

REVIEW

Cell entry strategy of clostridial neurotoxins

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Abstract

Tetanus neurotoxin and botulinum neurotoxins are the causative agents of tetanus and botulism. They block the release of neurotransmitters from synaptic vesicles in susceptible animals and man and act in nanogram quantities because of their ability to specifically attack motoneurons. They developed an ingenious strategy to enter neurons. This involves a concentration step via complex polysialo gangliosides at the plasma membrane and the uptake and ride in recycling synaptic vesicles initiated by binding to a specific protein receptor. Finally, the neurotoxins shut down the synaptic vesicle cycle, which they had misused before to enter their target cells, via specific cleavage of protein core

components of the cellular membrane fusion machinery. The uptake of four out of seven known botulinum neurotoxins into synaptic vesicles has been demonstrated to rely on binding to intravesicular segments of the synaptic vesicle proteins synaptotagmin or synaptic vesicle protein 2. This review summarizes the present knowledge about the cell receptor molecules and the mode of toxin-receptor interaction that enables the toxins' sophisticated access to their site of action.

Keywords: botulinum neurotoxin, exocytosis, ganglioside, protein receptor, synaptic vesicle, tetanus neurotoxin.

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Botulinum neurotoxins (BoNTs) are protein toxins produced by various strains of the spore forming bacteria *Clostridium botulinum*, *C. butyricum*, and *C. barati* and are regarded as the most hazardous natural substance known. To date seven antigenically distinguishable BoNTs (BoNT/A to BoNT/G) have been described in the literature. They exhibit significant amino acid sequence conservation. This is shared with another neurotoxin of clostridia, tetanus neurotoxin (TeNT) synthesized by *C. tetani* (Lacy and Stevens 1999). Together the toxins constitute the clostridial neurotoxin (CNT) family.

Poisoning by BoNTs mainly occurs upon oral ingestion and toxin uptake at intestinal epithelial cells, whereas TeNT is produced and released into the circulation by bacteria that germinated in infected tissue lesions. BoNTs cause a flaccid paralysis, which in severe intoxications can result in respiratory failure and death. TeNT causes the opposite symptoms because of its intraaxonal ascent from motoneurons into the spinal cord and action in inhibitory interneurons (Schiavo *et al.* 2000). The 50% lethal doses of CNTs range down to subnanogram amounts per kg of body weight for a variety of susceptible vertebrates (Gill 1982). Hence, because of the lack of immunization protection in the population BoNTs constitute a major bioweapon (Bigalke and Rummel 2005; Aron *et al.* 2006). On the other hand, during the past

two decades BoNT/A and B have become widely used therapeutics for the treatment of a variety of neurological disorders with still increasing numbers of indications (Davletov *et al.* 2005).

Each CNT is initially synthesized as ~150 kDa single chain protein, but subsequently cleaved by specific bacterial or host proteases. The resulting ~50 kDa light chain (LC) and ~100 kDa heavy chain (HC) remain attached via a single disulfide bond (Fig. 1). The LCs represent the active

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Abbreviations used: BoNT, botulinum neurotoxin; CNT, clostridial neurotoxin; GAL, galactose; HC, heavy chain; H_C, C-terminal half of HC; H_{CC}, C-terminal half of H_C; H_{CN}, N-terminal half of H_C; H_N, N-terminal half of HC; Lac-Cer, lactose-ceramide; LC, light chain; MPN, mice phrenic nerve hemidiaphragm; NAcGal, N-acetylgalactosamine; NAcNeu, N-acetylneuraminic acid; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SV2, synaptic vesicle protein 2; Syt, synaptotagmin; TeNT, tetanus neurotoxin.

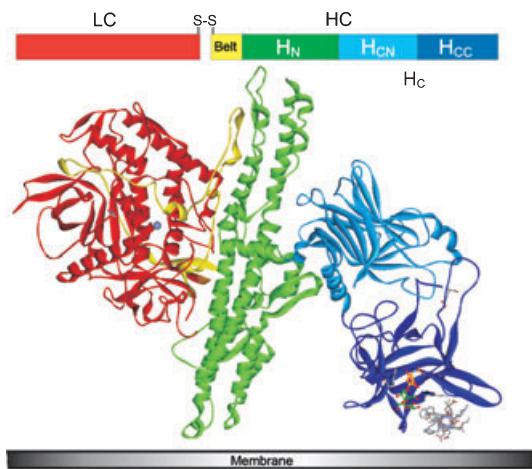


Fig. 1 Domain structure and receptor binding pockets of BoNT/B. Exemplary schematic and crystallographic presentation of the domain architecture of BoNT/B showing its probable perpendicular orientation to the membrane upon interaction with membrane anchored gangliosides and synaptic vesicle protein receptor synaptotagmin II (Syt-II). Structures of BoNT/B-sialyllactose complex (1F31.pdb) and BoNT/B H_C-fragment-Syt-II 7-61 complex (2NM1.pdb) are superimposed. Red ribbon, 50 kDa light chain (LC); grey sphere, Zn²⁺ coordinated in the active center of the endoprotease; yellow ribbon, belt wrapping around the LC; green ribbon, translocation domain (H_N); light blue ribbon, N-terminal half of H_C-fragment (H_{CN}); dark blue ribbon, C-terminal half of H_C-fragment (H_{CC}) harbouring the conserved ganglioside binding site and protein receptor binding site; green-orange ball and stick representation, bound sialyllactose; grey stick representation, bound Syt-II 17mer.

components. They operate as zinc endoproteases with strict substrate specificities. Once the target cell cytosol is reached, BoNT/B, D, F, G, and TeNT LCs proteolyze each a unique peptide bond of the synaptic vesicle protein VAMP (vesicle associated membrane protein)/synaptobrevin-2 (Schiavo *et al.* 1992, 1993a,b; Yamasaki *et al.* 1994). BoNT/A, C, and E each cleave an individual peptide bond of the pre-synaptic membrane protein SNAP-25 (synaptosome associated protein of 25 kDa) (Blasi *et al.* 1993b; Schiavo *et al.* 1993b; Binz *et al.* 1994; Vaidyanathan *et al.* 1999). BoNT/C is in addition capable of cleaving a specific peptide bond of the integral pre-synaptic membrane protein syntaxin-1 (Blasi *et al.* 1993a; Schiavo *et al.* 1995). The CNT substrate proteins are members of larger protein families that are collectively termed soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). Individual sets of them build the core for the different intracellular membrane fusion processes, and zippering of such SNAREs into parallel four-helix coiled-coil bundles is regarded to provide the driving force for membrane merger (Sutton *et al.* 1998; Jahn and Scheller 2006). Hence, cleavage of nerve terminal SNAREs results in the inhibition of neurotransmitter release into the synaptic cleft.

