Characterisation of the Cell Binding Domain of Clostridial Neurotoxins

Von der Naturwissenschaftlichen Fakultät der Universität Hannover
zur Erlangung des Grades Doktor der Naturwissenschaften
Dr. *rer. nat.*
genehmigte Dissertation von

Dipl.-Chem. Andreas Rummel

geboren am 06. November 1973 in Langenhagen

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Zusammenfassung

Die Gruppe der clostridiellen Neurotoxine (CNTs) besteht aus Tetanus Neurotoxin (TeNT) und den sieben serologisch unterschiedlichen Botulinus Neurotoxinen (BoNT/A-G). Sie werden als 150 kDa große, einkettige Proteine in *Clostridium tetani*, *C. botulinum*, *C. butyricum* und *C. baratii* produziert und gehören zu den bakteriellen AB Proteintoxinen. Die CNTs werden posttranslational in eine 50 kDa leichte Kette (LC; A-Teil) und eine 100 kDa schwere Kette (HC; B-Teil) hydrolysiert, die durch eine Disulfid-Bindung und nicht kovalente Wechselwirkungen miteinander verbunden bleiben. Die HC besteht aus drei Domänen, der 50 kDa amino-terminalen Domäne H_N, und zwei 25 kDa großen Domänen, H_{CN} und H_{CC}, aus welchen sich das carboxyl-terminale H_C-Fragment zusammensetzt. Das H_C-Fragment bindet hochspezifisch an Motorneurone und übernimmt die rezeptorvermittelte Endozytose der CNTs. Aufgrund der Ansäuerung der Endosomen insertiert die hydrophobe H_N-Domäne in die Vesikelmembran und die LC entfaltet sich partiell, um in das Zytosol transluziert werden zu können. Dort angekommen wird die Disulfid-Bindung reduziert, und die freigesetzte LC, eine Zn^{2+}-Endoprotease, kann spezifisch die Kernkomponenten der Vesikelfusionsmaschinerie hydrolysieren, so dass die Neurotransmitterfreisetzung unterbrochen ist. Die BoNTs blockieren die Azetylcholinfreisetzung in Motorneuronen, was zur Paralyse der Muskulatur führt (Botulismus). Im Gegensatz dazu wird TeNT in neutrale Vesikel sortiert und retrograd intraaxonal in inhibitorische Neuronen transportiert. Dort wird die Freisetzung von Glycin und γ-Aminobuttersäure (GABA) inhibiert, welches zu einer spastischen Paralyse der Muskulatur führt (Tetanus). Die hochspezifischen Interaktionen des H_C-Fragments mit seinen Rezeptoren, Polysialinsäuregangliosiden und größtenteils unbekannten Transmembranproteinen, sind auf molekularer Ebene nahezu unbekannt gewesen.

Unter Berücksichtigung der dreidimensionalen Struktur der CNTs wurden mittels zielgerichteter Mutagenese zwei Gangliosidbindungstaschen in TeNT und eine dazu homologe Stelle in BoNT/A und B identifiziert und detailliert charakterisiert. Die homologe Gangliosidbindungstasche ist in fast allen Serotypen konserviert und besteht aus folgendem Peptidmotiv: D/E….H….SXWY….G. Die Mutation einer einzigen Schlüsselaminosäure wie z.B. des Tryptophans führt zu einer drastischen Verminderung der Neurotoxizität von >95% im Mauszwerchfell-Testsystem. Massenspektroskopische Untersuchungen zeigten, dass BoNT/A und B lediglich ein einziges Gangliosidmolekül binden, während TeNT deren zwei koordiniert. Dies wäre ein Erklärungsansatz für die unterschiedliche Sortierung von TeNT und BoNTs im Motorneuron. BoNT/B benutzt die intravesikuläre Domäne der synaptischen Vesikelproteine Synaptotagmin I und II als zweiten Rezeptor. Das nahe verwandte BoNT/G zeigte ebenfalls eine Interaktion mit denselben Proteinabschnitten in GST-pull-down Experimenten. Die Neurotoxizität von BoNT/B und G im Mauszwerchfell Testsystem wurde durch Zugabe eines Peptids identisch zur intravesikulären Domäne von Synaptotagmin I bzw. II neutralisiert. Das Wissen der molekularen Interaktion der zwei Rezeptortypen innerhalb der H_{CC}-Domäne von BoNT/B und G versetzt den Fachmann damit in die Lage, einen hochaffinen, zweizähnigen Liganden zu konstruieren, welcher als Antagonist für akute BoNT Intoxikationen eingesetzt werden könnte.

Schlagworte: Botulinus Neurotoxin, Tetanus Neurotoxin, Gangliosid, H_C-Fragment, Rezeptor

Abstract

The group of clostridial neurotoxins (CNTs) consists of tetanus neurotoxin (TeNT) and the seven botulinum neurotoxin serotypes (BoNT/A-G). They are produced as 150 kDa single chain proteins by *Clostridium tetani*, *C. botulinum*, *C. butyricum* and *C. baratii* and belong to the bacterial AB protein toxins. The CNTs are posttranslationally hydrolysed into a 50 kDa light chain (LC; A-unit) and a 100 kDa heavy chain (HC; B-unit) which remain associated via a disulfide bond and non-covalent interactions. The HC is composed of three domains, a 50 kDa amino-terminal domain (H_N), and two 25 kDa domains, H_{CN} and H_{CC}, constituting the carboxyl-terminal H_C-fragment. The H_C-fragment binds specifically to motoneurons and mediates uptake of the CNTs via receptor mediated endocytosis. Upon acidification of the endosome the H_N-domain inserts into the membrane, the LC partially unfolds and is translocated into the cytosol. Here, following reduction of the disulfide bond the LC acts as Zn^{2+} dependent endoprotease specifically hydrolysing the core components of the vesicular fusion machinery thereby abrogating neurotransmitter release. While BoNTs block acetylcholine release in the motoneuron resulting in flaccid paralysis (botulism), TeNT is sorted into vesicles with neutral pH and intraaxonal retrogradely transported to inhibitory neurons to block glycine and γ-aminobutyric acid (GABA) release resulting in spastic paralysis (tetanus). The molecular understanding of the highly specific interaction of the H_C-fragment with its receptors, polysialo gangliosides and predominantly unidentified transmembrane proteins, is unknown. Rational site directed mutagenesis employing three dimensional structures of CNT H_C-fragments led to the identification and characterisation of two ganglioside binding sites in TeNT and one homologous pocket in BoNT/A and B. The homologous ganglioside binding pocket is mainly conserved throughout the CNTs and is formed by a D/E….H….SXWY….G peptide motif. Mutation of a single key residue like the tryptophane leads to a loss of more than 95% in neurotoxicity at mice phrenic nerve preparations. According to mass spectroscopy experiments BoNT/A and B bind only a single ganglioside molecule while TeNT bounds two of them. This may imply a reason for the different sorting of TeNT and BoNTs. Further on, BoNT/B employs the intravesicular domain of the synaptic vesicle protein synaptotagmin I and II as second receptor. The homologous BoNT/G was shown to interact similarly with synaptotagmin I and II in GST-pull-down assays. The neurotoxicity of BoNT/B and G in the mice phrenic nerve assay could be neutralised by addition of the intravesicular domain peptide of synaptotagmin I and II. The dual receptor interactions of BoNT/B and G occur in the H_{CC}-domain which would allow the design of potent bidentate binding inhibitors as antagonist for acute BoNT intoxication.

Keywords: Botulinum neurotoxin, Tetanus neurotoxin, ganglioside, H_C-fragment, receptor

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Abbreviations

α-SNAP	α soluble NSF attachment protein
a	year
aa	amino acid
ACE	angiotensin converting enzyme
BoNT/A-G	botulinum neurotoxin A-G
bp	base pair
BSA	bovine serum albumin
C. botulinum	*Clostridium botulinum*
CD	circular dichroism
CDC	Centers for Disease Control and Prevention
Cer	ceramide
CHO	Chinese hamster ovary
CMC	critical micellar concentration
CNT	clostridial neurotoxin
CT	cholera toxin
Da	Dalton
DNA	desoxyribonucleic acid
E. coli	*Escherichia coli*
Gal	galactose
FDA	Federal Food and Drug Administration
GABA	γ-aminobutyric acid
GD1a	NAcNeuα3Galβ3NAcGalβ4(NAcNeuα3)Galβ4GlcβCer
GD1b	Galβ3NAcGalβ4(NAcNeuα8NAcNeuα3)Galβ4GlcβCer
GD3	NAcNeuα8NAcNeuα3Galβ4GlcβCer
Glc	glucose
GM1	Galβ3NAcGalβ4(NAcNeuα3)Galβ4GlcβCer
GM3	NAcNeuα3Galβ4GlcβCer
GPI	glycosylphosphatidylinositol
GQ1b	NAcNeuα8NAcNeuα3Galβ3GalNAcβ4 (NAcNeuα8NAcNeuα3) Galβ4GlcβCer
GT1b	NAcNeuα3Galβ3GalNAcβ4(NAcNeuα8NAcNeuα3) Galβ4GlcβCer
GST	glutathion-S-transferase
GT-sepharose	glutathion-sepharose
HA	hemagglutination activity
HC	100 kDa heavy chain of clostridial neurotoxins
H_C	50 kDa carboxyl-terminal fragment of heavy chain
H_{CC}	25 kDa carboxyl-terminal domain of H_C-fragment
H_{CN}	25 kDa amino-terminal domain of H_C-fragment
H_N	50 kDa amino-terminal fragment of heavy chain
HEPES	4-(2-hydroxyethyl)-1-piperazinethansulfonic acid
HPLC	high performance liquid chromatography
IPTG	*iso*-propyl-1-thio-*β*-D-galactopyranoside
Lac	lactose, 4-*O*-β-D-galactopyranosyl-D-glucose
LC	50 kDa light chain of clostridial neurotoxins
LMW	low molecular weight
LT	*E. coli* heat-labile enterotoxin
Lubrol	polyoxyethylen-9-laurylether

MALDI-TOF	matrix assisted laser desorption and ionisation – time of flight
MLD	minimal lethal dose
MNT	maternal and neonatal tetanus
MPN	mouse phrenic nerve
mRNA	messenger ribonucleic acid
MS	mass spectroscopy
NAcGal	*N*-acetyl-galactosamine
NAcNeu	*N*-acetyl-neuraminic acid, sialic acid
Neu	neuraminic acid
NGal	galactosamine, 2-amino-2-desoxy-D-galactose
NGF	nerve growth factor
NTNH	non-toxic non-hemagglutination
NSF	*N*-ethylmaleinimid-sensitive factor
ORF	open reading frame
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate buffered saline
PC12	pheochromocytoma cells
PCR	polymerase chain reaction
pH	*potentia hydrogenii*
PMSF	phenylmethylsulfonylfluoride
PPMP	*D,L*-threo-l-phenyl-2-hexadecanoylamino-3-morpholino-propanol-HCl
RBS	ribosome binding site
RNA	ribonucleic acid
rpm	rounds per minute
sc	single chain
SEC	size exclusion chromatography
SDS	sodium dodecylsulfate
SNAP-25	synaptosome associated protein of 25 kDa
SNARE	soluble NSF attachment protein receptor
Syb	synaptobrevin, *q.v.* VAMP
Syt	synaptotagmin
TeNT	tetanus neurotoxin
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
VAMP	vesicle associated membrane protein, *q.v.* Syb
WHO	World Health Organization

Introduction

The group of clostridial neurotoxins (CNTs) consists of tetanus neurotoxin (TeNT) and the seven botulinum neurotoxin serotypes (BoNT/A-G). They are produced by *Clostridium tetani*, *C. botulinum*, *C. butyricum* and *C. baratii* and belong to the bacterial AB protein toxins. Their minimal lethal doses (MLD) range down to subnanogram amounts per kg body weight (Gill, 1982); consequentially they are the most potent natural toxins known.

