pre-incubated with various concentrations of the respective H_C-fragment for 5 min at 22°C, prior to addition to the hemidiaphragm preparation. Control measurements without any H_C-fragment were always conducted in parallel to the competition measurements for the same neurotoxin serotype.

Complex polysialo ganglioside-deficient mice

Mouse phrenic nerves were derived from 20 g Naval Medical Research Institute [NMRI; nomenclature Crl:NMRI(Han) (outbred)] mice (wild-type) and C57BL/6 mice lacking the genes B4galnt1 encoding β-1,4-*N*-acetylgalactosaminyltransferase (NAcGalT; EC 2.4.1.92) and/or St8sia1 encoding GD3-synthetase (GD3S – CMP-sialic acid:GM3 α-2,8-sialyltransferase; EC 2.4.99.8). Whereas neurons of NMRI mice contain the full set of complex polysialo gangliosides, the nerve cells of complex ganglioside-deficient mice contain only GM3 or predominantly GM1 and GD1a (Kawai *et al.* 2001). No difference in the paralytic half-time was observed when phrenic nerve hemidiaphragm preparations from NMRI and wild-type C57BL/6 mice were compared.

Co-purification of proteins from synaptic vesicles

A total of 10 µL of Streptactin-Superflow agarose beads were washed three times in HNE buffer and subsequently blocked for 2 h at 4°C in 360 µL HNE buffer supplemented with 2% bovine serum albumin. Blocked beads were washed three times in HNE buffer supplemented with 1% Triton X-100. SVs were purified as described previously (Takamori *et al.* 2006). H_C-fragments (15 µg) and SVs (25 µg of total protein) were added and the reaction volume was adjusted to 200 µL HNE buffer containing 1% Triton X-100. The samples were incubated overnight at 4°C and subsequently washed three times using HNE buffer supplemented with 1% Triton X-100. Bound H_C-fragments and associated proteins were released from the beads by incubation with 150 µL HNE buffer supplemented with 1% Triton X-100 and 10 mM Biotin for 3 h at 4°C. Samples were centrifuged with 2000 ×*g* and the supernatants containing the recovered H_C-fragments and bound proteins were subjected to SDS–PAGE and western blotting. The antibodies used in this study were rabbit anti-SV2A, rabbit anti-SV2B, rabbit anti-SV2C (dilution 1 : 5000; all from Synaptic Systems, Göttingen, Germany), mouse anti-Syt-I (clone Cl 41.1 dilution 1 : 5000; Brose *et al.* 1992), and mouse anti-Syt-II (clone 26, dilution 1 : 5000; BD Transduction Laboratories, Lexington, KY, USA).

Results

Reduced neurotoxicity of botulinum neurotoxin at ganglioside-deficient MPN hemidiaphragms

Complex polysialo gangliosides are required as acceptors for BoNTs. BoNT/F binds *in vitro* with similar affinity to the isolated gangliosides GT1b, GD1b and GD1a, under low ionic strength conditions (Ochanda *et al.* 1986), but so far it is unclear whether BoNT/F requires binding to gangliosides to exert its neurotoxicity. Therefore, phrenic nerve hemidiaphragms, derived from mice with a single St8sia1 KO or with a combined St8sia1/B4galnt1 double KO, were tested. The St8sia1 KO prevents synthesis of GD3S, resulting in expression of only GM3, GM2, GM1 and GD1a (Fig. 1a). The double KO mice lack GD3S and NAcGalT and thus express only GM3 (Kawai *et al.* 2001). Here, we show that the neurotoxicity of BoNT/F is reduced to 62% and 6% at GD3S KO and GD3S/NAcGalT KO MPN hemidiaphragms, respectively (Fig. 1b). Hence, the absence of the disialic acid moiety, such as found in GD1b and GT1b, only moderately impairs BoNT/F activity, whereas the lack of the terminal NAcGalβ3-1Galβ3-2NAcNeuα moiety found in GT1b and GD1a drastically decreases the activity of BoNT/F (Fig. 2a). The closely related BoNT/E showed a comparable loss of activity (80% and 9.5% neurotoxicity, respectively),