The LCs, however, were ordinary proteases, i.e. non-toxic molecules, without linkage to their HCs. Only this linkage

turns the LCs into dangerous agents, because the HCs ensure that the catalytic LCs come across their neuronal target cells and pass the plasma membrane of the target cell to reach the site of action, the cytosol. In order to fulfill these tasks, the HCs comprise two functional subunits, a ~50 kDa largely α -helical domain at the N-terminus, called H_N, and at the C-terminus a ~50 kDa fragment, H_C, in which the two ~25 kDa domains H_{CN} and H_{CC} can be confined (Fig. 1).

N-terminal half of HC translocates the LC from the uptake compartment across an intracellular membrane into the target cell cytosol. Evidence has been provided that an acidic milieu as prevailing in the endosomal compartment causes H_N to integrate into the membrane and to form a cation selective channel. This is most likely coupled to partial unfolding of LC, its entry into and transit through the H_N channel followed by its refolding in the cytosol, and release from H_N subsequent to reduction of the disulfide bridge (Koriatova and Montal 2003; Fischer and Montal 2007). It has been proposed that the so-called belt region (Fig. 1), a 54 amino acid segment at the N-terminus of H_N that wraps around the catalytic domain might act as chaperone assisting in LC unfolding, channel transit, and refolding (Brunger *et al.* 2007).

The function of the lectin like jelly role H_{CN}-domain of H_C that connects H_N and H_{CC} is still unresolved. Whereas the H_{CN}-domain of TeNT does not bind to rat primary dorsal root ganglionic cells (Figueiredo *et al.* 1995) and nerve growth factor-differentiated PC12 cells (Herreros *et al.* 2000a), a low affinity binding of H_{CN} of BoNT/A to phosphatidylinositol-monophosphate incorporated in sphingomyelin enriched microdomains of the immortalized motor neuron cell line NSC-34 was reported recently (Muraro *et al.* 2009). Nevertheless, a direct involvement of H_{CNA} in the translocation step could be ruled out lately (Fischer *et al.* 2008). On the other hand, the β -trefoil domain H_{CC} harbours the main features required for target cell recognition and internalization, as demonstrated for TeNT and several BoNTs (see below). It mediates highly specific binding to non-myelinated areas of cholinergic motor nerve terminals (Dolly *et al.* 1984). This target cell selectivity is the key aspect to explain the extreme toxicity of CNTs. The molecular mechanisms that enable CNTs to specifically enter motoneurons will be discussed in the following chapters.

Anchorage of CNTs via gangliosides

Long ago it was shown that the specific binding to peripheral nerve endings at the neuromuscular junction solely involves the H_C-fragment (Simpson 1984a,b, 1985; Evinger and Erichsen 1986; Fishman and Carrigan 1987; Lalli *et al.* 1999) and complex polysialo-gangliosides, glycosphingolipids that are found particularly in the outer leaflet of neuronal cell membranes (van Heyningen and Miller 1961; Simpson and Rapport 1971). The interaction of gangliosides with

CNTs was investigated for TeNT and several serotypes of BoNT in extensive studies (reviewed in Halpern and Neale 1995; Yowler and Schengrund 2004b). Whereas TeNT employs the disialo-carbohydrate structure as found in GT1b, GD1b and GQ1b (Holmgren *et al.* 1980; Angstrom *et al.* 1994) (Fig. 2a, highlighted in orange, NAcNeu-6 and NAcNeu-7), BoNT/A prefers binding to the terminal NAc-

Gal β 3-1Gal β (*N*-acetylgalactosamine – galactose) moiety of GT1b and GD1a (Takamizawa *et al.* 1986; Schengrund *et al.* 1991; Yowler and Schengrund 2004a) (Fig. 2a, highlighted in green, NAcGal-3 and Gal-4). However, TeNT, BoNT/A, B, C, E, and F display affinities in the upper nM range in various *in vitro* binding assays with immobilized polysialo-gangliosides *i.a.* depending on the ionic strength, whilst