Clostridial neurotoxins cause tetanus and botulism

Tetanus (Greek: to contract) was already described by Hippocrates 400 B.C., but not until the beginning of the 19th century the disease botulism (Latin: *botulus*, sausage) was recognised to be caused by a toxin derived from sausages (Kerner, 1817). The toxin's bacterial origin remained in the dark for almost another hundred years (van Ermengem, 1897; Burke, 1919).

The disease tetanus

Tetanus is caused by germination of Gram-positive, anaerobic spore-forming *Clostridium tetani* in infected tissue lesions. The bacteria start producing TeNT which is released into the blood stream and reaches the neuromuscular endplate via the circulation. After uptake into motoneurons TeNT is transported retrogradely to inhibitory neurons and blocks the release of glycine or γ-aminobutyric acid (GABA) which results in spastic paralysis of the muscles.

Fig. 1: Neonatal tetanus of a two week old baby (WHO *et al.*, 2000)

Thorough immunisation of the population with tetanus toxoid has nearly eradicated tetanus in industrialised countries (2 cases per 10 million inhabitants). In contrast, especially maternal and neonatal tetanus (MNT) remains endemic in the developing world. The World Health Organization (WHO) estimated that there were approximately one million deaths from tetanus worldwide in 1992. This included 580,000 deaths from neonatal tetanus, of which

210,000 were in South East Asia and 152,000 in Africa. In 1998, despite of the WHO's attempt to eliminate MNT, in 57 countries of the developing world approximately 289,000 cases of neonatal tetanus led to the death of 215,000 newborns (75% fatality rate; 14% of all newborns deaths) and 30,000 mothers died of maternal tetanus, while only one case of tetanus in newborns was reported in the U.S.A. (WHO *et al.*, 2000).

The disease botulism

In contrast, botulism is described by flaccid paralysis of the muscles because the BoNTs remain in the motoneuron after uptake and inhibit the acetylcholine release. Four clinical forms of botulism occur in humans: food borne botulism, wound botulism, infant botulism (infant intestinal colonisation) and, rarely, adult infectious botulism (adult intestinal colonisation). Food borne as well as the rare inhalation botulism is evoked by ingestion of food respectively aerosols containing BoNT progenitor toxins. Spores of various strains of *C.botulinum*, *C.butyricum* and *C.barati* germinate well in contaminated food under anaerobic conditions and the bacteria produce acid and protease resistant BoNT progenitor toxins. After passage of the gastrointestinal tract, resorption in the intestine and disassembly, the pure BoNT reaches the neuromuscular endplate via the circulation. Wound botulism is caused by germination of spore-forming *C. botulinum* in infected tissue lesions, nowadays preferably upon contaminated injections in drug addicts, leading to the release of BoNT progenitor toxin directly into the blood stream.

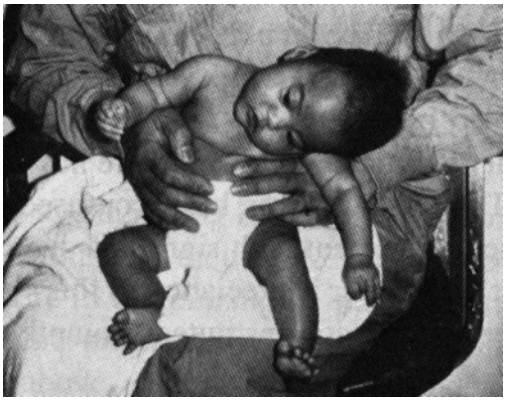

Fig. 2: Infant botulism (Simpson, 1989).

Adult and the more frequent infant intestinal botulism are caused upon the ingestion of spores and subsequent colonisation of the intestine with *C. botulinum*. Especially the infants' intestinal flora is very immature during the first six postnatal months allowing an easy colonisation. About 10% of the cot death cases seem to be related to infant botulism. A main source of *C. botulinum* spores is honey. The BoNT progenitor toxin is released directly from the bacteria into the gut and is subsequently resorbed. Due to the increasing number of

treatments with pharmaceutical compositions of BoNT, the risk of overdoses also leads to a few cases of botulism.

From 1989 through 2003 the 1,800 notified cases of botulism in the U.S.A. led to only 21 deaths. Due to high hygienic standards and clinical treatment the overall fatality rate of botulism decreased from 50% to <5% in industrialised countries in the last 60 years. In the developing world, botulism still remains a severe threat due to lack of hygiene and rare detection of symptoms followed by appropriate therapeutic treatment. 348 cases of food borne botulism (19.3%; average 23 cases/a; range 8-86 cases/a; fatality rate 7%), 225 cases of wound botulism (12.5%; average 15 cases/a; range, 0-30 cases/a, fatality rate 10%), 12 cases of adult infectious botulism (0.7%; average 1 case/a; range, 0-3 cases/a), 1211 cases of infant botulism (67.3%; average 81 cases/a; range, 50-104 cases/a, fatality rate <2%) and 7 cases of botulism of undetermined type were reported to the Centers for Disease Control and Prevention (CDC) in Atlanta, GA (Maslanka and Sobel, 2000-2004). In the U.S.A., 55% of food borne botulism are caused by BoNT/A and 35% by BoNT/E; the remaining food borne cases are almost equally divided between BoNT/B and F. Among cases of wound botulism, approximately 95% are caused by BoNT/A and 5% by BoNT/B while 40% of infant botulism are caused by BoNT/A and 69% by BoNT/B (Maslanka and Sobel, 2000-2004). BoNT/C1 and D do not initiate human food borne botulism but cause botulism in wildlife and domestic animals. Especially in hot summers outbreaks of avian botulism caused by BoNT/C1 are observed. However, humans are thought to be susceptible to these serotypes because they have caused botulism in primates (Gunnison and Meyer, 1930). BoNT/C1 has recently been shown to affect humans similarly to BoNT/A and B with respect to dose, mode and duration of action (Eleopra *et al.*, 2004). BoNT/G, produced by a bacteria species discovered in South American soil in 1969 (Giménez and Ciccarelli, 1970), has never been described to cause food borne botulism. The species differences in BoNT toxicity may be due to the individual compositions of progenitor toxins or different binding affinities to diverse distributed cell surface receptors. Despite these disagreements, the symptoms of intoxications by the various serotypes of BoNT closely resemble each other, because of a similar mode of action. However, there might be differences in the degree peripheral nerves are affected. Depending on the serotype and the dose ingested, the disease may last from a few days to several months. Intoxication with BoNT/A has the longest duration of three to six months, whereas a patient poisoned with BoNT/E, even if it is a high dose, will recover within a few days. Recovery occurs when the catalytic domain of the neurotoxin inside the nerve ending is degraded to

inactive fragments and membrane fusion will be reactivated (Erdal *et al.*, 1995; Adler *et al.*, 2001; Fernandez-Salas *et al.*, 2004).

Application of BoNTs - a janus-faced molecule

Use of BoNTs as pharmaceuticals

In the beginning of the 1970s crystalline BoNT/A complex was successfully tested by the surgeon Alan B. Scott and the microbiologist Edward J. Schantz for the treatment of hyperactive muscle disorders in monkeys and human beings (Scott, 1980; Schantz and Johnson, 1992). In December 1989 the BoNT/A complex, marketed as BOTOX®, was approved as orphan drug by the Federal Food and Drug Administration (FDA) to treat two eye muscle disorders (blepharospasm and strabismus). BOTOX® as well as the BoNT/B complex (Neurobloc®/Myobloc®) were approved in 2000 to treat cervical dystonia, a neurological movement disorder causing severe neck and shoulder muscle contractions. In 2002 BoNTs were approved as a cosmetic to temporarily improve the appearance of moderate to severe frown lines between the eyebrows and in 2004 to treat severe underarm sweating known as "primary axillary hyperhidrosis" (www.fda.gov). Subsequently, the American Society of Aesthetic and Plastic Surgery estimated that 2.27 million BoNT treatments were performed in the U.S.A. in 2003, which makes the BoNT market to be worth more than $ 900 million in 2003. Other BoNT/A complex preparations on the current world market include Dysport® (Ipsen, Inc., UK, to be marketed in the U.S. as Reloxin®, Inamed, Inc. early 2006), Linurase® (Prollenium, Inc., Canada), and an assortment of preparations from Asia, including CBTX-A® (Lanzhou Biological Products Institute, VR China), and Neuronox® (Medy-Tox, Inc., South Korea). Mentor Corporation plans to introduce a highly purified BoNT/A preparation (free of complex proteins) for cosmetic use in the U.S.A. in 2006, and a similar, highly purified BoNT/A (NT201/Xeomin®, Merz Pharma GmbH, Frankfurt/M.) was approved in Germany in June 2005. At present, more than 100 clinical applications are described for BoNT therapy and clinical trials investigate the use of BoNTs in migraine and pain. This rapid development highlights the importance to complete the basic knowledge of the mechanism of BoNTs action.

The misuse of BoNTs as biological warfare agent

Another application of BoNT was fortunately banned in 1972 by a United Nations' convention: the use for biological warfare. Nevertheless, although nearly 140 countries have signed the treaty, the former U.S.S.R as well as Iraq continued to produce BoNT/A as biological warfare agent. After the 1991 Persian Gulf War, Iraq admitted to the United

Nations' inspection team to having produced 19 m^3 of concentrated BoNT/A, of which approximately 10 m^3 were loaded into military weapons. These 19 m^3 of concentrated BoNT/A solution constitute approximately three times the theoretical amount needed to kill the entire current human population by inhalation (UN, 1995). Apart from state driven biological warfare the threat of bioterror attacks has dramatically heightened in the last decade terrorizing populations with toxins, causing panic and possible loss of lives. In 1990 the Japanese sect, Aum Shinrikyo, unsuccessfully dispersed aerosols containing BoNT and spores of *bacillus anthracis* at various locations in Japan (Wilkening and Seth Carus, 1998). In autumn 2001, in view of the terror attack of 11th September, letters containing spores of a laboratory *bacillus anthracis* strain were sent out to U.S. media personalities and politicians causing 22 incidences of anthrax poisoning, of which 19 cases had been confirmed claiming five lives and three cases had been suspected (Thompson, 2003). Later, the arrest of several Arabs with connections to the terror network of al quaida, who were trying to produce ricin toxin in northern London, was reported in January 2003. In light of these occurrences a theoretical model of a bioterror attack adding BoNT into the milk supply chain revealed that only 10 g of BoNT/A almost certainly leads to a loss of 500,000 lives (Wein and Liu, 2005). Theoretically, 10 g of BoNT/A are sufficient to kill 100 million adults. This illustrates the omnipresent threat by biological weapons and the need of research to take effective countermeasures such as screening for effective antagonists or improving detection systems.

