Current Topics in Microbiology and Immunology

Holger Barth *Editor*

Uptake and Trafficking of Protein Toxins

Springer

Current Topics in Microbiology and Immunology

Volume 406

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Springer

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ISSN 0070-217X ISSN 2196-9965 (electronic)
Current Topics in Microbiology and Immunology
ISBN 978-3-319-58891-9 ISBN 978-3-319-58893-3 (eBook)
DOI 10.1007/978-3-319-58893-3

Library of Congress Control Number: 2017955822

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Bacterial protein toxins of the AB-type are the most toxic substances known today. They cause a variety of severe diseases in humans and animals either after uptake of the isolated toxins into the body, e.g. in the context of food-borne diseases such as botulism, or after uptake of toxin-producing bacteria or their spores into the body in the context of infectious diseases. Here, the released toxins represent the virulence factors which cause the clinical symptoms. Clinically relevant examples for the latter are diphtheria, anthrax, *Clostridium* (C.) *difficile* associated diseases (CDIs) and further severe enteric diseases caused by clostridia that produce binary toxins. Moreover, some of these toxins (e.g. *C. botulinum* neurotoxins) or toxin-producing bacteria (e.g. *Bacillus anthracis*) are considered as biological warfare and play emerging roles in the context of bioterrorism.

The remarkable toxicity of AB toxins is due to their unique structure and mode of action: A specific binding/transport (B) subunit of the toxin mediates the transport of an enzymatically active (A) subunit into the cytosol of mammalian target cells. There, the A subunit modifies its specific cellular substrate molecule, which changes the morphology of cells or interferes with cell signaling. In any case, the substrate-modification leads to the clinical symptoms which are characteristic for each toxin-induced disease. This volume reviews the current knowledge on the cell surface receptors as well as the molecular mechanisms underlying cellular uptake and intracellular transport of *C. botulinum* neurotoxins, *C. difficile* toxins A (TcdA) and B (TcdB), the Rho-modulating *C. botulinum* C3 toxin, the binary clostridial ADP-ribosylating enterotoxins including *C. botulinum* C2 toxin, *C. perfringens* iota toxin and *C. difficile* CDT, the binary anthrax toxins and diphtheria toxin. After receptor-binding and internalization into cells by receptor-mediated endocytosis, the before mentioned toxins deliver their A subunits from acidified endosomal vesicles into the host cell cytosol. This transport across endosomal membranes is pH-driven and requires specific translocation subunits of the toxins that insert as pores into the endosomal membrane and facilitate the translocation of the respective A subunits into the cytosol. Moreover, for some toxins a crucial role of specific host cell factors during this membrane transport step was described in past years. ADP-ribosylating toxins such as diphtheria toxin and the binary

clostridial actin ADP-ribosylating toxins exploit the components of the cellular Hsp90 chaperone machinery including Hsp90, Hsp70, cyclophilins and FK506 binding proteins for the translocation of their A subunits across endosomal membranes.

Some chapters of this volume point out that a detailed understanding of the molecular mechanisms underlying this extremely efficient and highly sophisticated transport of bacterial protein toxins into the cytosol of mammalian cells is not scientifically interesting but also clinically relevant. The novel knowledge from this basic research can be directly transferred to develop and optimize novel compounds for the targeted pharmacological inhibition of the uptake of clinically important toxins into cells. By preventing the uptake of the A subunits into the host cell cytosol, the cells are protected from the cytotoxic effects caused by the toxins and therefore, the toxin-induced clinical symptoms should be prevented or at least decreased in humans and animals. Thus, novel specific anti-toxins as described in this issue might result in novel therapeutic strategies to prevent and/or cure some toxin-associated diseases including food-borne intoxications such as botulism as well as severe infectious diseases including diphtheria, anthrax and enteric diseases caused by clostridial toxins. Anti-toxins including pharmacological inhibitors of relevant host cell chaperones or multivalent and heterocyclic molecules which specifically bind into the translocation channels of the toxins and inhibit translocation of the A subunits directly inhibit the mode of action of some toxins, even after their internalization into cells. Therefore, such compounds could be combined with antibiotics to target the toxins in addition to the toxin-producing bacteria. This strategy might be of particular interest if the toxin-associated disease is caused by bacteria that are (multi-)resistant towards antibiotics.