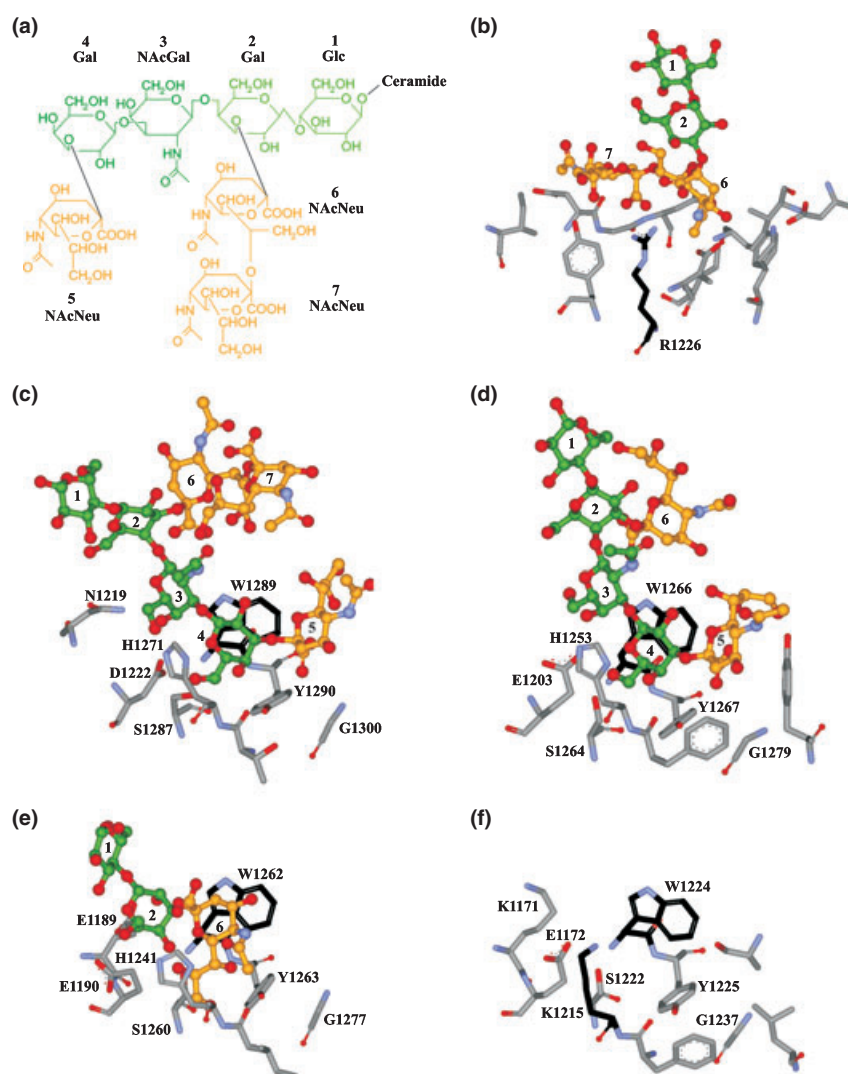


Fig. 2 Ganglioside binding of clostridial neurotoxins. Neurotoxin residues of binding sites are displayed as stick presentations and numbered whereas the bound carbohydrates are displayed in ball and stick presentation. (a) Chemical structure of polysialo ganglioside GT1b. (b) Binding of disialyllactose via its NAcNeu α 8-2NAcNeu α element (carbon scaffold in orange) to the sialic acid binding site in TeNT H_{CC}-domain (1YYN.pdb). R1226 mediating the main salt bridge to the carboxyl group of the terminal NAcNeu is highlighted in black. (c) GT1b- β bound via its terminal NAcGal β 3-1Gal β moiety to the lactose binding site of TeNT H_{CC}-domain (1Fv2.pdb). The hydrophobic side of the Gal rings packs parallel against the key residue W1289 highlighted in black. (d) Like in TeNT, synthetic GT1b binds via its terminal

NAcGal β 3-1Gal β moiety to the conserved ganglioside binding site within the BoNT/A H_{CC}-domain (2VU9.pdb). The hydrophobic side of the Gal rings packs parallel against the key residue W1266 highlighted in black. The 8-2NAcNeu α could not be resolved crystallographically. (e) In contrast, sialyllactose binds via its terminal Gal β 3-2NAcNeu α into the conserved ganglioside binding site of BoNT/B (1F31.pdb). The hydrophobic side of the NAcNeu ring packs parallel against the key residue W1262 highlighted in black. (f) BoNT/E displays a conserved ganglioside binding site including the key residue W1224 with the exception of the opposite lysine 1215 (both highlighted in black) (3ffz.pdb) (Kumaran *et al.* 2009) which substitutes the conserved histidine found in BoNT/A, B and TeNT.

CNTs show a much higher affinity ($K_D = 1.2$ nM) to synaptosomes prepared from neuronal tissue. At the cellular level, removal of sialic acid residues by neuraminidase treatment of cultured cells isolated from spinal cord (Bigalke *et al.* 1986) and adrenergic chromaffin cells (Marxen *et al.* 1989) reduced BoNT/A potency as well as TeNT action (Critchley *et al.* 1986). Conversely, bovine chromaffin cells lacking complex polysialo-gangliosides were rendered sensitive to TeNT and BoNT/A by pre-treatment with gangliosides (Marxen and Bigalke 1989; Marxen *et al.* 1991). In addition, a monoclonal antibody to GT1b antagonized the action of BoNT/A on rat superior cervical ganglionic neurons (Kozaki *et al.* 1998). The inhibition of ganglioside biosynthesis with fumonisin in primary spinal cord neurons or with D,L-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-propanol-HCl in the neuroblastoma cell line Neuro-2a resulted in insensitivity towards TeNT and BoNT/A, respectively (Williamson *et al.* 1999; Yowler *et al.* 2002). Employing a genetic approach, mice, deficient in NAcGal-transferase thus only expressing lactose-ceramide (Lac-Cer), GM3 and GD3, resisted treatment with TeNT and BoNT/A and B (Kitamura *et al.* 1999; Bullens *et al.* 2002). In addition, binding and entry of BoNT/A, B, E, and G was reduced in cultured hippocampal neurons of NAcGal-transferase knock out mice, but could be rescued by adding exogenous gangliosides (Dong *et al.* 2007, 2008). On the other hand, GD3-synthase knock-out mice expressing only Lac-Cer, GM3, GM2, GM1 and GD1a were resistant to TeNT, but kept their sensitivity towards BoNT/A, B and E (Kitamura *et al.* 2005). A combination of both gene knock outs resulted in GM3-only mice which display *i.a.* high resistance towards BoNT/B and G (Rummel *et al.* 2007). Also, GM3-synthase knock-out mice theoretically expressing only Lac-Cer are insensitive to BoNT/C (Tsukamoto *et al.* 2005). Hence, complex polysialo-gangliosides such as GD1a, GD1b and GT1b play a crucial role in the specific binding of CNT to neuronal cells.