Taxonomy of *C. botulinum*

The traditional classification of BoNT producing *C. botulinum* into seven strains (A-G) was determined by neutralisation with specific antitoxin. However, all CNTs releasing *clostridia* were first divided into seven different groups upon their diverse phenotypes and sequencing of the 16S RNA supported this classification (Tab. 1). Within one group the 16S RNA is 99.6-100% identical (Collins and East, 1998). Meanwhile, the whole genomes of *C. tetani* and *C. botulinum* Hall strain A (ATCC 3502) are deciphered allowing a systematic investigation of the organism in a proteomic approach (Brüggemann *et al.*, 2003); http://www.sanger.ac.uk/Projects/C_botulinum). The known nucleotide sequences of all CNTs exhibit *i.a.* a different localisation of the NT coding genes as well as a high A/T content (70%) typical for *Clostridium*. Furthermore, the CNTs share an aa homology of about 35% which points towards a horizontal gene transfer of the CNT encoding genes between the different groups of *Clostridia* (Minton, 1995).

Table 1: Groups of CNT producing *Clostridia.*

Species	Group	CNT produced	Proteolysis	Localisation of CNT gene
Clostridium botulinum	I	A, B, F	+	chromosome
Clostridium botulinum	II	B, E, F	-	chromosome
Clostridium botulinum	III	C1, D	-	bacteriophage
Clostridium botulinum	IV	G	+	plasmid
Clostridium butyricum	V	E	-	chromosome
Clostridium baratii	VI	F	-	chromosome
Clostridium tetani		TeNT	+	plasmid

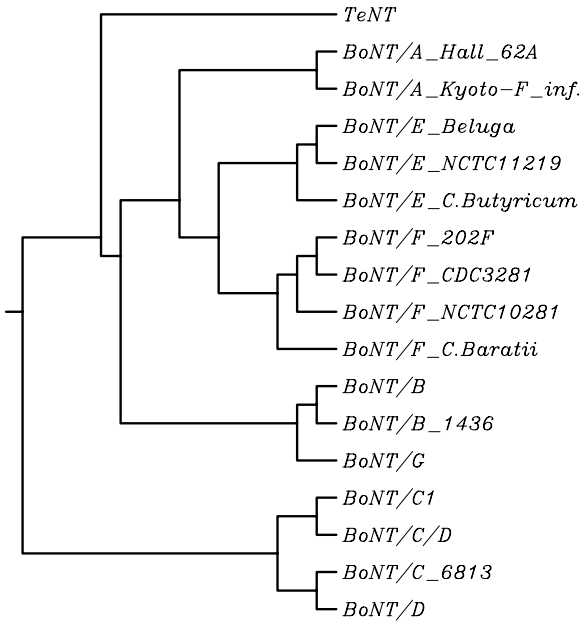

Fig. 3: Dendogram showing the phylogenetic differences of the 17 main CNT variants on the aa level (http://align.genome.jp).

It should be noted that the degree of similarity between CNTs variants differs significantly from the grouping of the host organisms (cp. Tab. 1 and Fig. 3). Furthermore, the neurotoxin encoding genes themselves seem to be assembled from sections of different sources (Niemann *et al.*, 1994).

BoNT progenitor toxins

The MLD of BoNTs dramatically depends on *i.a.* their molecular composition. *E.g.*, pure BoNT/A is about a factor 100,000 less toxic than the BoNT/A progenitor toxin by the oral route. The reason for this contradictory behaviour rests on the sophisticated assembly of the BoNT progenitor toxins released by *C. botulinum.* Upstream of the genes encoding the BoNTs, an open reading frame (ORF) for a 150 kDa protein and up to three ORFs for 17-70 kDa proteins with hemagglutination activity (HA) were found (Fig. 4).

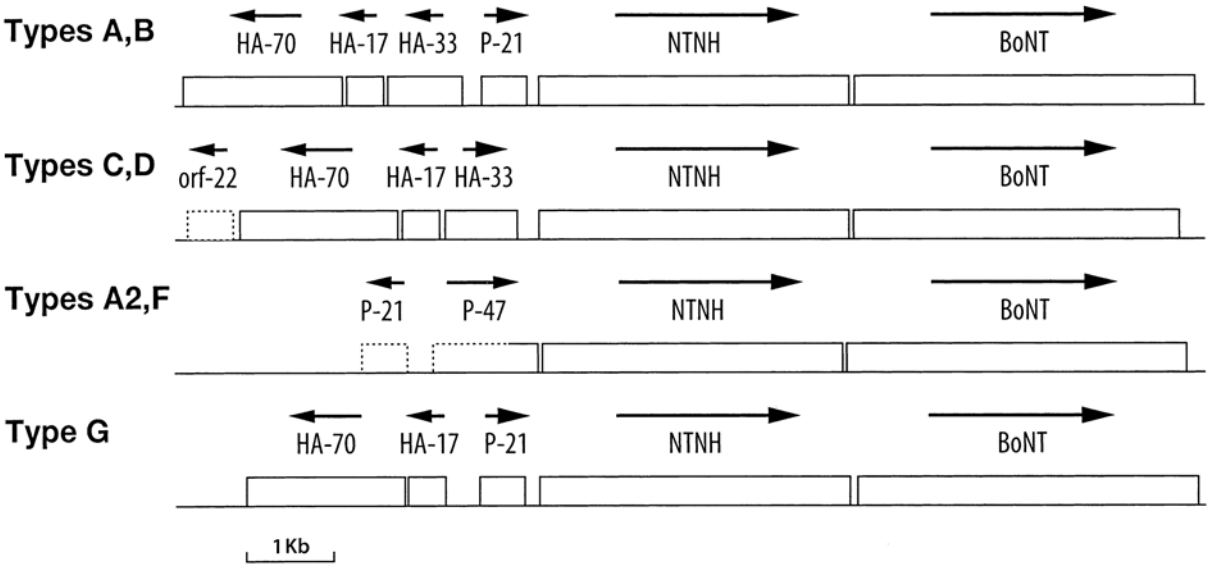

Fig. 4: Diagrammatic representation of the arrangement of genes encoding the progenitor toxin proteins consisting of BoNT, NTNH and HA (Collins and East, 1998).

The 150 kDa protein, called non-toxic non-hemagglutination (NTNH) protein, forms a 300 kDa complex with the BoNTs. This complex is one of the three forms of progenitor toxins known: the 12S M-Toxin (~280 kDa), consisting of one BoNT and one NTNH molecule; the 16S L-Toxin (~400 kDa), formed by association of M-Toxin with the three HA subcomponents, and the 19S LL-Toxin (~900 kDa) is composed of a homodimer of L-Toxin linked by HA-33. Accordingly, the L- and LL-Toxins exhibit hemagglutination activity, but the M-Toxin does not. However, the number of each HA subcomponent responsible for hemagglutination activity remains undetermined (Fujii, 1995). While BoNT/A occurs in all three different molecular forms, BoNT/C1 and D appear as L- and M-Toxins. BoNT/G is exclusively found as L-Toxin and BoNT/E and F are entirely composed of M-Toxin. The progenitor toxins, which are resistant to proteases and acids, dissociate immediately at physiological pH into neurotoxin and non-toxic components (Minton, 1995; Fujii, 1995). Thus, upon ingestion of the BoNT progenitor toxin, the protein complex resists the low pH in the stomach as well as the attack of pancreatic enzymes in the upper small intestine before it

is absorbed in the lower intestine. The HA proteins seem to act as adhesins, allowing the progenitor toxin to bind to intestinal epithelial cells and erythrocytes (Nishikawa *et al.*, 2004; Fujinaga *et al.*, 2004) most likely via terminal galactose molecules.

If pure BoNT/A is ingested, however, the neurotoxin loses most of its toxicity, because it is almost completely destroyed during the digestive tract passage. The same might be true if the neurotoxin is inhaled, because the surface of the mucous membrane in the lung is rich in protease activity. The pure neurotoxin does not lose its toxicity when applied parenterally, *e.g.* intraperitoneally, because a protection against proteases is not required in that case. For humans the calculated MLD of pure BoNT/A is approximately 0.3 ng/kg after intravenous application, 20 ng/min/m^3 after inhalation and 1 μg/kg following ingestion. These numbers allow only a rough estimation and were partly deduced from experiments with rodents (Cardella, 1964) and accidental intoxications in human (Naumann and Moore, 2003). Experiments with primates showed a higher toxicity, when BoNT entered the organism via the respiratory route (Franz *et al.*, 1993).

Three dimensional structure of CNTs

The elucidation of the crystal structures of BoNT/A and B (Lacy *et al.*, 1998; Swaminathan and Eswaramoorthy, 2000) illustrates that most likely all CNTs are composed of four functionally independent domains that perform individual tasks in the multi-step intoxication process (Fig. 5).

All CNTs are produced as ~150 kDa single chain (sc) proteins. A surface exposed peptide loop of eight to 27 aa, rich in lysine and arginine residues and framed between two cysteines, is posttranslationally hydrolysed either by clostridial or foreign host proteases (cp. Tab. 1). This process called nicking generates a ~100 kDa heavy chain (HC) and a ~50 kDa light chain (LC). Both chains remain associated by a single disulfide bond, which is bypassing the cleaved loop between LC and HC, non-covalent interactions and an HC derived peptide loop, called belt, wrapping around the LC. The HCs or B-units are responsible for neurospecific binding and uptake of the molecule for translocation of their LCs into the cytosol. The globular folded LC represents the catalytic domain or A-unit cleaving soluble NSF attachment protein receptor (SNARE) proteins. The HC is separated into the amino-terminal 50 kDa H_N domain and the carboxyl-terminal 50 kDa H_C-fragment. The H_N domain is highly α-helical and contains predominantly hydrophobic residues which are believed to be involved in the translocation step.