This volume includes eight chapters from experts in this field. I thank all contributors and I am confident that scientists from the fields of Toxinology, Toxicology, Pharmacology, Microbiology, Biochemistry and Cell Biology will enjoy this up-to-date resource.

Ulm, Germany Holger Barth

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Two Feet on the Membrane: Uptake of Clostridial Neurotoxins

Andreas Rummel

Abstract The extraordinary potency of botulinum neurotoxins (BoNT) and tetanus neurotoxin (TeNT) is mediated by their high neurospecificity, targeting peripheral cholinergic motoneurons leading to flaccid and spastic paralysis, respectively, and successive respiratory failure. Complex polysialo gangliosides accumulate BoNT and TeNT on the plasma membrane. The ganglioside binding in BoNT/A, B, E, F, G, and TeNT occurs via a conserved ganglioside-binding pocket within the most carboxyl-terminal 25 kDa domain H_{CC}, whereas BoNT/C, DC, and D display here two different ganglioside binding sites. This enrichment step facilitates subsequent binding of BoNT/A, B, DC, D, E, F, and G to the intraluminal domains of the synaptic vesicle glycoprotein 2 (SV2) isoforms A-C and synaptotagmin-I/-II, respectively. Whereas an induced α-helical 20-mer Syt peptide binds via side chain interactions to the tip of the H_{CC}-domain of BoNT/B, DC and G, the preexisting, quadrilateral β-sheet helix of SV2C-LD4 binds the clinically most relevant serotype BoNT/A mainly through backbone–backbone interactions at the interface of H_{CC} and H_{CN}. In addition, the conserved, complex N559-glycan branch of SV2C establishes extensive interactions with BoNT/A resulting in delayed dissociation providing BoNT/A more time for endocytosis into synaptic vesicles. An analogous interaction occurs between SV2A/B and BoNT/E. Altogether, the nature of BoNT-SV2 recognition clearly differs from BoNT-Syt. Subsequently, the synaptic vesicle is recycled and the bound neurotoxin is endocytosed. Acidification of the vesicle lumen triggers membrane insertion of the translocation domain, pore formation, and finally translocation of the enzymatically active light chain into the neuronal cytosol to halt release of neurotransmitters.

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Current Topics in Microbiology and Immunology (2017) 406:1–37
DOI 10.1007/82_2016_48

Published Online: 01 December 2016

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1 Introduction

Clostridial neurotoxins (CNT) exert the highest toxicity (parenteral LD_{50} ~1 ng/kg body weight) (Gill 1982) of all natural compounds due to their extraordinary target cell specificity. They bind specifically to nonmyelinated areas of cholinergic motor nerve terminals (Dolly et al. 1984). Here, gangliosides, complex poly sialic acid containing glycolipids, adhere the CNTs to the cell surface. A protease sensitive interaction of CNT with neuronal membranes provoked the dual receptor hypothesis postulating an interaction with gangliosides and a proteinaceous receptor (Montecucco 1986). In the last decade, enormous progress was seen on the identification of protein receptors and characterisation of the mode of receptor interaction which will be discussed here in detail.

2 How to Categorise CNT Variants: Serotypes and Subtypes

The family of CNT comprises tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNT). Whereas only a single isoform of TeNT was isolated and sequenced so far (Kitasato 1889; Eisel et al. 1986), a growing number of BoNT variants have been identified. Historically, they are grouped by the absence of cross-neutralisation in an animal bioassay by type-specific monovalent botulinum antitoxin (Leuchs 1910, 1919) into the seven serotypes BoNT/A-G. In 1895, Emile van Ermengem was the first to isolate a *Clostridium botulinum* strain (van Ermengem 1897) which later was ascribed as producing serotype BoNT/B (Leuchs 1910). In the following 75 years six further serotypes were discovered: BoNT/A in (1904), BoNT/C in 1922 (Bengtson 1922; Bengtson 1923), BoNT/D in 1928 (Meyer and Gunnison 1929), BoNT/E in 1937 (Hazel 1937), BoNT/F in 1960 (Møller and Scheibel 1960),