The mode of molecular CNT-ganglioside interaction

In a first approach to identify the ganglioside binding site in TeNT a cross-linking experiment employing ^{125}I -azido-GD1b and TeNT H_C -fragment led to radiolabelling of H1293 in the proximity of a large cavity within the H_{CC} -domain (Shapiro *et al.* 1997). The neighborhood of H1293 to the ganglioside binding pocket was confirmed in a mutagenesis study showing reduced *in vitro* binding of the TeNT H_C mutant H1293A to isolated ganglioside GT1b (Sinha *et al.* 2000). The mutation of the TeNT residue Y1290, forming the bottom of this cavity, to phenylalanine, serine and alanine also reduced the affinity to GT1b as well as binding to synaptosomal membranes (Sutton *et al.* 2001). Independently, the anticancer drug doxorubicin, an anthracycline antibiotic, could be docked *in silico* into this pocket and was

able to inhibit the binding of the TeNT H_C -fragment to liposome-integrated GT1b (Lightstone *et al.* 2000). CocrySTALLIZATION of the TeNT H_C -fragment and four carbohydrate subunits of GT1b revealed four distinct binding sites, including the one in the proximity of H1293, where a lactose molecule interacts with the residues D1222, T1270, S1287, W1289, Y1290 and G1300. A separate site comprising R1226 as the key residue coordinated either a molecule of NAcGal or sialic acid (*N*-acetylneuraminic acid, NAc-Neu). Two additional sites were identified in cocrystals with Gal or NAcGal (Emsley *et al.* 2000). However, the latter two sites were unlikely to function as binding pockets for polysialo gangliosides, because of insufficient space (Gal) for a complete ganglioside to bind or a too flexible carbon backbone (NAcGal). Isaacs and coworkers refined their cocrySTALLIZATION approach by using a synthetic GT1b- β analogue lacking the ceramide portion. Indeed, the terminal disaccharide NAcGal β 3-1Gal β bound to the lactose binding site next to H1293 (Fig. 2c), while the disialic acid branch of another GT1b- β molecule interacted with the sialic acid binding site comprising R1226 (Fotinou *et al.* 2001). Independent cocrySTALLIZATION of TeNT H_C -fragment with disialyllactose confirmed the binding of the disialic acid branch to the sialic acid binding site (Fig. 2b) (Jayaraman *et al.* 2005). Mutation of residues D1222, H1271 and W1289 in the lactose binding site led to reduced binding of TeNT H_C -fragment to GT1b in surface plasmon resonance experiments and nerve growth factor-differentiated PC12 cells (Louch *et al.* 2002). The importance of the lactose binding site in TeNT for binding and entry was conclusively demonstrated by the application of corresponding, recombinant full-length TeNT mutants using the mice phrenic nerve hemidiaphragm (MPN) assay leading to a 350-fold reduction in neurotoxicity in case of the single amino acid mutation W1289L (Rummel *et al.* 2003). Furthermore, these experiments demonstrated that the sialic acid binding site is also essential for TeNT action, as *e.g.* the TeNT mutant R1226F possesses a 70-fold reduced activity in the MPN assay. Mass spectroscopy experiments indicated simultaneous binding of two molecules GT1b to the TeNT H_C -fragment, but no ganglioside mediated cross-linking was observed in size exclusion experiments (Rummel *et al.* 2003). Based on these results the ganglioside specificities of the individual lactose and sialic acid binding sites could be biochemically refined confirming the above mentioned crystallographic studies by Fotinou *et al.* and Jayaraman *et al.* (Chen *et al.* 2008). Although binding of a ganglioside to the sialic acid binding pocket was shown, it is conceivable that there is subsequent substitution by or a direct interaction with glycosylphosphatidylinositol-anchored glycoproteins containing sialic acids (Herrerros *et al.* 2000b, 2001). At present, it is still unclear whether the two ganglioside binding sites of TeNT are a peculiarity of this neurotoxin and whether it relates to its retrograde intra-axonal transport.

The lactose binding site in TeNT is built by the peptide motif H.....SXWY.....G (Fig. 2c) which is conserved among the majority of CNTs. This cavity displays the typical features necessary for carbohydrate interaction found also in other protein toxins such as ricin and cholera toxin. An aromatic residue, preferable tryptophan or tyrosine, supplies the surface for the hydrophobic face of the sugar ring. Polar residues like glutamate, serine, or asparagine are located opposite to interact with the sugar hydroxyl groups. These polar interactions are supported by a histidine or like in BoNT/E by a lysine (Fig. 2f).

Cocrystallization studies with BoNT/B and sialyllactose (Fig. 2e) or doxorubicin suggested that the lactose binding site is the ganglioside binding pocket in BoNTs (Swaminathan and Eswaramoorthy 2000; Eswaramoorthy *et al.* 2001). Detailed mutational analyses defined the contribution of various residues within the homologous lactose binding pocket of BoNT/A and B. Again, the mutations of the aromatic key residues, W1266L and W1262L in BoNT/A and B, respectively, lead to dramatic reductions of neurotoxicity using the MPN assay (Rummel *et al.* 2004a). In contrast to TeNT, mass spectroscopy data revealed the binding of only a single GT1b molecule to the H_C-fragment of BoNT/A and B (Rummel *et al.* 2004a). Recently, these physiological and biochemical data were confirmed by a cocrystal of a synthetic GT1b-analogue bound to the H_C-fragment of BoNT/A (Fig. 2d) (Stenmark *et al.* 2008). Interestingly, whereas the mutational data of the lactose site suggest a shared ganglioside binding mode of BoNT/A and B differing to that of TeNT, the crystallographic results indicate that BoNT/A like TeNT interacts predominantly with the NAcGalβ3-1Galβ moiety whereas BoNT/B employs Galβ3-2αNAcNeu (Fig. 2c–e). Different crystallization conditions and the use of sialyllactose instead of GT1b could explain these discrepancies. Recently, the tryptophan residue 1268 of BoNT/G was shown to play a key role in ganglioside interaction (Rummel *et al.* 2007). Shortly thereafter, the conserved tryptophan of the ganglioside binding pocket in BoNT/C was identified as W1258 (Tsukamoto *et al.* 2008). Because of the high amino acid sequence divergence and the lack of crystal structures no homologous tryptophan has been identified as key residue for ganglioside binding of BoNT/D.

As BoNTs do not possess a second carbohydrate binding site, the question arises, whether protein receptors such as synaptotagmin (Syt)-II for BoNT/B (see below) bind in a pocket that is homologous to the sialic acid binding site within the H_{CC}-domain of TeNT. The different affinities of H_C-fragment hybrids generated of H_{CN}- and H_{CC}-domains of BoNT/B strains Okra and 111, respectively, to GT1b/Syt-II endowed liposomes point toward that direction (Ihara *et al.* 2003).