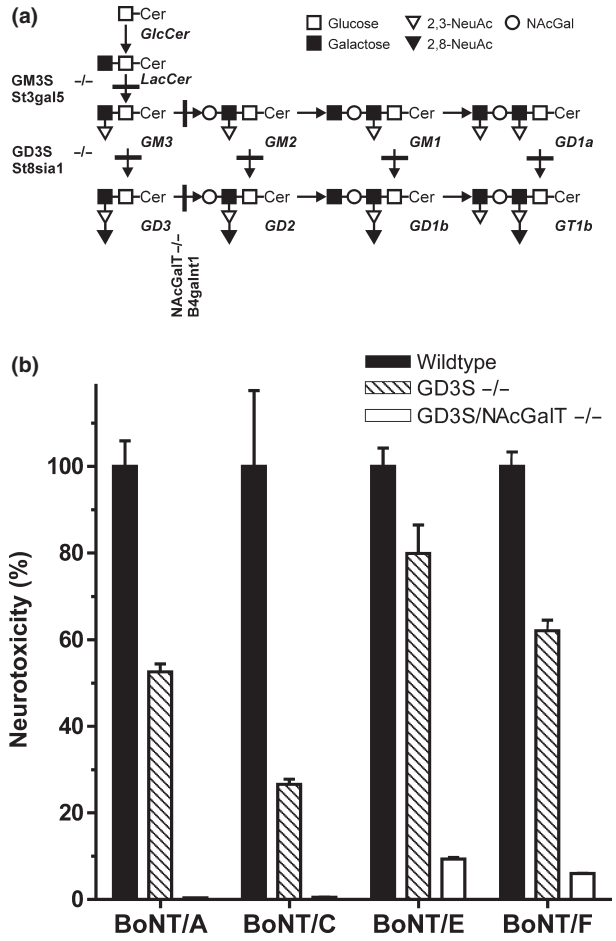


Fig. 1 (a) Schematic representation of the biosynthetic pathway for complex polysialo gangliosides. The scheme shows the enzymes GM3-synthetase (GM3S), GD3-synthetase (GD3S) and *N*-acetylgalactosaminyl-transferase (NAcGalT), which are involved in ganglioside biosynthesis, and their encoding genes, which are deleted in the respective knockout mice leading to an altered ganglioside expression pattern. (b) BoNT/A, C, E and F show reduced neurotoxicity when applied to phrenic nerve hemidiaphragm preparations obtained from single GD3S or combined GD3S/NAcGalT double knockout mice. These mice only express GM3, GM2, GM1 and GD1a or GM3, respectively (n = 3–7, mean ± SD).

indicating the necessity of a similar ganglioside expression pattern. Whereas the absence of GD1b and GT1b halved the activity of BoNT/A, an even more pronounced loss of activity was observed when GM3 was the sole ganglioside expressed: 0.5% neurotoxicity. Similar reductions in toxicity were observed for BoNT/C, using MPN hemidiaphragms preparations from the KO mice.

Identification of the ganglioside binding site in botulinum neurotoxin E and F

Having clarified the ganglioside-dependent activity of BoNT/F, it stands to reason that a ganglioside binding site should be present within the H_C-fragment of BoNT/F. However, until a short time ago, the lack of available crystal structures for both the BoNT/E and BoNT/F H_C-fragments required the calculation of homology models to allow rational site-directed mutagenesis. The C-alpha backbone of our BoNT/E H_C homology model (R846 to K1252) superimposes well with the recently published crystal structure of BoNT/E (P2-K1252; 407 residues used; root mean square deviation 2.33 Å) (Kumaran *et al.* 2009). Except for K1215 replacing the histidine, strictly conserved residues such as E1172, S1222, W1224 and Y1225 form the canonical ganglioside binding pocket in BoNT/E (Fig. 2c), as described for TeNT, BoNT/A and BoNT/B (Fotinou *et al.* 2001; Rummel *et al.* 2003, 2004b). Superimposition of the C-alpha backbone of our BoNT/F H_C model (I866 to E1277) fits very well with a BoNT/F H_C crystal structure (D868 to N1278; 398 residues used, root mean square deviation 1.88 Å) (Fu *et al.* 2009). Analysis of the BoNT/F H_C model revealed a pocket with a similar shape and location as the conserved ganglioside binding site in BoNT/A (Fig. 2b) (Binz and Rummel 2009). The BoNT/F pocket comprises H1241, E1195, S1248, W1250 and Y1251 (Fig. 2d) and thus contains the typical ganglioside binding site motif E…H…SXWY.

In BoNT/A, the mutants E1203L and W1266L displayed only 17% and 0.7% biological activity, respectively (Rummel *et al.* 2004b). Therefore, the role of the homologous tryptophan and glutamate residues in BoNT/E and F were analysed by site-directed mutagenesis (Fig. 2c–e). The BoNT/E mutants E1172A and W1224L showed a reduced neurotoxicity of 22.6% and 4.5% at MPN preparations, respectively. Analogously, the binding of radioactively labelled, *in vitro* translated H_CE E1172A and H_CE W1224L to rat brain synaptosomes was drastically decreased to 8.5% and 3.7% of wild-type levels, as well as the binding of *E. coli* derived H_CE mutants to isolated GT1b (Fig. 2e). Likewise, the H_CF mutants E1195A and W1250L displayed a diminished affinity to synaptosomes (31.6% and 20.5%, respectively), as well as a reduction of about 85% in binding to isolated GT1b compared with H_CF wild-type (Fig. 2e). Unfortunately, the low expression rates of the two recombinant full-length BoNT/F mutants precluded analysis of their biological activity in the MPN assay. Parallel to our examination of the ganglioside binding site in BoNT/E and F, the single site mutant W1258L of BoNT/C was generated. W1258 probably represents the tryptophan residue within the conserved E…H…SXWY motif and its mutation to alanine or phenylalanine strongly reduced the binding of H_C-fragments to rat brain synaptosomes (Tsukamoto *et al.* 2008). Mutation of W1258 to leucine also decreased the binding of the H_C-fragment to rat brain synaptosomes by 45% and to isolated GT1b by 33%. Furthermore, the neurotoxicity of BoNT/C W1258L declined to 5.6% compared with wild-type BoNT/C (Fig. 2e). In summary, mutation of the conserved tryptophan to the aliphatic amino acid leucine clearly reduced binding of BoNT/C, E and F to