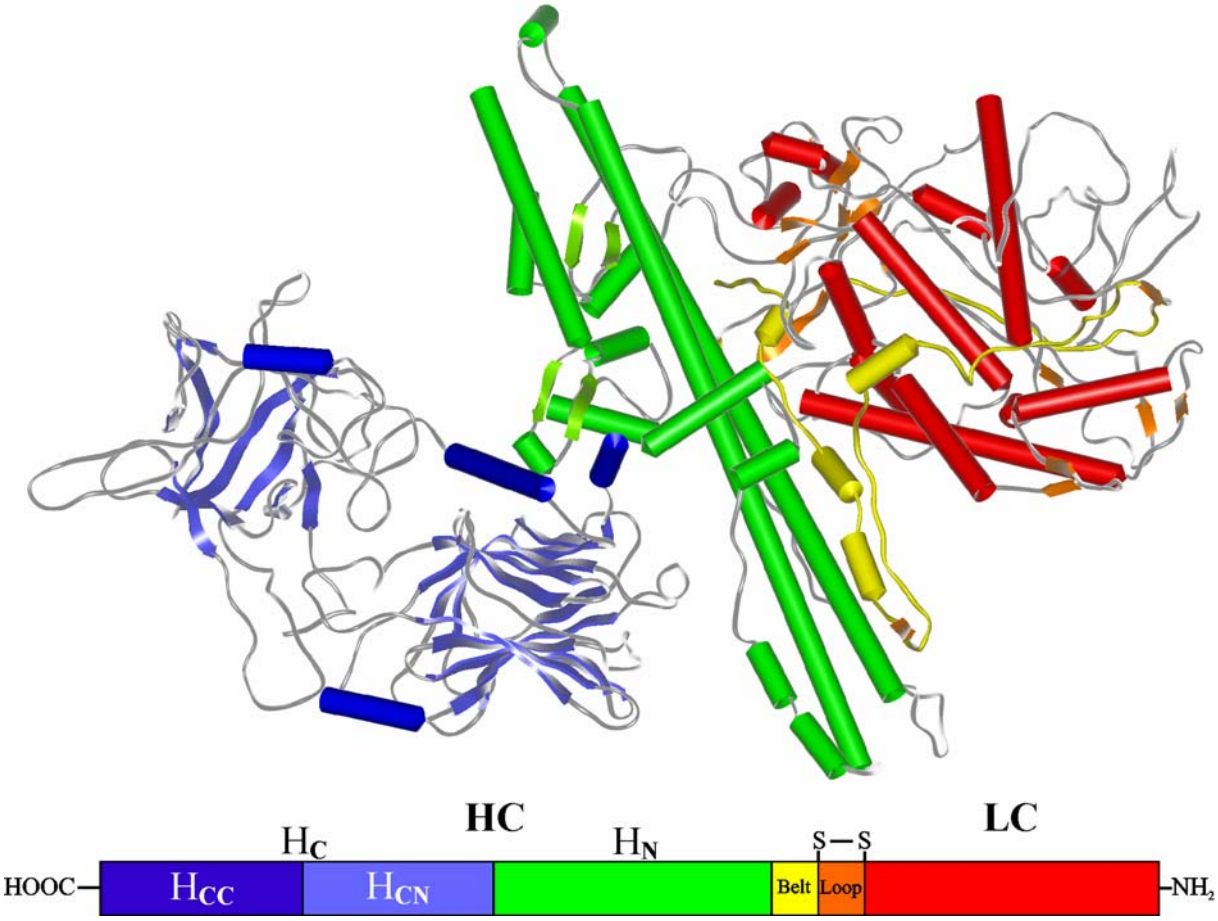

Fig. 5: Crystal structure of native BoNT/B (1EPW.pdb; top) and schematic domain arrangement of CNTs (bottom).

The crystal structure analysis of the TeNT H_C-fragment identified two 25 kDa domains within H_C, an amino-terminal lectin-like jelly-roll domain (H_{CN}, residues 865-1110) and a carboxyl-terminal β-trefoil domain (H_{CC}, residues 1110-1315) (Umland *et al.*, 1997; Knapp *et al.*, 1998). In a sequence and structural alignment study within the less conserved H_{CC} domains of TeNT and BoNT/A, B, E, F, and G (23% aa sequence similarity on average), 12 structurally conserved regions, each of five residues, were identified as β-trefoil forming segments; 44 of these 60 amino acids are highly conserved (Ginalski *et al.*, 2000). This sequence homology was confirmed by the tertiary structure found in the crystals of BoNT/A and B (Lacy *et al.*, 1998; Swaminathan and Eswaramoorthy, 2000). The characteristics of β-trefoil folds allow a high sequence variation outside the 12 five aa clusters (cp. Tab. 2). Accordingly to the four domain structure, LC, HC and H_C can be easily isolated as native/clostridial derived proteins by proteolysis and chromatography while the H_N domain is very insoluble. Upon recombinant expression of CNTs in *E. coli*, only LC and H_C-fragments are isolated in high yields, but hardly HC and H_N (Rummel Diplomarbeit, 1999).

Mode of action of CNTs

Following entry of the CNT into the circulation, the H_C-fragment specifically binds to glycolipid receptors on the surface of neuronal cells (Fig. 6). Subsequent internalisation via receptor-mediated endocytosis brings the BoNTs into the endosomal compartment of motoneurons (Bigalke and Shoer, 2000). Here, an acidic environment below pH 6.5 (M. Montal, personal communication) allows the structural rearrangement and concomitant insertion of the H_N-domain into the endosomal membrane. At the same time the LC is partially unfolded (Koriazova and Montal, 2003). Upon reduction of the disulfide bond, the LC functions as a Zn^{2+} dependent endopeptidase in the cytosol. TeNT is sorted in neutral vesicle and transported retrogradely to inhibitory neurons where a similar mechanism occurs.

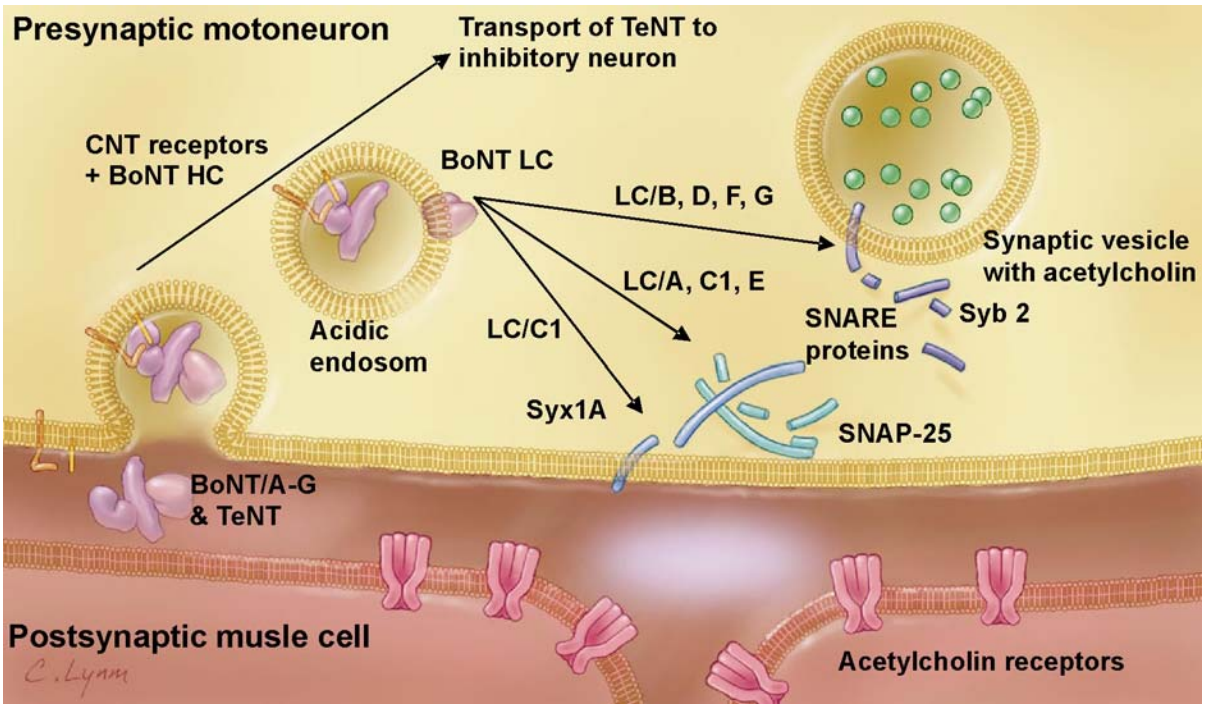

Fig. 6: Mode of action of BoNTs at the cholinergic motoneuron (modified from Arnon *et al.*, 2001).

CNT LCs specifically cleave SNARE proteins

CNT LCs exclusively hydrolyse members of the three SNARE families. VAMP (vesicle associated membrane protein)/synaptobrevin represents the substrate for BoNT/B, D, F, G, and TeNT, whereas BoNT/A, C, and E cleave SNAP-25 (synaptosome associated protein of 25 kDa) (Fig. 7). Except for BoNT/B and TeNT, which share the same cleavage site, hydrolysis occurs at unique positions. BoNT/C1 is additionally capable of hydrolysing syntaxin1A. Cleavage of any neuronal SNAREs results in inhibition of the fusion of synaptic vesicles with the presynaptic membrane, thus a blockade of neurotransmitter release (Niemann *et al.*, 1994; Schiavo *et al.*, 2000).

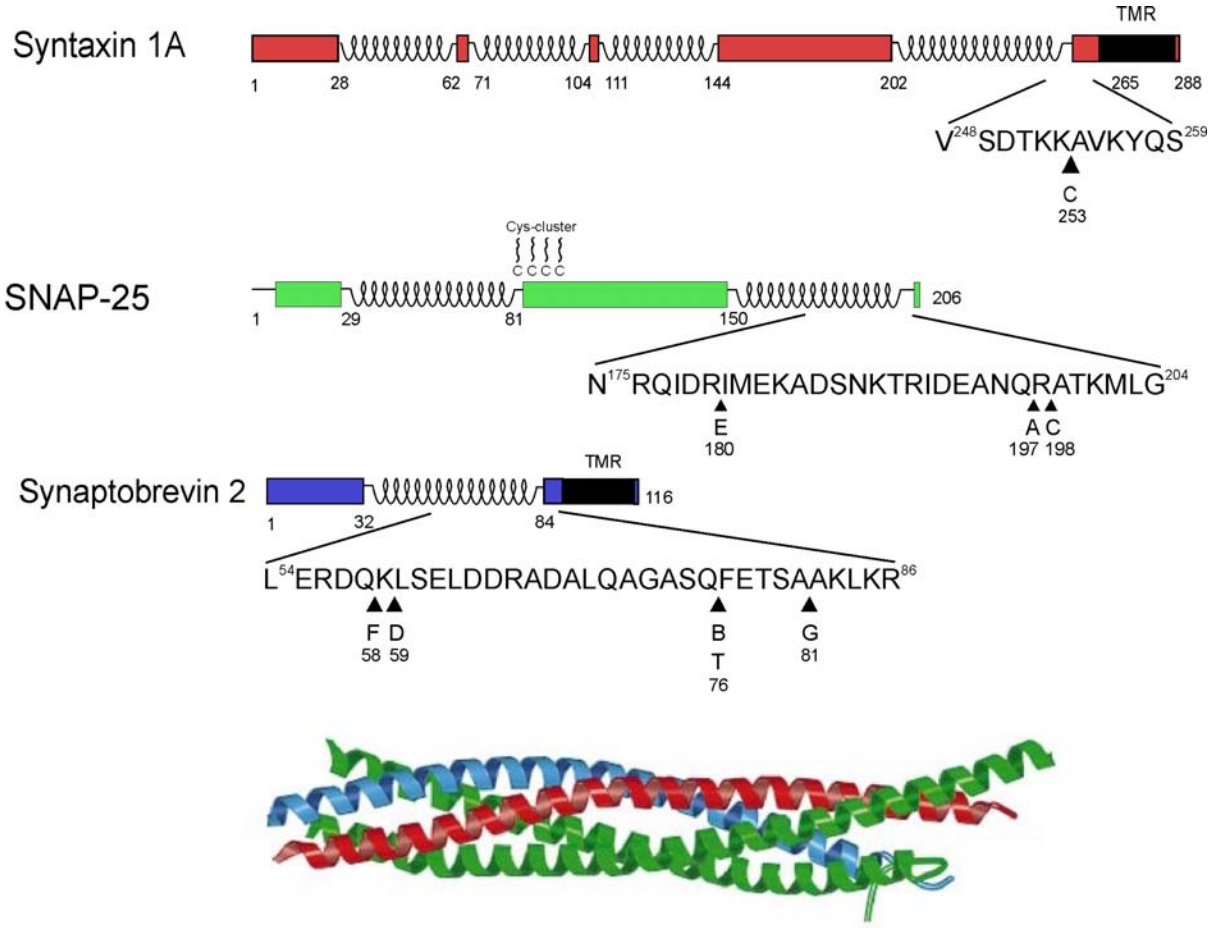

Fig. 7: The SNARE proteins are specific substrates for the CNT LCs. Schematic drawing of the SNARE proteins structure and LC cleavage sites (top). Crystal structure of the coiled coil bundle of the core SNARE complex (bottom; modified from R.B. Sutton *et al.*, 1998).