The dual receptor binding concept

Despite the nearly exclusive presence of G1b-series gangliosides on neuronal cells, many evidence argued against

gangliosides as the sole receptors of CNTs at nerve terminals like the much higher affinity *in vivo* compared to that observed in binary interaction studies *in vitro* (summarized in Montecucco 1986) or the marked reduction of TeNT binding to rat brain membranes upon protease pre-treatment (Lazarovici and Yavin 1986; Pierce *et al.* 1986). To account for these findings, a dual-receptor-model involving two sequential binding steps was proposed (Montecucco 1986). It suggests that the toxins bind initially to the abundant polysialo gangliosides, which act as low affinity receptors that accumulate CNTs in the plane of the plasma membrane surface (Fig. 3). Then the neurotoxins simply linger in that position until they get access to the sparsely distributed protein receptor(s) or move laterally within the membrane, still bound to low affinity receptors, to specific sites thereby increasing the chance of contact with the protein receptor. Simultaneous interaction with ganglioside and protein receptor may be considered as high affinity binding and be requisite for the subsequent specific endocytosis step of the neurotoxin (Fig. 3).

Harnessing protein receptors for uptake

The nature of protein receptors remained obscure for a long time, although meaningful information towards a class of proteins that might act as receptors had been available. It had been shown that stimulation of neurons accelerated the uptake of BoNT/A (Simpson 1985; Black and Dolly 1986; Keller *et al.* 2004) and provoked an earlier onset of the blockade of neurotransmission (Hughes and Whaler 1962; Simpson 1980; Schmitt *et al.* 1981). As stimulation causes increased rates of exo- and endocytosis of synaptic vesicles, it was suggestive that luminal segments of integral synaptic vesicles proteins, becoming temporarily exposed on the cell surface at the synaptic cleft on neurotransmitter release, might mediate the binding and internalization of BoNTs. However, classical biochemical separation techniques of total rat brain protein, not selective searches among synaptic vesicle proteins, provided further support for this hypothesis. Chemical cross-linking combined with immunodetection and N-terminal sequencing discovered that Syt-I bound to BoNT/B (Nishiki *et al.* 1994). Subsequent binding studies using Syt reconstituted in GT1b laden lipid vesicles showed that BoNT/B exhibits a ten-times higher affinity to isoform II of Syt (Nishiki *et al.* 1996). The two homologous type I membrane proteins of synaptic vesicles (Fig. 4a) (Perin *et al.* 1990; Geppert *et al.* 1991) are supposed to link synaptic vesicle fusion to Ca²⁺ influx (Chapman 2002; Sudhof 2002). However, the physiological role of this neurotoxin-Syt interaction for BoNT/B internalization was not shown. This was achieved years later by Dong and colleagues who conclusively demonstrated by means of loss-of-function and gain-of-function approaches employing PC12 cells that both Syts can mediate the entry of BoNT/B (Dong *et al.* 2003).

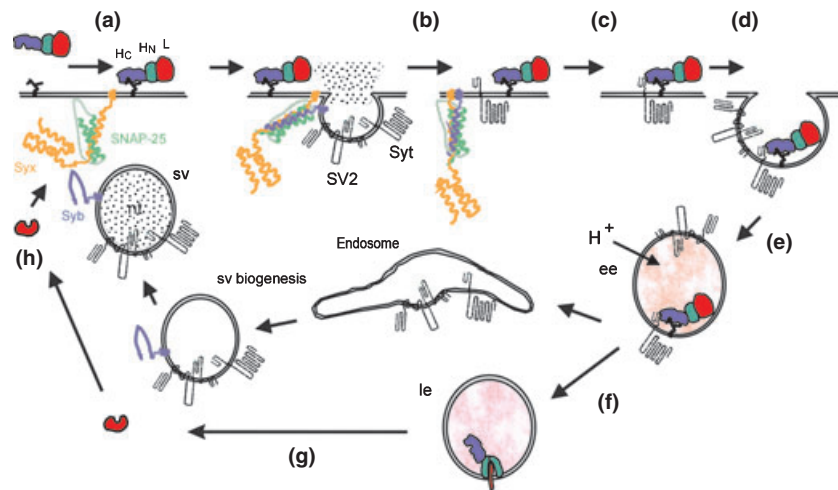


Fig. 3 Model for the entry of BoNTs into nerve cells. (a) BoNTs bind initially to a ganglioside molecule on the cell surface. (b) The intravesicular parts of synaptic vesicle proteins become exposed on the plasma membrane upon SNARE mediated neurotransmitter release. (c) BoNTs access their protein receptor (exemplified here for Syt) possibly by means of lateral diffusion while bound to the ganglioside receptor. (d) Having bound the protein receptor, the neurotoxins become endocytosed via retrieval of synaptic vesicles. (e) The lumen of the recycling vesicle becomes acidified via the action of the vesicular

proton pump. (f) Acidification provokes a structural rearrangement in the neurotoxins, whereby the H_N -domain forms a channel through the vesicular membrane. (g) The LCs pass the channel subsequent to partial unfolding and are released to the cytosol following reduction of the disulfide bond. (h) Ultimately, the LCs cleave their target SNARE(s), Synaptobrevin (Syb, light blue), Syntaxin (Syx, orange), or SNAP-25 (light green), and thus block the synaptic vesicle cycle which they had exploited for cell entry. ee, early endosome; le, late endosome; sv, synaptic vesicle.

Binding experiments with Syt truncation mutants further revealed that the interaction with BoNT/B is imparted at most by the 20 amino acids of the luminal domain adjoining to the transmembrane domain (Fig. 4a). Consistent with this finding, Syt-II fragments containing the luminal domain blocked the uptake of BoNT/B at rat phrenic motor nerve terminals and also neutralised, when administered together with gangliosides, the toxicity of BoNT/B in mice (Dong *et al.* 2003).