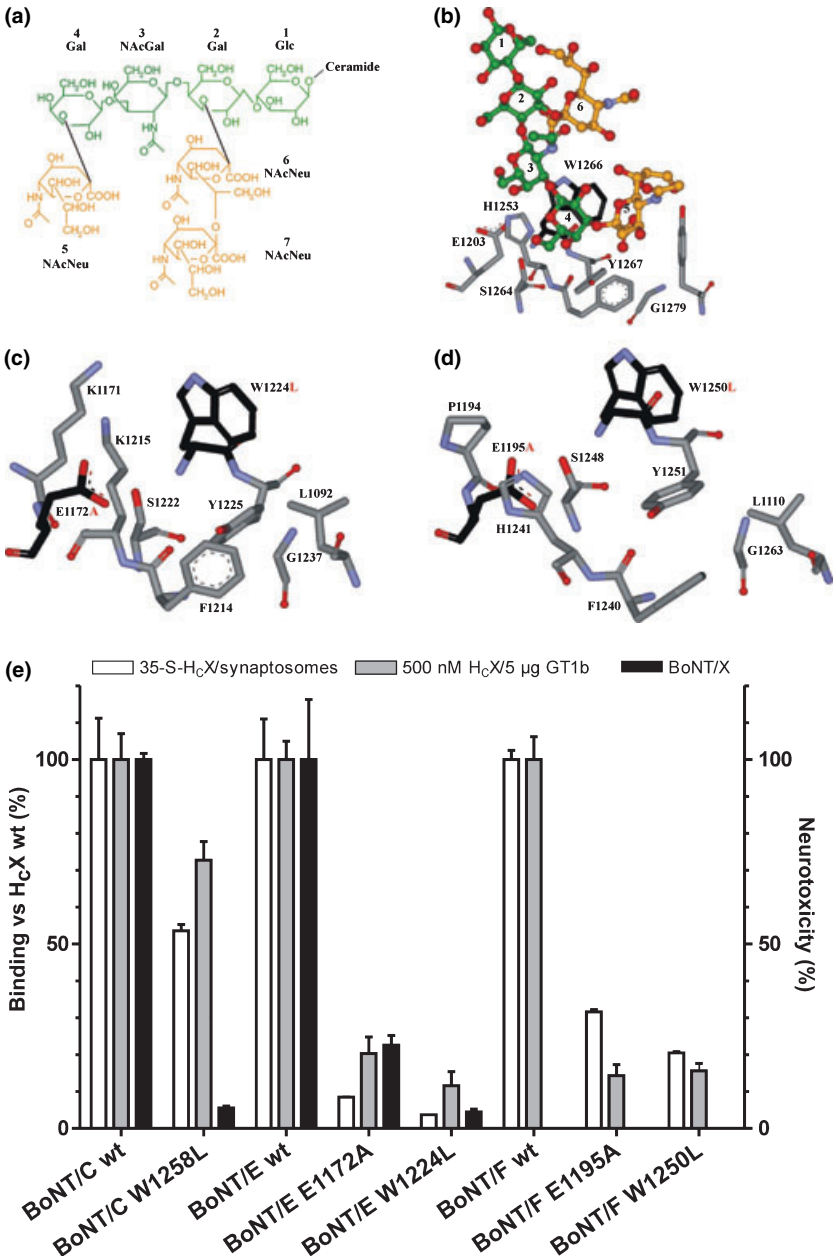


Fig. 2 (a) Chemical structure of ganglioside GT1b. Sialic acids (*N*-acetylneuraminic acid; NAcNeu) are highlighted in orange, the aldohexoses galactose (Gal), *N*-acetylgalactosamine (NAcGal) and glucose (Glc) in green. (b) Insight into the co-crystal structure of GT1b (ball and stick representation) bound in the conserved ganglioside binding pocket of BoNT/A H_C (stick representation; 2VU9.pdb) (Stenmark *et al.* 2008). GT1b associates with the hydrophobic side of its terminal Gal against the key residue W1266. The conserved residues E1203, H1253 and S1264 complete the interaction with GT1b via hydrogen bonding. Homology models of BoNT/E (c) and BoNT/F (d) reveal a pocket similar to this conserved ganglioside binding site, which contains the typical E…H…SXWY motif. The key residues tryptophan and glutamate, which were subjected to mutagenesis, are highlighted by a black carbon backbone. (e) Analysis of single site mutants of BoNT/C, E and F. The corresponding *in vitro* translated, ^{35}S-labelled H_C-fragments were bound to rat brain synaptosomes for 2 h at 0°C and quantified by autoradiography following SDS–PAGE. The recombinant H_C-fragments, expressed and purified from *E. coli*, were bound at 22°C for 2 h to 5 µg of isolated GT1b immobilised on microtitre plates and detected by streptactin conjugated alkaline phosphatase. Recombinant full-length BoNT/C and E mutants were analysed in the MPN assay and the remaining paralytic half-time was converted by a corresponding power function to percentage neurotoxicity versus wild-type (n = 3–5, mean ± SD).

isolated gangliosides and neuronal membranes, as well as drastically decreased the neurotoxicity of BoNT/C and E at their physiological targets. Thus, BoNT/E and F also possess a conserved ganglioside binding site.

Identifying receptor families using competition assays

A number of earlier studies employed iodinated BoNT/A–F to inhibit binding of native BoNT/A–F and TeNT to rat brain synaptosomes (Habermann 1976; Kozaki 1979; Williams *et al.* 1983; Murayama *et al.* 1984; Evans *et al.* 1986; Wadsworth *et al.* 1990; Yokosawa *et al.* 1991). However, these seven studies lacked 26 of the 49 possible combinations of cross-competition and did not investigate the mechanism of BoNT uptake into motor neurons. Furthermore, because binding of BoNTs to synaptosomes mainly depends on toxin-ganglioside interactions, it does not provide information about possible protein receptors for the toxins (Rummel *et al.* 2004b). Therefore, a competition assay approach was utilised to narrow down both the number and type of unidentified protein receptors for BoNT/C, D and F, as well as for TeNT. At the beginning of our study it was known that BoNT/B and G share Syt-II as a protein receptor (Dong *et al.* 2003; Rummel *et al.* 2004a). Employing a GST pull-down assay we tested whether either scBoNT/B or G bound to GST-Syt-II 1–61 could be displaced by the addition of increasing concentrations of the H_C-fragment of BoNT/B