All LCs contain a conserved HExxH motif, typical for Zn^{2+} metalloproteases as thermolysin and angiotensin converting enzyme (ACE). Two histidine residues and a conserved glutamate further downstream coordinate the zinc cation, while the glutamate in the HExxH motif positions a water molecule for hydrolysis into the empty coordination space of zinc. In case of BoNT/A, the conserved R362 and Y365 are also directly involved in the catalytic mechanism (Binz *et al.*, 2002). Recently, the cocrystal structure for BoNT/A LC and its substrate SNAP-25 was solved, visualising the extensive enzyme-substrate interaction surface which explains their high specificity (Breidenbach and Brunger, 2005).

Gangliosides as receptors for CNTs

The specific binding to peripheral nerve endings at the neuromuscular junction solely involves the 50 kDa carboxyl-terminal half of the HC, the H_C-fragment (Simpson, 1984a; 1984b; 1985; Evinger and Erichsen, 1986; Fishman and Carrigan, 1987; Lalli *et al.*, 1999) and complex polysialo gangliosides, glycosphingolipids that are found particularly in membranes of neuronal cells (van Heyningen and Miller, 1961; Simpson and Rapport, 1971a). The interaction of gangliosides with CNTs was investigated for TeNT and several serotypes of BoNTs in extensive studies (Halpern and Neale, 1995; Yowler and Schengrund, 2004). These studies revealed that the disialo carbohydrate structure as found in GD1b is essential for the binding of most of the CNTs. Furthermore, it was shown that TeNT, BoNT/A, B, C, E, and F displayed affinities in the high nM range in various *in vitro* binding assays with immobilised polysialo gangliosides, whilst binding experiments of CNTs to neuronal tissue like synaptosomes revealed much higher affinities (K_D = 1.2 nM).

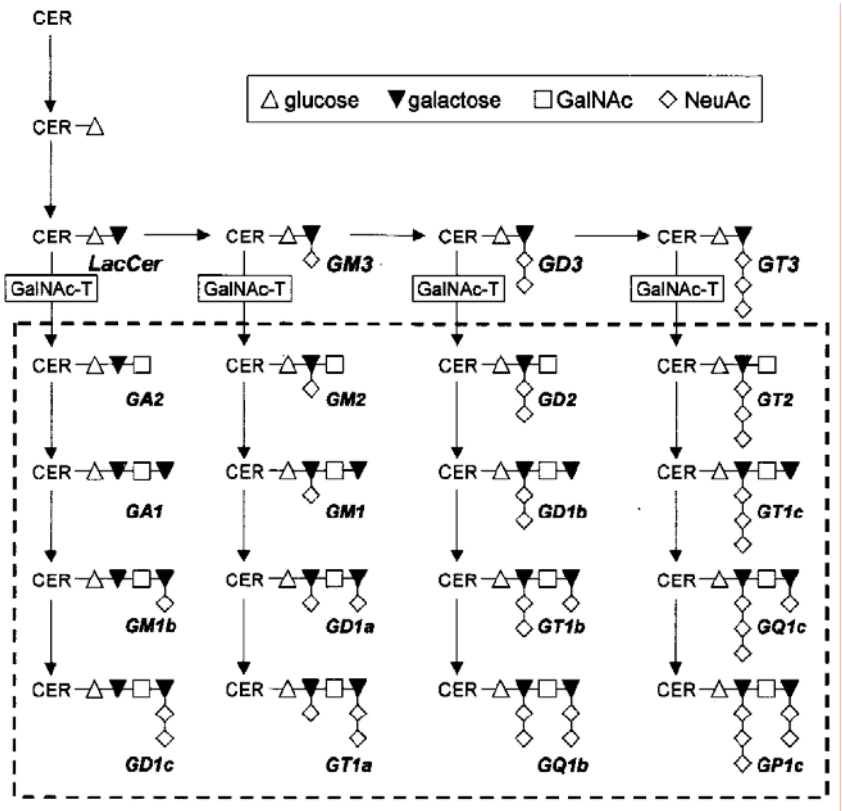

Fig. 8: Ganglioside biosynthesis. Disruption of the GalNAc-transferase gene in mice results in the absence of all the complex gangliosides within the dashed rectangle (Takamiya *et al.*, 1996).

At the cellular level, the cleavage 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) was shown to reduce BoNT/A potency as well as TeNT action (Critchley *et al.*, 1986). Conversely, bovine chromaffin cells lacking the complex polysialo

gangliosides were rendered sensitive to TeNT and BoNT/A by incubation with gangliosides (Marxen *et al.*, 1989; Marxen *et al.*, 1991). In addition, a monoclonal antibody to GT1b was shown to antagonize the action of BoNT/A on rat superior cervical ganglion neurons (Kozaki *et al.*, 1998). Recently, the inhibition of ganglioside biosynthesis with fumonisin in primary spinal cord neurons and with PPMP in the neuroblastoma cell line Neuro2a resulted in insensitivity to TeNT and BoNT/A, respectively (Williamson *et al.*, 1999; Yowler *et al.*, 2002).

Employing a genomic approach, mice, deficient in *NAcGal-transferase* thus only expressing GD3 (Fig. 8), were reported to resist treatment with TeNT and BoNT/A, B and E (Kitamura *et al.*, 1999; Bullens *et al.*, 2002), whereas *GD3-synthetase* knock-out mice lacking GD2, GD1b, GT1b and GQ1b were only resistant to TeNT but kept their sensitivity towards BoNT/A, B and E (Kitamura *et al.*, 2005). Hence, complex polysialo gangliosides like GD1a, GD1b and GT1b play an important role in mediating the first cell contact and for the specific binding to neuronal cells.

Postulation of a protein as second receptor for CNTs

Nevertheless, the discrepancy in affinity between binding of CNTs to isolated gangliosides and neuronal tissue prompted postulations of a second receptor component. The protease-sensitive binding of BoNT/A and TeNT to rat brain synaptosomes (Kitamura, 1976; Dolly *et al.*, 1982; Pierce *et al.*, 1986; Lazarovici and Yavin, 1986) resulted in a dual receptor model. First, the polysialo gangliosides were considered to accumulate CNTs on the plasma membrane surface. Then, the CNTs simply wait in that position until binding to their thinly distributed protein receptor(s) is enabled or move laterally within the membrane while still bound to low affinity receptors 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 prerequisite for the subsequent specific step of endocytosis (Montecucco, 1986; Niemann *et al.*, 1991).

Several studies demonstrated accelerated uptake of TeNT (Simpson, 1985) and BoNT/A (Black and Dolly, 1986) upon electrical stimulation into hemidiaphragm preparations as well as of BoNT/A and E upon K^+ stimulation into spinal cord neurons (Keller *et al.*, 2004). Also an earlier onset of the blockade of neurotransmission on nerve stimulation upon application of BoNT/A (Hughes and Whaler, 1962) and TeNT (Schmitt *et al.*, 1981) was observed. As nerve stimulation causes increased rates of exo- and endocytosis of synaptic vesicles, one can assume that synaptic vesicle proteins, which become temporarily exposed on the cell surface at the synaptic cleft upon neurotransmitter release, are involved in the binding

and uptake of CNTs. Indeed, the synaptic vesicle membrane protein synaptotagmin (Syt)-I fulfilling these criteria (Perin *et al.*, 1990; Geppert *et al.*, 1991) was identified as BoNT/B interacting protein of rat brain synaptosomes employing cross-linking experiments (Nishiki *et al.*, 1993; Nishiki *et al.*, 1994). The current 13 isoforms of the Syt family are supposed to trigger vesicular fusion upon Ca^{2+} entry (Chapman, 2002; Südhof, 2002). The recombinant isoforms Syt-I and Syt-II incorporated in GD1a or GT1b endowed liposomes interacted *in vitro* with BoNT/B (Nishiki *et al.*, 1996b) as well as Syt-II stably expressed in CHO cells (Nishiki *et al.*, 1996a). Use of recombinant deletion mutants of Syt-II demonstrated that only the amino-terminal domain, which is extracellularly exposed upon exocytosis, plus the transmembrane region retains BoNT/B binding activity (Kozaki *et al.*, 1998). Recently, the finding that Syt-I and Syt-II mediate the entry of BoNT/B was confirmed by means of loss-of-function and gain-of-function approaches employing PC12 cells. Furthermore, results of GST-pull-down assays narrowed the BoNT/B binding segment of Syt-I and Syt-II down to the 20 juxtamembrane aa of the intravesicular domain. A peptide derived of the Syt-II interacting segment blocked uptake of BoNT/B at rat phrenic nerve-hemidiaphragm motor terminals. It also neutralized the toxicity in mice when administered together with gangliosides. Neither binding of BoNT/A and E to Syt-I and Syt-II nor their uptake were observed (Dong *et al.*, 2003).

The diverse sites of action of BoNTs and TeNT, leading to truly opposite symptoms, are caused by a different sorting of BoNTs and TeNT after their uptake into presynapses at the neuromuscular junction. While BoNTs reach the acidic endosomal compartment in the presynapse, TeNT travels in various vesicles with neutral lumen inside the axon of motoneurons towards the spinal cord (Deinhardt and Schiavo, 2005). The molecular basis of the different sorting in peripheral neurons is not established so far, but could possibly be associated with different proteinaceous receptors of TeNT and BoNTs. Recently, glycosylphosphatidylinositol-(GPI)-anchored glycoproteins associated in rafts were identified in spinal cord cells and purified motoneurons as specific receptors for the H_C-fragment of TeNT (Herreros *et al.*, 2000b; Munro *et al.*, 2001). In NGF differentiated PC12 cells the GPI-anchored glycoprotein Thy-I was determined as binding partner of TeNT H_C-fragment (Herreros *et al.*, 2001). However, the relevance of gangliosides and protein receptors for the uptake and sorting process of TeNT are not yet deciphered in detail.

Two carbohydrate binding sites in the H_{CC}-domain of Tetanus neurotoxin are required for toxicity.

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Two Carbohydrate Binding Sites in the H_{CC}-domain of Tetanus Neurotoxin are Required for Toxicity

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Tetanus neurotoxin binds *via* its carboxyl-terminal H_C-fragment selectively to neurons mediated by complex gangliosides. We investigated the lactose and sialic acid binding pockets of four recently discovered potential binding sites employing site-directed mutagenesis. Substitution of residues in the lactose binding pocket drastically decreased the binding of the H_C-fragment to immobilized gangliosides and to rat brain synaptosomes as well as the inhibitory action of recombinant full length tetanus neurotoxin on exocytosis at peripheral nerves. The conserved motif of $S^{1287}XWY^{1290}…G^{1300}$ assisted by N1219, D1222, and H1271 within the lactose binding site comprises a typical sugar binding pocket, as also present, for example, in cholera toxin. Replacement of the main residue of the sialic acid binding site, R1226, again caused a dramatic decline in binding affinity and neurotoxicity. Since the structural integrity of the H_C-fragment mutants was verified by circular dichroism and fluorescence spectroscopy, these data provide the first biochemical evidence that two carbohydrate interaction sites participate in the binding and uptake process of tetanus neurotoxin. The simultaneous binding of one ganglioside molecule to each of the two binding sites was demonstrated by mass spectroscopy studies, whereas ganglioside-mediated linkage of native tetanus neurotoxin molecules was ruled out by size exclusion chromatography. Hence, a subsequent displacement of one ganglioside by a glycoprotein receptor is discussed.