This report boosted BoNT protein receptor identification studies targeted at synaptic vesicle proteins. In fact, an inspection whether further BoNTs utilize Syt for cell entry revealed an interaction of BoNT/G with both Syt-I and Syt-II *in vitro* as well, whereas neither of the remaining BoNTs bound to Syts (Rummel *et al.* 2004b). It was concluded that either Syt acts as receptors for BoNT/G, as pre-incubation of the toxin with the luminal domain of Syt-I or Syt-II drastically decreased its activity at MPN preparations. Support for this conclusion is also based on the degree of sequence conservation among H_{CC} -domains, which mediate receptor binding (Rummel *et al.* 2004b). Similarity calculations based on H_{CC} -domain amino acid alignments resulted in 43.8% identity between BoNT/B and G, but significantly lower identity scores between them and every other BoNT (Fig. 5). The relatedness of their binding domains is also reflected in the fact that binding of BoNT/G is dependent on the membrane juxtaposed segment of Syts, too (Rummel *et al.* 2004b). However, the binding properties differ in one

striking aspect. In contrast to BoNT/B, BoNT/G exhibits lower but similar affinities to both Syts *in vitro* (Rummel *et al.* 2007). Final confirmation for activity dependent uptake and Syt mediated cell entry of BoNT/G was provided by mouse hippocampal Syt knockout neurons and restoration of toxin sensitivity by Syt expression in those neurons (Dong *et al.* 2007).

Targeted searches for receptors of the remaining BoNTs among synaptic vesicle proteins soon succeeded again. Immunoprecipitation and pull-down experiments employing isolated synaptic vesicle proteins discovered the interaction between the large luminal domain of the synaptic vesicle protein 2 (SV2) and BoNT/A (Dong *et al.* 2006; Mahrhold *et al.* 2006). The function of this integral membrane glycoprotein with twelve putative transmembrane domains of which three closely related isoforms exist (Fig. 4b) (Bajjalieh *et al.* 1992, 1993; Feany *et al.* 1992; Janz and Sudhof 1999) is likely linked to synaptic vesicles priming or rendering primed vesicles fully Ca^{2+} responsive (Xu and Bajjalieh 2001; Custer *et al.* 2006; Chang and Sudhof 2009). SV2C probably exhibits the highest affinity and its isolated large luminal domain (the fourth luminal segment, LD4) inhibited binding and entry of the toxin into hippocampal neurons and motor nerve terminals (Dong *et al.* 2006) and efficiently decreased the neurotoxicity of BoNT/A (Mahrhold *et al.* 2006), thus evidencing the significance of the interaction for BoNT action. Elegant experiments including loss-of-function and gain-of-function studies in hippocampal

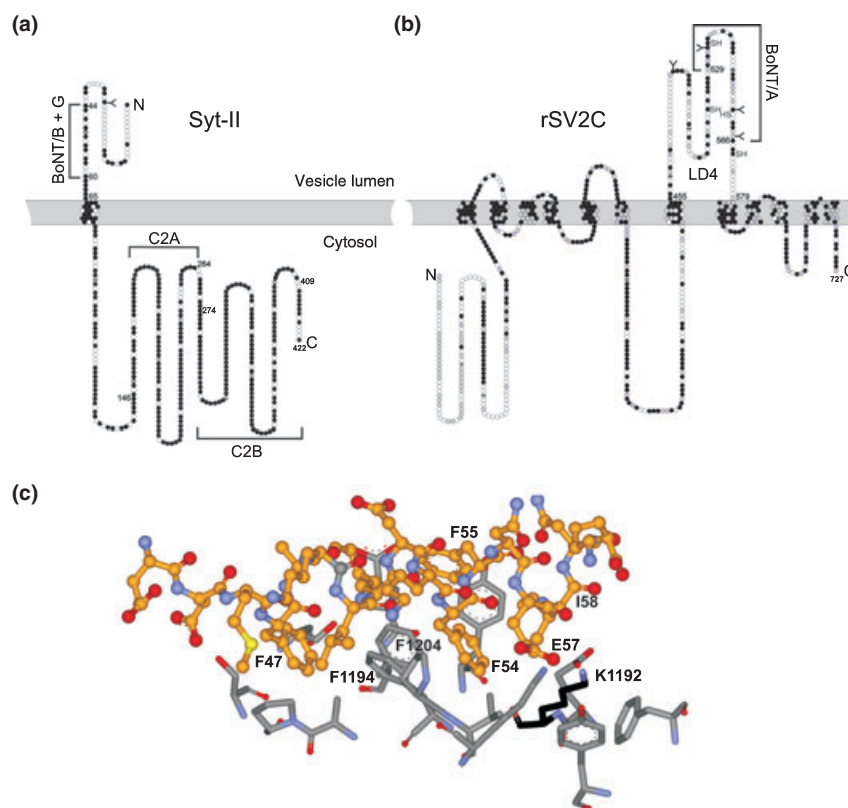


Fig. 4 Membrane topology of botulinum neurotoxin protein receptors. (a) Syt-II is a type I membrane protein consisting of 422 amino acids. The 65 N-terminal amino acids are localized in the lumen of synaptic vesicles. The cytosolic part forms two C₂-domains (indicated by brackets; C₂A: aa 146–264; C₂B: aa 274–409). Residues being conserved in Syt-I are shown in black, and the segment mediating binding of BoNT/B and G is indicated by a bracket. ‘Y’ represents a *N*-glycosylation site, which does apparently not contribute to binding of BoNT/B. (b) SV2C represents a membrane glycoprotein of 727 amino acids with 12 putative transmembrane domains. Strictly conserved residues are shown in black, those conserved in SV2A or SV2B in grey. The C-terminal part (indicated by a bracket) of the large luminal

domain (LD4) which comprises 125 amino acids and four *N*-glycosylation sites (indicated by ‘Y’, of which sites 1–3 are conserved in SV2A and SB2B) mediates the interaction with BoNT/A and E. SH denotes cysteine residues that may form disulfide bridges. Note that the binding of BoNT/E to SV2A and B strictly depends on glycosylation of the third *N*-glycosylation site. Part B is modified from (Janz and Sudhof 1999). (c) Binding of the transmembrane juxtaposed 17mer peptide of the intraluminal domain of Syt-II in an α -helical conformation (amino acids in orange ball and stick presentation) to the saddle like site in the distal tip of the H_{CC}-domain of BoNT/B (residues in grey stick presentation) (2nm1.pdb). K1192 forming an important salt bridge to E57 of Syt-II is highlighted in black.

neurons (Dong *et al.* 2006), as well as RNAi experiments using wild-type PC12 and Neuro-2a cells as well as transiently SV2 expressing PC12 and Neuro-2a knockdown cell lines verified that all three SV2 isoforms can act as physiological receptors for BoNT/A (Dong *et al.* 2006; Mahrhold 2008). Yet, to date it is not clear which SV2 isoform is crucial for entry into peripheral motoneurons, as those express all three isoforms (Dong *et al.* 2006).