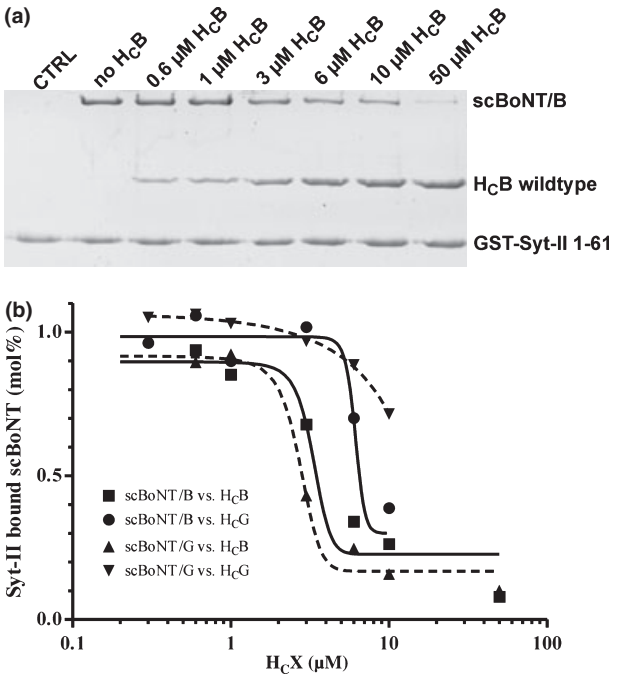


Fig. 3 (a) An example of a gel stained with Coomassie Blue following SDS–PAGE. The gel illustrates the analysis of scBoNT/B bound to immobilised GST-Syt-II 1–61 and its successive displacement by the addition of increasing concentrations of H_CB wild-type. (b) Quantification of the four GST-Syt-II 1–61 competition experiments, which used scBoNT/B, scBoNT/G, H_CB wild-type and H_CG wild-type. The data points were fitted using sigmoidal regression curves.

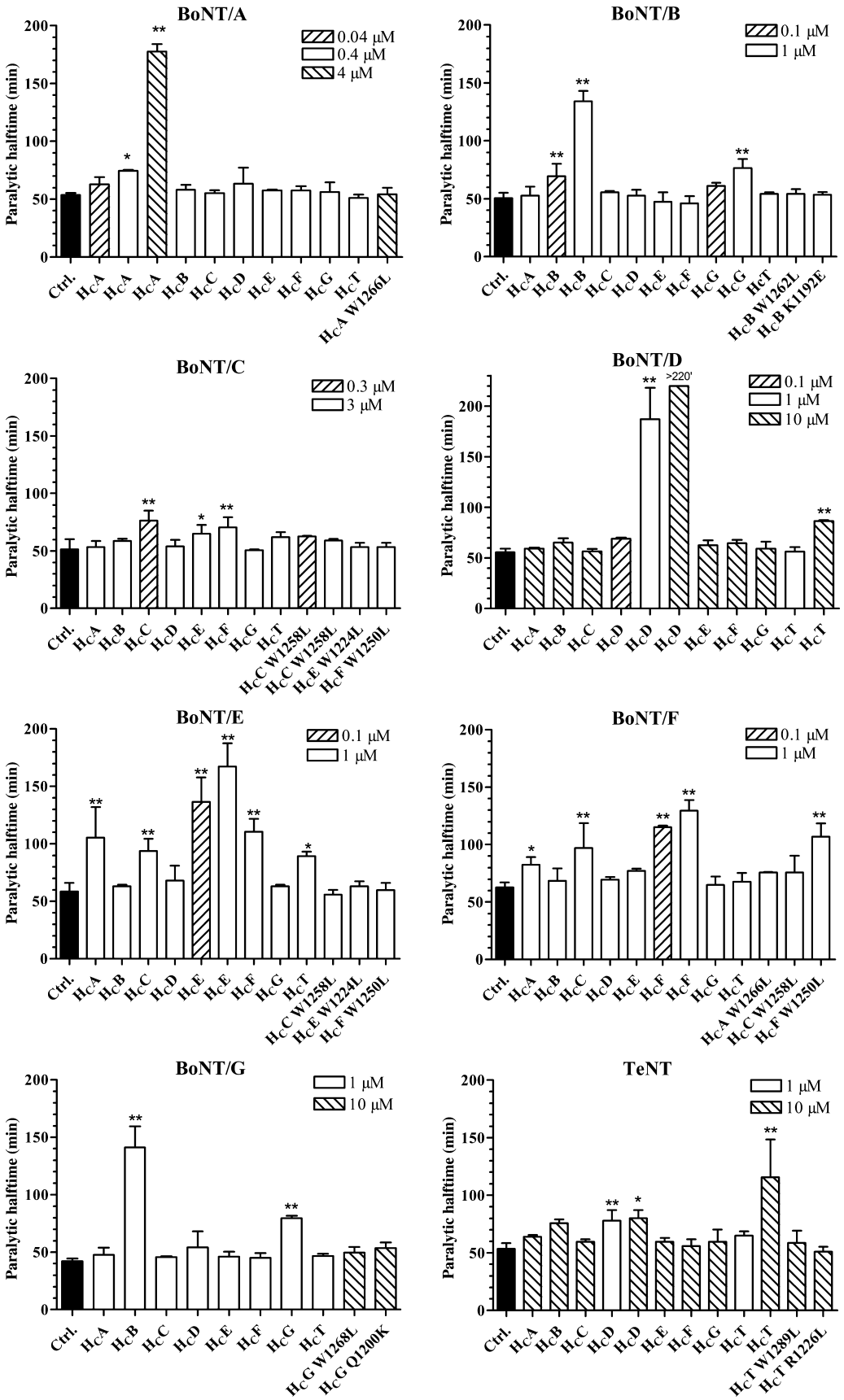
or G (Fig. 3a); 50% of Syt-II bound scBoNT/B was displaced by 3.5 µM H_CB and 6.5 µM H_CG, respectively. Half-maximal binding of scBoNT/G to Syt-II was obtained by the addition of 2.5 µM H_CB and > 10 µM H_CG (Fig. 3b). The lower concentrations of H_CB needed to displace either scBoNT/B or scBoNT/G, when compared with H_CG, reflect the high affinity of BoNT/B to Syt-II (K_d = 34 nM) (Jin *et al.* 2006) and the weak binding of BoNT/G to Syt-II (Rummel *et al.* 2007). Further, and more interestingly, these competition experiments confirm that the non-toxic H_C-fragment is able to displace binding of a different BoNT serotype to their common protein receptor.