Keywords: recombinant tetanus neurotoxin; H_C-fragment; ganglioside binding site; phrenic nerve toxicity assay; MALDI-TOF mass spectroscopy

Introduction

Tetanus neurotoxin (TeNT) and the seven botulinum neurotoxins (BoNTs, serotypes A-G), the causative agents of the diseases of tetanus and botulism, are produced as ~150 kDa single chain (sc) proteins in *Clostridium tetani* and *Clostridium botulinum*. Subsequently, each toxin is cleaved by proteases into a ~100 kDa heavy chain (HC) and a ~50 kDa light chain (LC), which remain associated through a single disulfide bridge, non-covalent interactions, and an HC-derived peptide loop wrapped around the LC. Whereas the LCs act as zinc-dependent endopeptidases which exclusively hydrolyze certain members of the three soluble NSF attachment protein receptor (SNARE) families, abrogating the fusion of synaptic vesicles with the presynaptic membrane,[1–3] the HCs are the agents of specific binding to neurons, uptake

Abbreviations used: BoNT, botulinum neurotoxin; Cer, ceramide; CD, circular dichroism; CMC, critical micellar concentration; CNTs, clostridial neurotoxins; CT, cholera toxin; Gal, galactose; GD1b, Galβ3NAcGalβ4-(NAcNeuα8NAcNeuα3)Galβ4GlcβCer; Glc, glucose; GT1b, NAcNeuα3Galβ3NAcGalβ4(NAcNeuα8NAcNeuα3)-Galβ4GlcβCer; HC, heavy chain; H_C, carboxyl-terminal fragment of the HC; H_{CC} and H_{CN}, carboxyl and amino-terminal domains of the H_C-fragment, respectively; H_N, amino-terminal fragment of the HC; LC, light chain; LT-I, *E. coli* type I heat-labile enterotoxin; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MPN, mouse phrenic nerve; NAcGal, *N*-acetylgalactosamine; NAcNeu, sialic acid; scTeNT, single chain TeNT; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; SNARE, soluble NSF attachment protein receptor; TeNT, tetanus neurotoxin.

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by receptor-mediated endocytosis, and translocation of the LCs into the cytosol where the LCs gain access to their substrates.

The HC can be subdivided into two functionally distinct domains of ~50 kDa each. The largely α-helical amino-terminal half (H_N-domain) provides the translocation apparatus for the delivery of the LC from the endosome into the cytosol. This process is presumably triggered by the acidic endosomal pH. The carboxyl-terminal domain, referred to as the H_C-fragment, mediates the highly specific binding of clostridial neurotoxins (CNTs) to nerve terminals at the neuromuscular junction. While BoNTs act locally at motorneurons causing flaccid paralysis, TeNT is transported retrogradely within the axon to inhibitory neurons in the spinal cord to evoke spastic paralysis.

Isolated H_C-fragments retain full binding affinities. It is well established that CNT H_C-fragments bind gangliosides,[4–6] a class of glycosphingolipids that are found particularly in membranes of neuronal cells. Recently, the role of gangliosides was pinpointed by inhibiting their biosynthesis with fumonisin in primary spinal cord neurons; this showed an insensitivity to TeNT.[7] Similarly, NAcGal-transferase knock-out mice were reported to resist treatment with TeNT and BoNT/A, B, and E.[8] The interaction of gangliosides with CNTs was investigated for TeNT and several serotypes of BoNTs in extensive studies.[9] These studies revealed that the disialo carbohydrate structure as found in GD1b is essential for the binding of TeNT and that TeNT, BoNT/A, B, C, E, and F showed affinities in the high nM range in *in vitro* binding assays with immobilized polysialo gangliosides, whilst binding experiments of CNTs to neuronal tissue revealed much higher affinities ($K_d = 1.2$ nM). This and other discrepancies, such as protease-sensitive binding of TeNT to neurons,[10,11] resulted in a two-receptor model according to which polysialo gangliosides are considered to accumulate CNTs on the plasma membrane surface, and protein receptors to mediate specific endocytosis.[12,13] This hypothesis was supported by the demonstration of the binding of BoNT/A, B, and E to synaptotagmin II in the presence of GT1b[14–18] and the recent identification of GPI-anchored glycoproteins in neuronal rafts as specific receptors for the H_C-fragment of TeNT.[19–21]

The crystal structure analysis of the TeNT H_C-fragment identified two domains within H_C, an amino-terminal lectin-like jelly-roll domain (H_{CN}, residues 865–1110) and a carboxyl-terminal β-trefoil domain (H_{CC}, residues 1110–1315).[22,23] Deletion mutagenesis studies revealed that the TeNT H_{CC}-domain binds to gangliosides and neuronal cells even more efficiently than the complete H_C-fragment,[24] whereas no binding could be detected for the H_{CN}-domain.[25] Moreover, the H_{CC}-domain constitutes the section that mediates retrograde intra-axonal transport in chicken spinal cord cells[26] and, as demonstrated by cross-linking experiments, interacts with the GPI-anchored glycoprotein receptor.[27]

A cross-linking experiment employing [^{125}I]azido-GD1b and the TeNT H_C-fragment led to radiolabeling of H1293 in the proximity of a large cavity within the H_{CC}-domain.[28] This cavity was also favored as the ganglioside binding site in computer-aided docking studies with putative inhibitors, in which doxorubicin was identified as a competitive inhibitor affecting the binding of the TeNT H_C-fragment to liposome-integrated GT1b.[29] A recent co-crystallization of the TeNT H_C-fragment and four carbohydrate subunits of GT1b exhibited four distinct binding sites, including the one in the proximity of H1293, where lactose interacts with the residues D1222, T1270, S1287, W1289, Y1290, and G1300 (Figure 1).[30] A separate site comprising R1226 as the key residue coordinated either a molecule of sialic acid or *N*-acetylgalactosamine (NAcGal; Figure 1). Two additional sites were identified in co-crystals with galactose (Gal) or NAcGal. However, the latter two sites are unlikely to function as binding pockets for polysialo gangliosides, due to insufficient space (Gal) or a high flexibility of the carbon backbone (NAcGal).

Our study aimed to clarify the molecular interactions between the TeNT H_C-fragment residues of the proposed binding pockets and ganglioside GT1b. This is a prerequisite for a rational design of binding inhibitors to prevent the neurotoxic action following acute intoxication. We applied site-directed mutagenesis, to either sterically block binding pockets with bulky side groups, or to replace amino acid residues that appear to maintain ionic, H-bond, or ring stacking interactions. We show that two ganglioside molecules associate simultaneously with the TeNT H_C-fragment and demonstrate through binding studies and toxicity assays that both the lactose and the sialic acid binding pockets of the H_C-fragment are vital for the toxicity of TeNT.

Results and Discussion

Although complex gangliosides are well characterized as one class of receptor molecules for CNTs, little is known about their interaction with the H_C-fragments. Recent co-crystallization approaches, employing the TeNT H_C-fragment and various carbohydrate elements of gangliosides, exhibited four distinct binding sites in its carboxyl-terminal β-trefoil domain.[30] However, the functionality of these sites in *in vivo* interactions with gangliosides remained unclear. Furthermore, depending on the crystallization conditions and the resolution, X-ray structures provide only limited insights into biochemical interactions between molecules.

To examine the significance of the lactose and sialic acid binding sites of the TeNT H_C-fragment for receptor binding, we generated two classes of

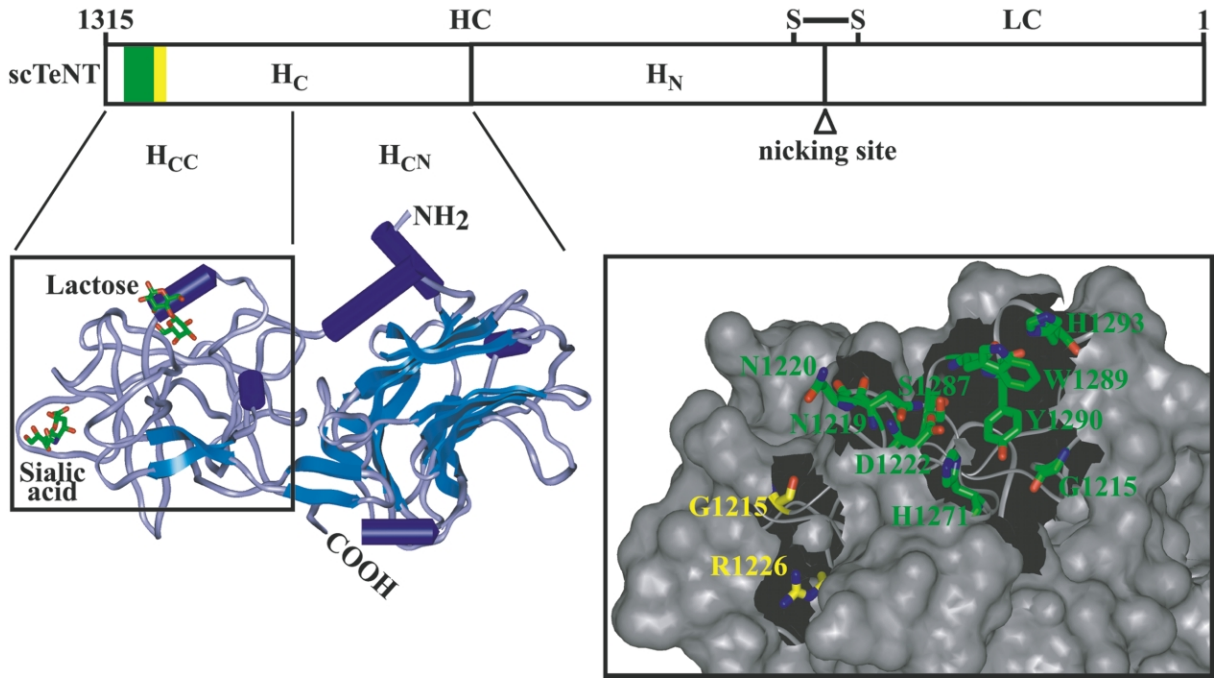

Figure 1. Upper panel: schematic drawing of TeNT and designation of its domains. The nicking site between LC and HC is depicted. Regions forming the sialic acid and lactose-binding sites are highlighted by yellow and green bars, respectively. Lower left panel: secondary structure presentation of the TeNT H_C-fragment. α-Helices are presented as dark blue cylinders, β-strands as light blue arrows, and the sialic acid and lactose bound to the H_C-fragment, as identified by co-crystallization experiments, are depicted in the stick model. Lower right panel: magnified surface plot of the H_{CC}-domain. Amino acid residues that presumably interact with sialic acid or lactose are shown in the stick model in yellow or green, respectively.

mutants. Employing molecular modeling, the first class of mutants was designed to block access to the binding cavities. G1215 was converted to phenylalanine in the sialic acid binding pocket, while in the lactose-binding site, H1271 and G1300 were replaced by tryptophan or phenylalanine, respectively. In the second approach, side groups of amino acids, presumed to be involved in ionic, H-bond, or ring-stacking interactions, were isosterically modified, so as to suspend that particular interaction without affecting intramolecular interactions.