and BoNT/G in 1970 (Gimenez and Ciccarelli 1970). Of them, BoNT/A, B, E, and F are predominantly causing foodborne (intoxication), infant (colonisation of colon) and wound (infection) botulism in humans. BoNT/C and BoNT/D mainly evoke botulism in birds and cattle, respectively, while BoNT/G is rarely causing botulism. It took almost a century to decipher their encoding DNA sequences (CDS) (Binz et al. 1990; Whelan et al. 1992a, b; Thompson et al. 1990; Hauser et al. 1990; Poulet et al. 1992; East et al. 1992; Campbell et al. 1993) which revealed 37–69 % between-serotype difference (Niemann et al. 1994). Recombination events in the BoNT gene (Smith et al. 2015) led to the occurrence of interserotype mosaic BoNT like BoNT/CD and BoNT/DC (Moriishi et al. 1996a, b) which questions the universal validity of the historic serotype definition (Moriishi et al. 1989). Very recently, a novel mosaic BoNT was isolated (Barash and Arnon 2014) which can only be neutralised by anti-BoNT/A antibodies (Maslanka et al. 2016), although its CDS revealed high homology to BoNT/A only for the C-terminal third, the cell-binding domain H_C (Dover et al. 2014; Gonzalez-Escalona 2014). The remaining part of the mosaic BoNT is considered as a novel toxinotype designated H, but the next closest relative BoNT/F caused its temporary naming as BoNT/FA, although anti-BoNT/F antiserum does not neutralise it (Maslanka et al. 2016; Pellett 2016).

In course of BoNT gene sequencing studies also genetic variants of BoNT/A, B, E, and F were identified (Poulet et al. 1992; Thompson et al. 1993; Willems et al. 1993; Hutson et al. 1994, 1996; East et al. 1998; Santos-Buelga et al. 1998). Enormous progress in sequencing technology allowed publication of the first *C. botulinum* genome (Sebaihia et al. 2007). Subsequent worldwide systematic efforts in genome sequencing of *C. botulinum* strain collections and screening for novel clinical and environmental isolates boosted the number of genetic BoNT variants to >40 (Hill and Smith 2013; Peck and Smith 2016). They can differ up to 36 % in amino acid (AA) sequence as in case of BoNT/F variants. In contrast, apart from the two BoNT/CD and DC mosaics, no other variants of the serotypes BoNT/C, D, G as well as TeNT are known. Functional differences such as in antigenicity led to the introduction of the term subtype: a genetic BoNT variant with minimum 2.6 % difference in AA sequence (Smith et al. 2005). An alternative phylogenetic approach defines subtypes as corresponding to clades formed by the clustering of bont sequences (Hill et al. 2007; Raphael et al. 2010; Chen et al. 2007). Applying either of these definitions, the currently identified genetic variants have been ascribed to the subtypes BoNT/A1-A8 with 2.9–15.6 % between-subtype differences, BoNT/B1-B9 (1.6–7.3 %), BoNT/E1-E12 (0.9–10.9 %), and BoNT/F1-F9 (3.0–36.2 %) (Hill and Smith 2013; Peck and Smith 2016; Kull et al. 2015; Wangroongsarb et al. 2014; Kalb et al. 2012; Raphael et al. 2012; Weedmark et al. 2014; Giordani et al. 2015; Sikorra 2016; Smith et al. 2015; Mazuet et al. 2015). It is highly plausible that the between-subtype differences will also cause functional diversity, e.g., with respect to receptor recognition.