To further validate these findings, the *in vitro* competition approach was transferred to the MPN hemidiaphragm preparation, which is an established *ex vivo* assay that allows one to determine the biological activity of BoNTs (Dreyer and Schmitt 1981; Schmitt *et al.* 1981). Importantly, in this assay, the receptor expression and presentation resemble that found physiologically and allows for the inhibition of CNT uptake.

The inhibition of neurotoxicity of all eight CNTs was systematically analysed by addition of the eight non-toxic H_C-fragments (Fig. 4). As expected, increasing concentrations of H_CB and H_CG specifically prolonged the time of paralysis for both BoNT/B and BoNT/G, indicating a competition for their protein receptors Syt-I and Syt-II. None of the other six H_C-fragments inhibited the neurotoxicity of BoNT/B and BoNT/G. Further, neither H_C-fragments with deactivated ganglioside binding sites (H_CB W1262L; H_CG W1268L) nor with disabled Syt binding sites (H_CB K1192E and H_CG Q1200K) (Rummel *et al.* 2004b, 2007) were able to interfere with uptake of the full-length BoNT/B and G. Hence, H_C-fragments non-competent for ganglioside binding do not enrich on the membrane surface and cannot compete for protein receptor binding, whereas H_C-fragments with deactivated protein receptor binding sites accumulate on the membrane but do not subsequently interfere with protein receptor binding of the BoNTs.

Only H_CA reduced the neurotoxicity of BoNT/A, whereas the remaining H_C-fragments, as well as the mutant H_CA W1266L that lacks ganglioside binding (Rummel *et al.* 2004b), did not interfere with BoNT/A neurotoxicity (Fig. 4). Also, spot checks, using an order of magnitude higher concentrations of each wild-type H_C-fragment, did not yield any competition with BoNT/A (data not shown), presumably because BoNT/A is the only serotype displaying high affinity interactions with all three SV2 isoforms (Dong *et al.* 2006; Mahrhold *et al.* 2006). On the other hand, 1 µM H_CA was able to interfere with the activities of BoNT/E and BoNT/F, respectively. Identical concentrations of H_CE, H_CF and, surprisingly, H_CC interfered with the uptake of BoNT/E, but only H_CC and H_CF significantly increased the paralytic half-time of BoNT/F. Conversely, H_CE and H_CF decreased the neurotoxicity of BoNT/C. These competitions are specific, because equal or higher concentrations of the mutants H_CA W1266L, H_CC W1258L, H_CE W1224L and H_CF W1250L, which all possess a deactivated ganglioside binding site, did not cause a significant increase in the paralytic half-times of BoNT/C, E and F. Only 1 µM of the mutant H_CF W1250L significantly prolonged the time to half-maximal paralysis by BoNT/F, but identical concentrations of H_CF wild-type led to stronger inhibition (Fig. 4).

Fig. 4 Systematic analysis of the effect produced by the addition of all H_C-fragments on uptake of the seven BoNT and TeNT in the MPN hemidiaphragm assay. H_C-fragment and BoNT were mixed together for 5 min at 22°C prior to addition to the hemidiaphragm. Control measurements without any H_C-fragment (n = 5–14) (black column) were always conducted in parallel to competition measurements (n = 3–5) for the same neurotoxin serotype. Data are presented as the mean ± SD. Calculation of statistical significance by one-way ANOVA, for all measurements acquired per serotype, revealed a $p < 0.0001$ for all serotypes except BoNT/C ($p = 0.0006$). Post-analysis by Dunnett's multiple comparison test calculated the individual significance for each competition measurement compared with the control measurement (black column) (*$p < 0.05$ and **$p < 0.001$).