In the meantime it has become clear that internalization of the close relative BoNT/E by neurons also occurs in an activity dependent fashion (Keller *et al.* 2004; Lawrence *et al.* 2007; Dong *et al.* 2008) and is mediated by a synaptic vesicle protein (Dong *et al.* 2008). Co-immunoprecipitation experiments, assays employing hippocampal neurons of SV2A/B knockout mice, gain of toxin sensitivity by

expression of SV2 isoforms in SV2 knockout hippocampal neurons, as well as the reduced toxicity in SV2B knockout mice revealed that the *in vivo* toxicity of BoNT/E relies on SV2A and SV2B, but not on SV2C (Dong *et al.* 2008). Contrasting earlier findings for BoNT/A, its interaction with the large luminal domain of SV2 proved to be strictly dependent upon *N*-glycosylation of N573 in SV2A, the C-terminal of three conserved *N*-glycosylation sites among the LD4 of SV2 (Fig. 4b). A closer examination of the SV2-BoNT/A interaction demonstrated that glycosylation at N573 in SV2A may enhance binding affinity to BoNT/A, too. Interestingly, mapping studies showed that unlike binding of BoNT/B and G to Syt the interaction of BoNT/A and E with SV2 requires a segment of up to 40 amino acids of the ~125 residues comprising LD4 (Fig. 4b).

H _{CC} A	H _{CC} B	H _{CC} C	H _{CC} D	H _{CC} E	H _{CC} F	H _{CC} G	H _{CC} T	X	protein receptor
-----	24.5	18.7	16.3	31.5	34.8	22.5	22.1	H _{CC} A	SV2A, B, C
-----	19.2	18.4	24.5	21.9	43.8	25.2		H _{CC} B	Syt-I, II
-----	23.0	17.4	19.3	19.2	15.7			H _{CC} C	?
-----	19.6	18.2	19.9	16.3				H _{CC} D	?
-----	39.7	22.3	19.6					H _{CC} E	SV2A*, B*
-----	19.8	16.0						H _{CC} F	?
-----	23.8							H _{CC} G	Syt-I, II
-----								H _{CC} T	?

Fig. 5 Amino acid sequence similarities among BoNTs and TeNT. Identity values of aligned H_{CC}-domain sequences calculated using the MegAlign 4.04 program (DNASTAR, Madison) are depicted. Scores above 30% are highlighted in white digits on black background. To date identified protein receptors are shown in the right column. Note that BoNT/B and G as well as BoNT/A, E, and F are most similar to each other and that they share protein receptors. SV2 marked by an asterisk indicates that *N*-glycosylation is requisite for interaction with neurotoxin.

Together, the current knowledge about BoNT protein receptors bears out the view that they generally employ luminal segments of synaptic vesicle proteins to enter nerve cells. However, retrograde transport of BoNT/A in central neurons and its subsequent transsynaptic transfer into afferent neurons was recently reported (Antonucci *et al.* 2008). At present, the molecular machinery that directs the toxin into that alternative uptake and transport route is unclear. In addition, there is dispute about the involvement of protein receptors and uptake via the synaptic vesicle recycling pathway for BoNT/C, D, and F. BoNT/F uptake at cultured hippocampal neurons has been reported to occur independently of synaptic activity (Verderio *et al.* 1999). On the other hand, the BoNT/F H_{CC}-domain exhibits 39.7% and 34.8% sequence conservation with BoNT/E and A, respectively. Except for the BoNT/B-BoNT/G score, those are the highest similarity scores among CNT H_{CC}-domains. As BoNT/A and E H_{CC}-domains are also 31.5% identical, it is imaginable that BoNT/F harnesses SV2 for cell entry (Fig. 5), too. Published data on BoNT/C and D are even more puzzling. Tsukamoto and colleagues concluded from results of filter absorbance assays that proteins are not a receptor component, as protease treatment or boiling of solubilised rat brain synaptosomes had no effect on binding of these toxins (Tsukamoto *et al.* 2005). Regarding BoNT/C, these data are consistent with the observation that this toxin failed to pull-down synaptic vesicle proteins, whereas under identical experimental conditions BoNT/A and B exhibited

interactions with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate stable synaptic vesicle protein complexes (Baldwin and Barbieri 2007). Clearly, thorough studies are required to clarify this issue. If entry of BoNT/C, D, or F turns out to depend upon proteinaceous receptors, it will be interesting to find out whether different proteins than the synaptic vesicle proteins Syt or SV2 act as receptors.