3 How Are CNT Molecules Structured?

Each CNT is initially synthesised as ~150 kDa single chain protein, which is 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 and non-covalent interactions mediated by a HC-derived peptide loop wrapping around the LC within the substrate cleft. The LC represents the active component which operates as zinc endoproteases with strict substrate specificities (Binz 2013). Their apo structures have been all determined (reviewed in (Brunger and Rummel 2009)). The structural differences among the LC are mostly limited to solvent-exposed loops and potential substrate interaction sites. Without linkage to their HC the LC are ordinary proteases, i.e., nontoxic molecules, but become highly poisonous agents upon linkage. The HC ensure that the catalytic LC come across their neuronal target cells and conquer the plasma membrane to reach the site of action, the cytosol. In order to fulfil these tasks, the HC comprise two functional subunits, a ~50 kDa largely α–helical domain at the N-terminus, called H_N, and at the C-terminus the ~50 kDa H_C-fragment, in which the two ~25 kDa domains H_{CN} and H_{CC} can be defined. The attachment of H_C to H_N is rigid in case of BoNT/A and B (Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000), but flexible in BoNT/E (Kumaran et al. 2009). Nevertheless, pH-induced binding of the respective nontoxic non-hemagglutinin (NTNHA) to either BoNT/A or BoNT/E causes a 140° rearrangement of H_C via the H_N-H_C linker (Eswaramoorthy et al. 2015; Gu et al. 2012). Structural comparison among the H_C-fragments of BoNT/A [PDB code: 2VUA (Stenmark et al. 2008) 4RJA (Benoit et al. 2014)], B [2NM1 (Jin et al. 2006)], C [3R4S (Strotmeier et al. 2011)], DC [4ISR (Berntsson et al. 2013)], D [3OBT (Strotmeier et al. 2010)], E [3FFZ (Kumaran et al. 2009)], F [3FUQ (Fu et al. 2009)], G [2VXR (Stenmark et al. 2010)], and TeNT [3HMY (Chen et al. 2009)] showed that there is a varying twist between H_{CN} and H_{CC} culminating in H_{CC} by about 17.2°. Nevertheless, separate pairwise structure comparisons of all eight H_{CN}- and H_{CC}-domains demonstrated that the structures are conserved within each domain. H_{CN} folds as lectin-like jelly role, whereas H_{CC} builds up a β-trefoil domain which is assembled by 60 conserved residues in 12 conserved structural motifs which comprise six β-strands forming a barrel and six β-strands forming hairpins that close the bottom of the barrel (Ginalski et al. 2000). In spite of the conserved core structure, large structural differences are found in many surface-exposed loops. Five of such areas reside in H_{CN} and nine loops in the H_{CC}-domain (Fig. 1) providing sufficient degree of freedom to accommodate each H_C to specific receptor structures.

The function of the H_{CN} domain connecting H_N and H_{CC} is still not fully resolved. A low affinity binding of BoNT/A H_{CN} to phosphatidylinositol monophosphate incorporated in sphingomyelin enriched microdomains of the immortalised motor neuron cell line NSC-34 was reported (Muraro et al. 2009). Very recently, some contribution to the protein receptor binding of BoNT/A and E was exhibited (Yao et al. 2016; Mahrhold et al. 2013). Nevertheless, a direct

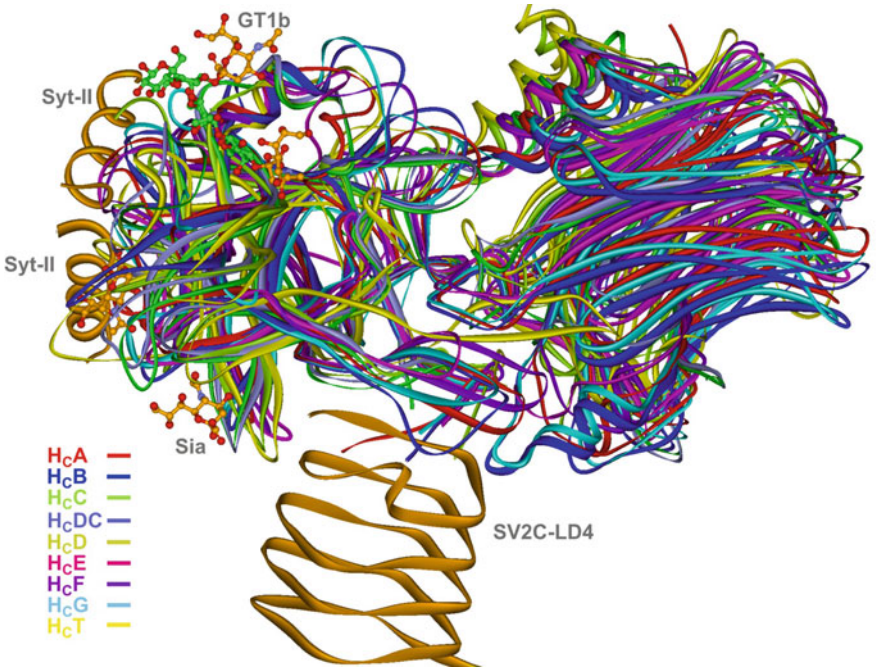


Fig. 1 Superimposition of the H_C-fragment crystal structures of TeNT, seven BoNT serotypes and BoNT/DC. Rat Syt-II (*orange ribbon*) bound to H_CB (*dark blue ribbon*, PDB code 2NM1) was superimposed with H_CA (*red ribbon*) in complex with GT1b (ball & stick, 2UV9) and human SV2C (*orange ribbon*, 4RJA), H_CC (*dark green*) in complex with sialic acid (*ball & stick*, 3R4S), H_CDC (*grey blue*) in complex with mouse Syt-II (*orange ribbon*, 4ISR), H_CD (*light green*) in complex with sialic acid (ball & stick, 3OBT), H_CE (pink, 3FFZ), H_CF (*orange*, 3FQU), H_CG (*light blue*, 2VXR) and H_CT (*yellow ribbon*) in complex with disialyllactose (*ball & stick*, 1YYN)