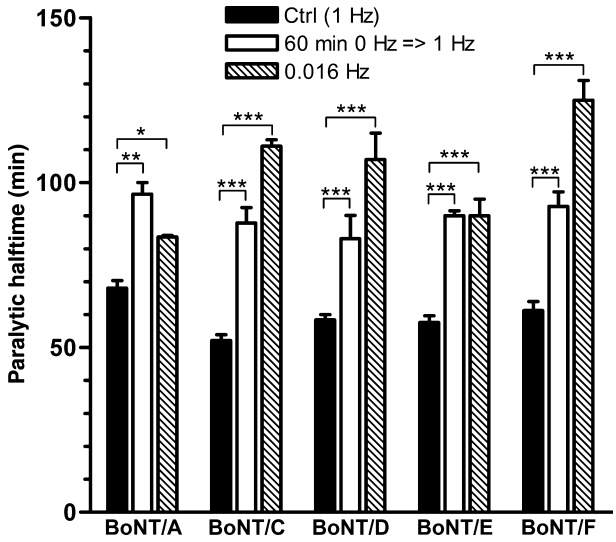


Fig. 5 Variation of electrical stimulation influences the uptake of BoNT/A, C, D, E and F in the MPN hemidiaphragm assay. The two stimulation paradigms studied (addition of BoNT at 37°C initially in the absence of stimulation followed after 60 min by stimulation at 1 Hz or continuous stimulation with 0.016 Hz), clearly prolonged the time of paralysis when compared with control, indicating an accelerated uptake of BoNT/A, C, D, E and F. Calculation of statistical significance was by an unpaired *t*-test; $*p < 0.05$, $**p < 0.001$ and $***p < 0.0001$.

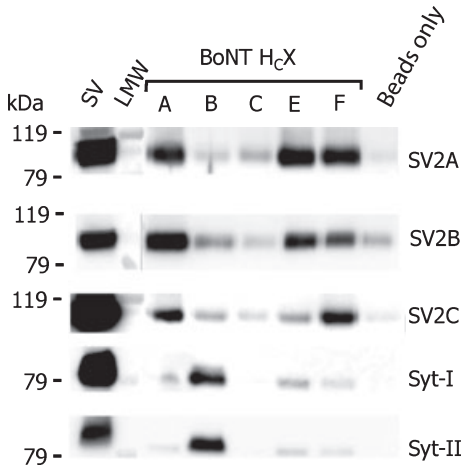


Fig. 6 Co-purification of proteins from detergent-solubilised synaptic vesicles with One-strep-tagged H_C-fragments derived from BoNT/A, B, C, E and F. BoNT H_C-fragments (15 μg each) were incubated with highly purified SVs solubilised with Triton X-100 (25 μg total protein) in a total volume of 200 μL and subsequently purified using Streptactin-Superflow beads. Co-purified proteins were visualised by western blotting using specific antibodies against the three isoforms of the synaptic vesicle glycoprotein 2 (SV2A, B and C) and synaptotagmin-I and -II (Syt-I and Syt-II). The lane labelled 'beads only' displays background binding of SV proteins to Streptactin-Superflow beads.

BoNT/D, which displays the lowest amino acid sequence conservation among all BoNT H_C-fragments, is efficiently inhibited by increasing concentrations of its own H_C-fragment, as well as by 10 μM H_CT. Up to 10 μM of the other H_C-fragments did not influence the paralytic half-time of BoNT/D.

Although the MPN is not the physiological site of action for TeNT, it does constitute the physiological site of entry for this toxin. Thus, the uptake of TeNT was analysed, showing not only inhibition at high concentration of H_CT but also by similar (and even lower) concentrations of H_CD. None of the other serotypes interfered with the uptake of TeNT when used at concentrations of 1 μM (data not shown) or 10 μM (Fig. 4). Deactivation of ganglioside binding (using the mutants H_CT W1289L or R1226L) (Rummel *et al.* 2003) prevents inhibition of full-length TeNT uptake. In summary, TeNT and BoNT/D show an unexpected cross-competition at high concentrations.

Stimulation-dependent uptake of botulinum neurotoxin

Increased electrical stimulation amplifies the uptake of BoNT/A and subsequently accelerates the blockade of neurotransmitter release. This result was confirmed for BoNT/A, which showed an increase in paralytic half-time either upon lowering the stimulation frequency from 1 to 0.016 Hz or upon a 1 h stimulation break and subsequent 1 Hz stimulation (Fig. 5). The same procedures were applied for BoNT/C, D, E and F. Employing a 1 h stimulation break, the paralytic half-time increased for all the serotypes tested by more than 25 min. If the stimulation was reduced to a constant 0.016 Hz, the paralytic half-time was prolonged by 42 min in the case of BoNT/E and by 63 min for BoNT/F. Hence the four serotypes BoNT/C, D, E and F show a similar behaviour to BoNT/A. As the largest luminal domain of the SV2 protein is the site of interaction for BoNT/A and E, one can deduce that the remaining receptor structures for BoNT/C, D and F are also part of SVs.

Interaction of SV2A, SV2B and SV2C with botulinum neurotoxin F H_C

To identify the SV structures functioning as protein receptors for BoNT/C and F, highly purified SVs were solubilised by Triton X-100 and separately incubated with equal amounts of BoNT/A, B, C, E and F H_C-fragments. The isoforms of SV2 (known to be protein receptors for BoNT/A and E) and Syt-I and Syt-II (utilised by BoNT/B and G) were purified by binding to the respective H_C-fragments, subjected to SDS–PAGE and detected by western blot (Fig. 6). In contrast to a recent report (Fu *et al.* 2009), BoNT/B H_C only precipitated Syt-I and Syt-II, as expected. BoNT/A H_C specifically co-purified SV2A, SV2B and SV2C from the detergent preparation, whereas BoNT/E isolated only SV2A and SV2B from the Triton X-100 solubilisate. In contrast to BoNT/E, the H_C-fragment of BoNT/F bound to all three