Mutation of the lactose and sialic acid interaction sites affects the binding of the H_C-fragment to isolated GT1b and synaptosomes as well as the neurotoxicity of the full length TeNT

To assess the influence of the various mutations introduced into the H_C-fragment on the properties of TeNT, three different assays were performed. (i) The ability of the full length TeNT to poison isolated motorneurons was analyzed employing mouse phrenic nerve (MPN) toxicity tests. (ii) Binding to nerve cell membranes was studied by means of rat brain synaptosome preparations. (iii) The competency to interact with isolated ganglioside was examined by ELISA.

For MPN assays, all mutants and the wild-type TeNT were expressed in *Escherichia coli* under biosafety level 2 containment as single chain (sc) molecules fused to a carboxyl-terminal StrepTag. Recombinant wild-type scTeNT displayed 34% toxicity, as compared to native TeNT, while nicked recombinant wild-type TeNT proved to be equally potent (data not shown). Therefore, all TeNT mutants were evaluated following trypsin activation. The measured paralytic halftimes and the corresponding toxicities of the various mutants are presented in Table 1.

Synaptosome binding assays were performed employing ^{35}S-labeled H_C-fragments under physiological buffer conditions, and the amount of bound H_C-fragments was quantified by phosphor imaging. After two hours of incubation at 0 °C, 65(±9)% of the total wild-type H_C-fragment was associated with synaptosomal membranes. The binding of mutated H_C-fragments was specified as the percentage of the wild-type value (Figure 2). Binding of the radiolabeled H_C-fragments proved to be specific, since it was saturable, and the *in vitro* translated protein could be displaced by the micromolar addition of either recombinant wild-type H_C-fragment or native TeNT (data not shown).

For interaction studies with isolated gangliosides, GT1b was used, as earlier experiments on TeNT attributed the highest binding affinities to

Table 1. Biological activities of wild-type and mutated TeNT in the MPN toxicity assay

Recombinant nicked[a] TeNT	Paralytic halftime $t_{1/2}$[b] (minutes) at 65 nM	Paralytic halftime $t_{1/2}$[b] (minutes) at 327.9 nM	Toxicity *versus* wild-type TeNT[c] (%)
scTeNT wild-type	60.0 ± 7.9		34.9 ± 4.6
TeNT wild-type	43.8 ± 4.7		100[d]
Sialic acid binding site mutants			
TeNT G1215F	76.7 ± 3.5		15.6 ± 0.7
TeNT R1226F	>180	98.0 ± 15.6	1.4 ± 0.2
TeNT R1226L	>180	97.0 ± 4.2	1.4 ± 0.1
Lactose-binding site mutants			
TeNT N1219I	171.5 ± 16.3		1.1 ± 0.1
TeNT N1220I	66.7 ± 3.5		24.8 ± 1.3
TeNT D1222L	131.0 ± 5.7		2.6 ± 1.3
TeNT S1287A	>180	71.5 ± 10.6	3.9 ± 0.6
TeNT W1289G	>180	138.5 ± 13.4	0.4 ± 0.1
TeNT W1289L	>180	157.0 ± 18.4	0.3 ± 0.1
TeNT Y1290F	65.7 ± 3.2		26.0 ± 1.3
TeNT Y1290A	>180	129.5 ± 3.5	0.5 ± 0.1
TeNT H1271A	>180	139.5 ± 14.8	0.4 ± 0.1
TeNT H1271W	>180	142.0 ± 7.1	0.4 ± 0.1
TeNT G1300F	100.7 ± 4.2		6.3 ± 0.3
TeNT H1293A	138.5 ± 0.7		2.2 ± 0.1

[a] Full length scTeNT proteins were nicked between LC and HC by trypsin immobilized on agarose beads (0.002 units/μg TeNT) for one hour at 30 °C.
[b] Data are means ± S.D. ($n = 3–4$).
[c] To a five point dose-response-curve of TeNT wild-type a power function was fitted ($y = 154.18x^{-0.3014}$, $R^2 = 0.999$). For each mutant the correlative concentration of wild-type was calculated and set in ratio.
[d] Toxicity of wild-type is set to 100%.

this trisialo ganglioside.[31,32] GT1b was immobilized on microtiter plates and incubated with the various H_C-fragment mutants at low ionic strength. The amount of bound mutated protein was quantified by ELISA employing a monoclonal H_C-fragment specific antibody and expressed as the percentage of binding of wild-type H_C-fragment (Figure 3).

In the sialic acid binding pocket, the formation of a salt bridge between the carboxyl group of sialic acid and the guanidine group of R1226 is the most prominent feature. This interaction can be found in a comparable manner in both the co-crystals with sialic acid and GT1b-β.[30,33] Its importance is highlighted by the drastic drop in toxicity to 1.4% and the almost complete loss of binding in the synaptosome assay and ELISA upon mutation of R1226 to leucine or phenylalanine. Placing a bulky phenyl ring at the opening of the binding pocket, as implemented by TeNT-G1215F, reduced the toxicity to 15% in the MPN assay, the binding to 18% in the synaptosome assay and approximately to 50% in the ELISA compared to the wild-type values. The mutation interferes with H-bonding between D1214 and N1216 and sialic acid, and partially shields the central R1226 residue, according to molecular modeling analyses. These data provide the first biochemical evidence for the necessity of the sialic acid binding site to retain

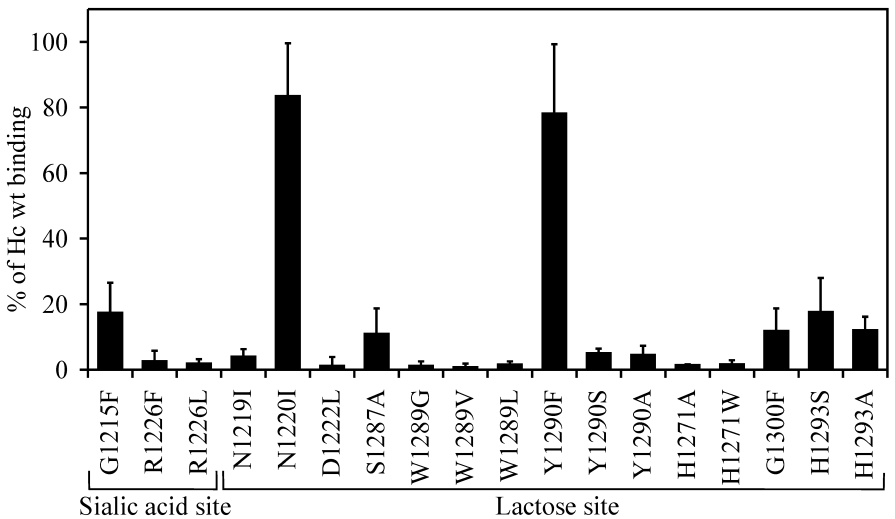

Figure 2. Binding of *in vitro* translated ^{35}S-labeled H_C-fragment mutants to rat brain synaptosomes. Binding experiments were performed in physiological buffer for two hours at 0 °C. Washed pellet fractions were subjected to SDS-PAGE and the amount of bound [^{35}S]H_C-fragment mutants was quantified by phosphor imaging and depicted as percentage of wild-type binding.

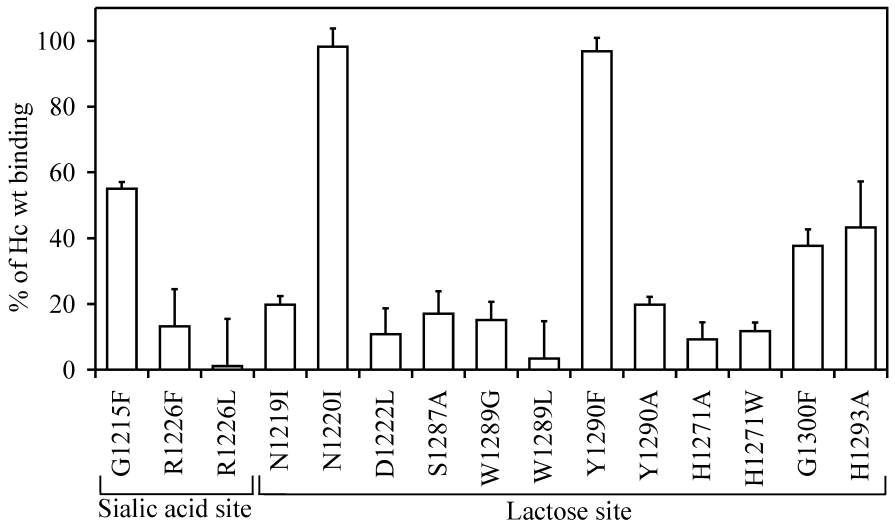

Figure 3. Binding of TeNT H_C-fragment mutants to 1 μg ganglioside GT1b immobilized on microtiter plates. The amount of bound H_C-fragments was determined, using a monoclonal TeNT H_C-fragment specific antibody, and illustrated as percentage of wild-type binding.

the neurotoxicity of TeNT. Interestingly, comparison of the sialic acid binding site of TeNT with the active sites of viral neuraminidases revealed similar structural features.[34,35]

Mutations designed to sterically block access to the lactose-binding pocket, i.e. replacement of the H1271 imidazole side group by a bulky indole, and the addition of a benzyl function in place of the G1300 hydrogen, resulted in a dramatic loss in toxicity. 0.4% and 6.3% residual toxicity were measured for H1271W and G1300F, respectively. Furthermore, those mutations also severely interfered with binding to synaptosomes and gangliosides, resulting in 2% and 12% or 7% and 38% residual binding, respectively.

The mutant TeNT-H1271A displayed barely detectable toxicity. Consistently, this elimination of the imidazole ring caused a drastic loss in the binding to synaptosomes and diminished the affinity for GT1b to less then 20%. These results indicate important interactions between sugar hydroxyl groups of GT1b and the imidazole ring, although no interactions between H1271 and lactose could be observed in the co-crystal formed with the H_C-fragment.[30] Our findings support the data of the GT1b-β/H_C-fragment co-crystallization according to which the freely rotatable imidazole ring of H1271 forms H-bonds between ND1 and O-4 and O-6 of Gal4.[33] Hence, it does not expose its π-electron system towards the pocket as does W1289, due to a coordination by the backbone carbonyl oxygen of N1272.

Mutations of D1222 to leucine and of the neighboring S1287 to alanine strongly decreased the biological activity at the MPN as well as binding to synaptosomes and immobilized GT1b. This agrees with the formation of important H-bonds, as predicted by the lactose co-crystal. In contrast, S1287 unexpectedly showed no interaction with GT1b-β in the H_C-fragment co-crystal.[33] Another important discrepancy turns up in the polar interaction between N1220 and O-3 of the Glc portion, and in the passive role of the adjacent N1219 as found in the lactose co-crystal. We determined that the mutation of N1220 to isoleucine hardly influenced the TeNT binding characteristics and only moderately affected toxicity, whereas the mutation N1219I led to a nearly inactive neurotoxin due to its low affinity to gangliosides. This result demonstrates the flexibility of the loop, including residues F1218 to D1222, which allows N1220 to move out of the binding area and N1219 to shift towards the carbohydrate binding site and corroborates the side-chain arrangement observed in the GT1b-β/H_C-fragment co-crystal.[33]

As signified by the discrepancies between our biochemical and the structural data described above, these analyses reveal that lactose as a mimicking agent for the carbohydrate structure of gangliosides is limited. In addition, the up to 890 times lower concentration of GT1b-β used for co-crystallization[33] and the lack of competition between wild-type H_C-fragment and lactose, NAcGal, or sialic acid in the range of 0.3 to 300 mM on binding to rat brain synaptosomes (data not shown) substantiate this conclusion.