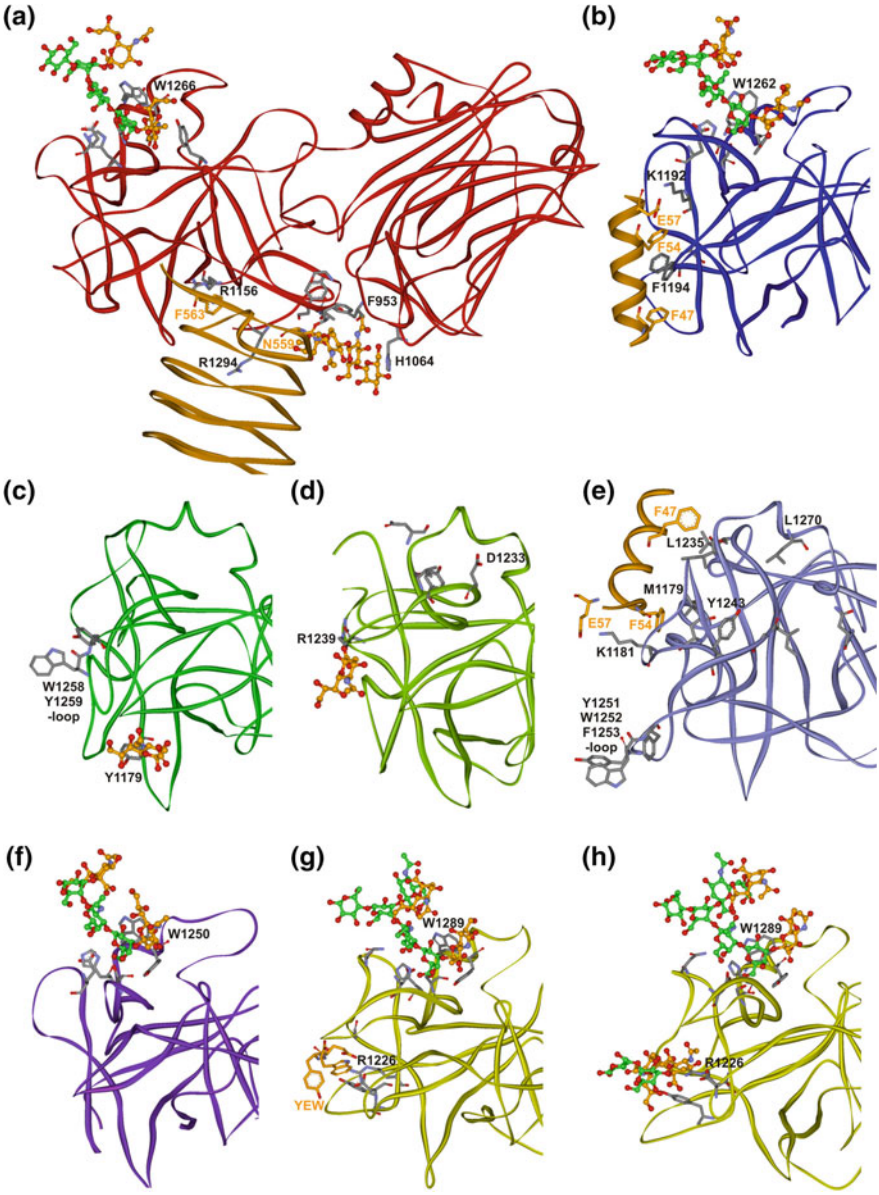
involvement of H_{CN} of BoNT/A in the translocation step could be ruled out lately (Fischer et al. 2008). On the other hand, the H_{CC}-domain harbours the main features required for target cell recognition and internalisation (see below).