isoforms of SV2, but no specific interaction with Syt-I or Syt-II was detected, explaining the inhibition of BoNT/F action by BoNT/A H_C, as well as of BoNT/E activity by BoNT/F H_C, at the MPN. Whereas SV2A was isolated in similar amounts by H_CA, H_CE and H_CF, SV2B showed by far the highest affinity to BoNT/A H_C followed by BoNT/E H_C and finally BoNT/F H_C. Despite the cross-competition of BoNT/C with BoNT/E and F, no interaction with any of these five synaptic vesicle proteins was discovered for BoNT/C. This is in line with an earlier finding that BoNT/C does not immunoprecipitate SV2 or Syt-I from 3-[N-(3-Cholanamidopropyl)-dimethylammonio]-1-propansulfonat or Triton-solubilised SVs (Baldwin and Barbieri 2007). However, with respect to the stimulation-dependent uptake of BoNT/C, it is reasonable to speculate that an interaction with other SV proteins takes place.

Discussion

Kitamura *et al.* (1999) were the first to demonstrate that the neurotoxicity of TeNT, BoNT/A and B was dependent on gangliosides, through their use of the time-to-death method with mice deficient in NAcGalT. More recently, it was shown that the binding and entry of BoNT/E into hippocampal neurons cultured from the same NAcGalT KO mice was impaired by the expression of only GM3 and GD3 (Dong *et al.* 2008). Employing GD3-synthase KO mice expressing GM3, GM2, GM1 and GD1a (Fig. 1a), TeNT exhibited a 10-fold higher activity compared with the NAcGalT KO mice in the time-to-death assay, whereas the sensitivity towards BoNT/A, B and E was surprisingly equal to wild-type mice (Kitamura *et al.* 2005). In contrast, using GD3S KO MPN hemidiaphragms the activity of BoNT/A, C, E and F decreased by 20–75% (Fig. 1b). As it has been reported that these serotypes efficiently interact with GT1b and/or GD1b (Yowler and Schengrund 2004). Hence the observed reductions in neurotoxicity have to be caused by the lack of GD1b and GT1b and cannot completely be compensated by the presence of GD1a. The combination of GD3S and NAcGalT gene KO resulted in GM3-only mice which display high resistance towards both BoNT/B and G (Rummel *et al.* 2007), and, as shown here, also towards BoNT/E and F. Furthermore, the neurotoxicity of BoNT/A and C was drastically reduced by > 99%. In conclusion, the physiological activities of BoNT/A, C, E and F clearly depend on expression of GT1b and/or GD1b and GD1a, whereas GM3 cannot rescue their absence.

Consequently, the ganglioside requirements of BoNT/E and F necessitate corresponding ganglioside binding sites. Based on homology models, two key residues of a putative ganglioside binding site were identified in both BoNT/E and F and substituted by site-directed mutagenesis. Exchange of the conserved tryptophan by an aliphatic leucine in BoNT/C, E and F H_C-fragments prevents inhibition of BoNT/C and E in the MPN assay. These mutants also display drastically decreased binding to the isolated ganglioside GT1b and to synaptosomal membranes. In a parallel study, the identical mutants BoNT/F H_C E1195A and H_C W1250L also lacked binding to isolated GT1b and in addition, H_CF W1250L is hardly taken up into hippocampal neurons (Fu *et al.* 2009). Furthermore, the neurotoxicity of the full-length mutants BoNT/E W1224L and BoNT/C W1258L was severely reduced, demonstrating the important interaction between the conserved tryptophan and the hydrophobic side of the terminal carbohydrates in ganglioside molecules. The results obtained with the mutant BoNT/C W1258L confirm and extend earlier findings using H_CC W1258A and H_CC W1258F in competition binding assays using synaptosomal membranes (Tsukamoto *et al.* 2008). In conclusion, the location and function of the ganglioside binding site within the H_{CC} domain of BoNT/C, E and F and its amino acid composition is very similar to that found in BoNT/A, B and G (Rummel *et al.* 2004b, 2007).

The finding that BoNT/B and G share Syt-I and Syt-II as protein receptors prompted the use of a competition assay to narrow down the number of protein receptor families. *In vitro*, defined Syt-II–BoNT complexes could be dissociated by the addition of an H_C-fragment derived from another serotype but which also interacts with the same protein receptor, forming a new 'protein receptor–BoNT H_C complex'. This approach was transferred to the MPN hemidiaphragm *ex vivo* assay to investigate the binding and subsequent uptake of BoNT at their physiological site of action. Systematic analysis of all eight CNTs confirmed that BoNT/B and G share the Syt-I and Syt-II isoforms as their protein receptors. Moreover, BoNT/A was not inhibited by any serotype, because BoNT/A can interact with either SV2A, B, or C (Dong *et al.* 2006; Mahrhold *et al.* 2006), preventing BoNT/E H_C (which employs *N*-glycosylated SV2A and B as protein receptors (Dong *et al.* 2008)) from inhibiting BoNT/A uptake. In fact, exclusive saturation of SV2A and B binding sites by BoNT/E H_C allows BoNT/A to enter the neuron via the accessible SV2C. In contrast, BoNT/A H_C impaired the uptake of BoNT/E, because of its action in 'blockading' the luminal domain 4 in both SV2A and B. Seemingly analogous, BoNT/F was unilaterally inhibited by BoNT/A H_C, although another report could not demonstrate this inhibition (Fu *et al.* 2009). Our finding points towards an involvement of both SV2A and SV2B as protein receptors for BoNT/F, which is further supported by the fact that the H_C-fragment of BoNT/F is able to compete with BoNT/E, just like BoNT/A H_C (Fig. 7). Application of the BoNT/F H_C mutant W1250L (which possesses a deactivated ganglioside binding site) does not result in inhibition of BoNT/E, emphasising the specificity of the BoNT/E–BoNT/F H_C competition. On the other hand, BoNT/E H_C does not significantly impair the neurotoxicity of BoNT/F, which requires the involvement of the remaining SV2C as a