Mode of the toxin-protein receptor interaction

The mode of interaction of BoNT/B with its protein receptor Syt-II has been addressed by two parallel cocrystallization approaches. In one study full-length BoNT/B was cocrystallised with a 20mer peptide that corresponded to the proposed interacting segment of Syt-II (Chai *et al.* 2006). In the second study, a recombinant fusion protein consisting of the H_C-fragment of BoNT/B linked via a strep affinity tag to the luminal domain of Syt-II was crystallised (Jin *et al.* 2006). Analysis of both crystals revealed that Syt-II involving amino acids 44–60 bound to a saddle like crevice at the distal tip of the H_C-fragment in the direct neighbourhood of the ganglioside binding pocket (Fig. 4c). Interestingly, the unstructured luminal domain of Syt-II formed an α -helix upon binding to BoNT/B. Its interaction with the toxin relies mainly on hydrophobic interactions via two adjacent pockets on the surface of BoNT/B, but also involves major salt bridges formed by Syt-II-E57. Yet another parallel study employing computer-assisted binding pocket predictions and mutational analyses identified the protein receptor binding site of BoNT/B to reside at the distal tip of the molecule (Rummel *et al.* 2007). This study also evidenced that Syt-I and Syt-II bind to the equivalent place in BoNT/G, though only the general shape of the pocket is conserved according to a homology model calculated for the H_C-fragment of BoNT/G, but not the amino acid residues forming the surface of that binding area. As determined by isothermal titration calorimetry, Syt-I has an at least two orders of magnitude lower affinity for BoNT/B compared to Syt-II (Jin *et al.* 2006), though it displays only two conservative replacements of residues at positions that were shown to be involved in the Syt-II/BoNT/B interaction, M47 instead of Syt-II F55 and L50 instead of Syt-II I58 (Fig. 4c). Substitution of methionine and leucine in Syt-I by phenylalanine and isoleucine, respectively, increased the binding affinity of Syt-I, and the double mutation converted Syt-I to a Syt-II-like high affinity receptor (Jin *et al.* 2006). This result substantiates a conserved binding mode for both Syt isoforms. It is presently not solved whether Syts are bound by BoNT/G in the same manner and whether the positioning of Syt is maintained at all. Mutations in Syt-II showed comparable effects on binding of BoNT/G except that Syt-II-E57K did not bind to BoNT/B, but exhibited wild-type like binding affinity in the case of BoNT/G (Rummel and Binz, unpublished results). Replacement of BoNT/G-Q1200, the counterpart

of BoNT/B-K1192, which forms a major salt bridge with Syt-II-E57, by the equivalent lysine residue of BoNT/B did not improve binding to Syt-II, but dramatically diminished binding (Rummel *et al.* 2007). These findings evidence at least subtle differences in the mode of receptor binding among BoNT/B and BoNT/G.

Although ganglioside and protein receptor binding sites are in close proximity (Fig. 1) they function independently and do not require pre-formation of a ganglioside/protein receptor complex (Rummel *et al.* 2007). Moreover, mutants of BoNT/B with both the ganglioside and Syt binding sites (individually or in combination) deactivated do not exhibit appreciable toxicity excluding any significant contributions of other cell surface molecules to binding and entry of BoNTs (Rummel *et al.* 2007). Taken together, these results support the dual-receptor concept nearly 20 years after it had been proposed.

As outlined above there is evidence from crystallization and mutagenesis studies that TeNT exhibits a second binding site for saccharides. This pocket was later on also shown to accommodate the tripeptide YEW (Jayaraman *et al.* 2005). It is located in the area that corresponds to the Syt binding site of BoNT/B and G, but smaller and chemically different containing several hydrophilic and basic residues. Although it is not known at present, whether this site mediates interaction with a protein receptor for TeNT, it may at least suggest that CNTs generally exhibit a second receptor binding at the distal tip of the cell binding domain. As TeNT was reported to interact with a 15 kDa *N*-glycosylated glycosylphosphatidylinositol-anchored protein (Herreros *et al.* 2001; Munro *et al.* 2001), the different configuration of this site may allow for the binding to sialic acid of this glycoprotein. In line with this assumption, BoNT/A, B and G appear to interact via non-glycosylated segments of their protein ligands SV2 or Syt (Dong *et al.* 2003, 2006; Rummel *et al.* 2004b; Mahrhold *et al.* 2006).

The positioning of the toxin on the membrane may be important for the subsequent endocytosis and translocation steps. Geometric restrictions are imposed by the simultaneous adherence to the ganglioside and the protein receptor (Jin *et al.* 2006). Different binding modes are conceivable. It has been suggested that the predominant negatively charged molecular surfaces of BoNT/B H_N favor a perpendicularly orientation of the translocation domain and thus bending of Syt into a membrane tangential direction (Fig. 1). In this scenario, four solvent-exposed lysine residues being conserved among Syt-I and Syt-II might interact with the phospholipid headgroups of the membrane (Jin *et al.* 2006). This view has recently been supported by modeling GT1b into the Syt-II/BoNT/B complex based on its binding to the ganglioside binding pocket of BoNT/A (Stenmark *et al.* 2008). This model further predicts an extended loop of BoNT/B that contains a very hydrophobic tip (G1246-F-1249) and points out between the ganglioside and Syt

binding pockets to be additionally involved in the interaction with the membrane. Further experiments are now needed to clarify how this binding setup governs the translocation domain to contact and insert into the membrane for channel formation.

Conclusions and future perspectives

Results especially attained during the past six years have enormously advanced the knowledge about the interaction of botulinum toxins with their neuronal target cells. It was demonstrated that BoNT/A, B, E, and G exploit the synaptic vesicle recycling pathway by means of binding to the synaptic vesicle proteins SV2 or Syt in order to reach the nerve cell cytosol. Cocrystal structures of BoNTs with saccharide portions of their ganglioside co-receptor and of BoNT/B with its protein receptor Syt-II unveiled details of the mode of interaction at the atomic level. These new achievements provided the source for understanding the unparalleled potency of CNT and for the development of toxin countermeasures like efficient binding inhibitors against intentionally released botulinum neurotoxins.

On the other hand, there are still several key questions to be answered. The probably most exciting task is the identification of the receptor that mediates the uptake of TeNT at peripheral neurons. This molecule is expected to be no synaptic vesicle protein, as uptake and routing of the toxin onto transcytotic pathways are probably linked. A no less interesting question is to clarify whether BoNT/C, D, or F are also taken up via binding to luminal segments of synaptic vesicle proteins or whether they can enter motoneurons in the absence of synaptic activity. If the latter was true, it will be a challenging job to define the class of receptors mediating their cell entry. Furthermore, the different interaction of BoNT/A and E with the twofold membrane anchored LD4 of SV2 compared to binding of BoNT/B at the type I transmembrane protein Syt-II has to be unraveled. Also efforts need to be directed to the function of the hardly investigated H_{CN}-domain, especially to find out at what stage its action is required.

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