4 How Do Complex Polysialo Gangliosides Accumulate CNT on the Neuronal Membrane?

TeNT was first identified to bind polysialo gangliosides, glycosphingolipids that are found particularly in the outer leaflet of neuronal cell membranes (van Heyningen 1959; van Heyningen and Miller 1961). A decade later BoNT/A, B, D or E were de-toxified by preincubation with gangliosides, especially GT1b (Simpson and Rapport 1971a, b). Extensive overlay binding assays employing ganglioside mixtures separated by thin layer chromatography demonstrated binding of BoNT/A, B,

C, E, and F to GT1b, GD1b, and GD1a with varying affinities (Kozaki et al. 1987; Takamizawa et al. 1986; Tsukamoto et al. 2005; Kamata et al. 1986; Ochanda et al. 1986; Kitamura et al. 1980). BoNT/A, B, and E adhered to GT1b better than to GD1a and much less to GM1, and as the ionic strength increased, less binding was observed (Schengrund et al. 1991). Employing surface plasmon resonance (SPR), however, BoNT/A bound to isolated GT1b when the ionic strength was increased from 0.06 to 0.16 with a similar K_D ($\sim 10^{-7}$ m) for each ionic strength (Yowler and Schengrund 2004). Use of isolated, individual gangliosides coated on polystyrene microtiter plates complemented the understanding of ganglioside preference. TeNT prefers the b-series gangliosides GT1b, GD1b, and GQ1b (Chen et al. 2008; Angstrom et al. 1994; Rummel et al. 2003). Isolated GT1b also binds BoNT/A, B and with higher affinity BoNT/G (Rummel et al. 2004; Schmitt et al. 2010). In addition, BoNT/G interacts equally well with GD1a, 10-fold weaker with GD1b, 250-fold weaker with GM3 and hardly with GM1a (Willjes et al. 2013), somewhat similar to BoNT/F which predominantly binds GD1a and GT1b but hardly GD1b or GM1 (Fu et al. 2009). Comparing different serotypes, GD1a is bound best by BoNT/F, followed by BoNT/E and A (Benson et al. 2011) thereby supporting the GD3S-KO mice data (Rummel 2013). In contrast, BoNT/C is efficiently immobilised by GD1b and to a lesser extent by GT1b and GD1a while the closely related mosaic serotype BoNT/DC preferentially binds GM1 and much weaker GD1a, but hardly GT1b and GD1b (Karalewitz et al. 2010). BoNT/D, like TeNT, displays a ganglioside preference for GT1b, GD1b, and GD2 pinpointing the requirement of the disialyl moiety (Kroken et al. 2011). Furthermore, MALDI-TOF MS demonstrated binding of isolated GT1b to BoNT/A, B, and D (Strotmeier et al. 2010; Rummel et al. 2004). Co-crystallisation studies exhibited that BoNT/A binds GT1b-oligosaccharide (Stenmark et al. 2008), BoNT/B interacts with sialyllactose (Swaminathan and Eswaramoorthy 2000) and GD1a-oligosaccharide (Berntsson et al. 2013), BoNT/C complexes two, BoNT/D and DC one sialic acid molecule (Strotmeier et al. 2010, 2011; Karalewitz et al. 2012), BoNT/F interacts with GD1a-oligosaccharide (Benson et al. 2011) and TeNT binds lactose, GT1b-analogue, GT2-oligosaccharide and disialyllactose (Chen et al. 2009; Emsley et al. 2000; Fotinou et al. 2001; Jayaraman et al. 2005) (Fig. 2).

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). Also binding of BoNT/C to neuroblastoma cell lines as well as rat brain synaptosomes was diminished upon neuraminidase treatment (Tsukamoto et al. 2005; Yokosawa et al. 1989) indicating interactions between sialic acid moieties and BoNT/A, C and TeNT. Conversely, bovine chromaffin cells lacking complex polysialo gangliosides were rendered sensitive to TeNT and BoNT/A by pretreatment with gangliosides (Marxen and Bigalke 1989; Marxen et al. 1991). In addition, a monoclonal antibody to GT1b antagonised the action of BoNT/A on rat superior cervical ganglions (Kozaki et al. 1998). The inhibition of ganglioside biosynthesis with fumonisin in primary spinal cord neurons or with D, L-threo-l-phenyl-2-hexadecanoylamino-3-morpholino-propanol in the mouse



neuroblastoma cell line Neuro-2a resulted in insensitivity toward TeNT and BoNT/A, respectively (Williamson et al. 1999; Yowler et al. 2002).

Employing a genetic approach, mice lacking the genes encoding NAcGal-transferase and/or GD3-synthetase were created. NAcGal-transferase deficient mice only expressing lactose ceramide (Lac-Cer), GM3 and GD3