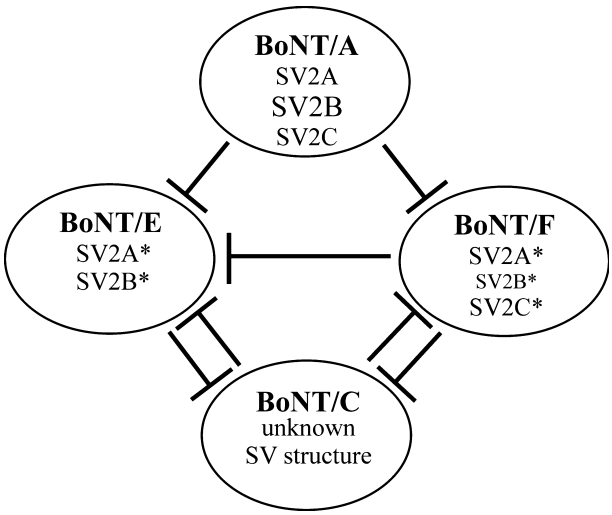


Fig. 7 A network of 'cross-competition' interactions exists for BoNT/A, C, E and F. The font size of SV2X indicates the relative affinity of the individual isoform for each serotype. *Indicates that *N*-glycosylation is required for the interaction.

receptor (Fig. 7). This statement is clarified by the ability of BoNT/F to co-precipitate all three isoforms of SV2 (Fig. 6). But why does BoNT/F H_C not inhibit BoNT/A, as it uses the same vesicular proteins as receptors? While the respective H_C-fragments display similar affinities to SV2A and SV2C, BoNT/A H_C co-purifies much more SV2B than BoNT/F H_C does (Fig. 6). The higher affinity of BoNT/A to SV2B seems to be the 'escape route' that allows BoNT/A uptake in the presence of BoNT/F. Moreover, the accelerated uptake of BoNT/E and BoNT/F into motor neurons on increased electrical stimulation (Fig. 5) as well as a high sequence identity of 40% within their H_{CC} domains (Binz and Rummel 2009) backs the involvement of the membrane spanning SV2 protein. Furthermore, in a parallel study BoNT/F H_C co-precipitated Syt-I and unspecified SV2 isoforms from Triton-solubilised SVs which partially overlaps with our findings, but deglycosylation seems to interrupt such interactions (Fu *et al.* 2009). In conclusion, several results show that SV2A, SV2B and SV2C are protein receptors for BoNT/F.

Surprisingly, BoNT/C cross-competed with BoNT/E and F (Fig. 7), while neither the involvement of a proteinaceous receptor in binding of BoNT/C to synaptosomes (Tsukamoto *et al.* 2005), nor co-precipitation of the SV2 proteins, Syt-I or synaptophysin by BoNT/C, could be demonstrated (Baldwin and Barbieri 2007). On the other hand, accelerated blockade of neurotransmitter release caused by enlarged BoNT/C internalisation upon increased electrical stimulation of the MPN (Fig. 5) confirmed an earlier finding (Simpson 1982), and implicates SV incorporated structures in the binding and uptake of BoNT/C. Nevertheless, we could not detect an interaction of BoNT/C with any of the SV proteins currently known to act as BoNT receptors (Fig. 6). Then again, only BoNT/E and F, which both require glycosylated SV2 as a receptor, are inhibited by BoNT/C, excluding a general inhibition of BoNT binding to gangliosides as being the first binding step. Possibly, BoNT/C interacts with carbohydrate containing structures integrated in SVs which are found in proximity to SV2. However, extensive investigations beyond the scope of this work are required to characterise the BoNT/C receptor.

Botulinum neurotoxin D is reported to be the only non-human pathogenic serotype (Coffield *et al.* 1997). This characteristic could be attributed to the low amino acid sequence identity in its H_{CC} domain (< 20%) when compared with BoNT/A, B, E, F and G. Accordingly, it was reported that BoNT/D does not require a protein receptor and binds to phosphatidylethanolamine instead of gangliosides, which would imply a different uptake mechanism (Tsukamoto *et al.* 2005). In contrast, uptake of BoNT/D is accelerated upon increased electrical stimulation of motor neurons, indicating the possible involvement of SV structures (Fig. 5). On the other hand, apart from high concentrations of TeNT H_C no other BoNT serotype could inhibit the neurotoxicity of BoNT/D, effectively excluding the identified BoNT binding regions on SV2 and Syt as interaction sites. The mutual inhibition of TeNT and BoNT/D opens up the possibility of an involvement of carbohydrate structures as receptors, because it is believed that TeNT interacts with the sialic acids of glycosylated glycosylphosphatidylinositol-anchored proteins during uptake (Herreros *et al.* 2001; Munro *et al.* 2001).

In conclusion, we demonstrated that both BoNT/E and F contain a ganglioside binding site at a conserved location in their H_{CC} domains and that BoNT/F requires gangliosides for exerting its neurotoxicity. Furthermore, BoNT/F employs SV2A, SV2B or SV2C as a protein receptor, whereas BoNT/C, D and TeNT utilise individual, yet still unidentified, receptor structures.

Acknowledgement

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