The conservative exchange of Y1290 for phenylalanine yielded a TeNT with 25% toxicity and retained almost wild-type binding characteristics, whereas removal of the phenyl ring, yielding A1290, or its replacement by serine, displayed hardly detectable toxicity and only residual binding below 20% in both binding assays. These results suggest an inferior role of the hydroxyl group in ligand interaction, but emphasize the importance of the phenyl ring for the functioning of this site (see below).

The substitution of W1289, situated above Y1290, by aliphatic amino acid residues (leucine, valine, glycine) generated nearly non-toxic molecules and caused the most severe reduction in binding among all mutations at the lactose-binding pocket. Thus, W1289 plays a dominant role in ganglioside interaction at the lactose-binding site. Supposedly, its indole group, defining one wall of the binding cavity, allows a sugar ring to stack parallel with

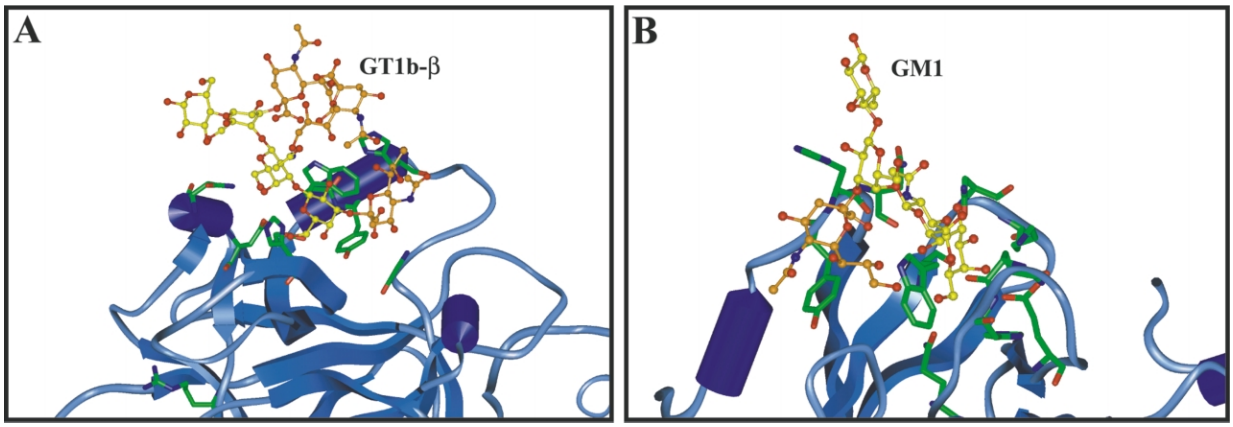

Figure 4. Co-crystal structures of TeNT and CT with gangliosides. Similar interactions are present between the characteristic tryptophan residues and the terminal galactoses of GT1b-β within the TeNT lactose binding site (A; modified from 1FV2.pdb[33]), and GM1 and the CT binding unit (B; modified from 2CHB.pdb[39]), respectively. Carbon atoms of protein residues are colored in green, of ganglioside sugars in yellow and of sialic acids in orange. The bidental binding mode of GM1 to CT additionally employs the sialic acid branch, which is sterically impossible in the case of TeNT.

the aromatic ring system. Our data approve this hydrophobic ring stacking interaction that was postulated due to a tryptophan fluorescence quench upon ganglioside binding to the corresponding site of BoNT/A,[36,37] a theoretical docking study,[29] and two co-crystallization approaches.[30,33]

Earlier cross-linking experiments employing GD1b implicated H1293 in ganglioside interaction.[28] Contrariwise, its mutation to serine or alanine did not affect the binding to motorneurons.[38] In our hands, these exchanges resulted in a significant reduction in binding to neuronal cell membranes to 18% and 12%, respectively. In line with this result, toxicity of TeNT-H1293A was drastically diminished. However, a direct involvement of H1293 in carbohydrate binding can largely be ruled out, since its imidazole ring is not a part of the binding cavity lining but is shielded by W1289 (see Figure 1). The impairment in binding affinity by removal of the imidazole group can rather be explained by the displacement of W1289, whose side group is positioned by the parallel oriented H1293 imidazole, thus by a lack of coordination *via* ring stacking interactions.

Comparison of the lactose-binding site with ganglioside binding domains of other bacterial protein toxins shows that the AB_5 toxins cholera toxin (CT) and *E. coli* type I heat-labile enterotoxin (LT-I) interact in a bidental mode with the monosialo ganglioside GM1.[39,40] Contrary to the salt bridge between the carboxyl group of NAcNeu and R1226 in TeNT, the sialic acid branch of GM1 binds *via* its *N*-acetyl group to Y12 of CT (Figure 4(B)). The terminal Gal, however, interacts with residues analogous to amino acid residues in the lactose-binding site of the TeNT H_{CC}-domain that constitute a similarly shaped second binding cavity (Figure 4). Further similarities can be found in the type-II ribosome-inactivating proteins (RIPs) ricin and ebulin, disulfide-linked heterodimers composed of an enzymatic A chain and a lectin B chain, which specifically interacts with terminal Gal in glycolipids and glycoproteins. The B chains fold as two β-trefoil domains, like that of the TeNT H_{CC}-domain, and their crystal structures revealed two binding pockets for Gal.[41,42]

Altogether, single mutations within the investigated carbohydrate binding sites generate TeNT molecules nearly lacking any binding affinity. Thus, the data clearly indicate that both the lactose and the sialic acid binding site are essential for receptor interaction and the toxicity of TeNT. This conclusion relies on analyses in three different assay systems that provided consistent results. The effects of the individual mutations measured for GT1b interaction substantially agreed with those for synaptosome binding. The impacts on binding, however, proved to be in general less pronounced compared to the results of the MPN toxicity assay, but the proportion of the effects among the individual mutations was largely maintained in all three assay systems. The stronger effects, observed in the MPN toxicity test, might be ascribed to the lower availability of receptor molecules.

Spectroscopic structure analyses of mutated H_C-fragments

To exclude the possibility that the individual mutations had affected structural elements of the H_C-fragment, far-UV CD spectra were recorded. The spectra of all mutants showed a single minimum at 208 nm, typical of proteins rich in β-sheet conformation and a low α-helical content (data not shown). The spectra of all H_C-fragment mutants, except for Y1290A, were essentially indistinguishable from the wild-type trace and secondary structure calculations, employing the methods of CONTIN, SELCON3, and CDSSTR,[43] predicted 3(±1)% α-helix, 37(±3)% β-sheet, 22(±2)% turns, and 38(±3)% random coil for wild-type TeNT H_C-fragment and the various mutants. These values are in line with the crystal structure of the TeNT H_C-fragment (5% α-helix,

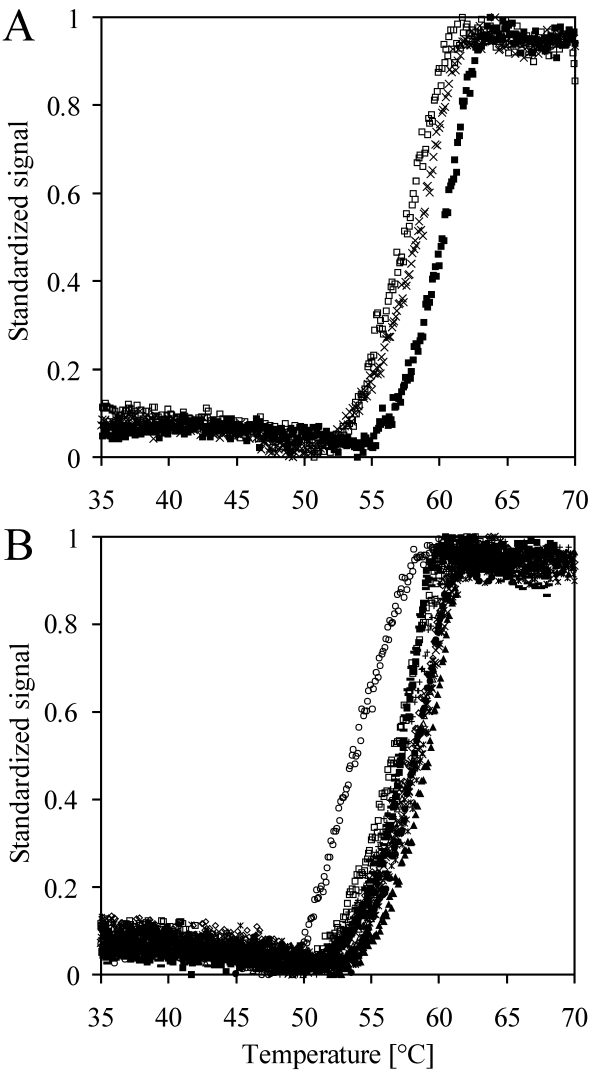

Figure 5. Temperature-induced denaturing of the wild-type TeNT H_C-fragment and various mutants. The CD signal at 220 nm was monitored at protein concentrations of 75 μg/ml and a temperature increase of 0.9 °C/minute from 25 °C to 80 °C. Analysis of mutants of the sialic acid binding site (A): H_C-wild-type (×), R1226L (■), G1215F/R1226F (□) or the lactose-binding site (B): H_C-wild-type (×), N1220I (+), N1219I/D1222L (◇), S1287A (−), W1289G (▲), W1289L (△), Y1290F (●), Y1290A (○), H1271A (■), H1271W/G1300F (□), and H1293A (∗).

35% β-sheet, 60% turns/random coil).[22] Interestingly, the β-sheet content of the Y1290A mutant was reduced by 6% while the α-helical content was doubled to 7% (see below).

To inspect the conformation of the mutated H_C-fragments in more detail, temperature-induced denaturing measurements were conducted. Changes in the molar ellipticity at 220 nm were recorded as a function of temperature. The traces are well described by steeply sigmoidal curves, which provide the melting temperatures (t_m) at their inflection points. The traces obtained for the wild-type H_C-fragment and the double mutant G1215F/R1226F were virtually identical, yielding t_m-values of 58.4 °C, and 57.6 °C, respectively. These were within the inaccuracy of about 0.8 °C for measurements of the same sample (Figure 5(A)). H_C-R1226L exhibited a slightly higher t_m-value of 60.3 °C, probably due to novel hydrophobic interactions between L1226 and P1212 as revealed by molecular modeling analysis. Spectra of the mutants of the lactose-binding site displayed an array of curves with t_m-values ranging from 56.6 °C to 59.4 °C, thus differing by less than 1.5 °C from the wild-type value (Figure 5(B)). Only the H_C-fragment mutant Y1290A showed a significantly lower melting temperature of 53.7 °C, matching the already observed overall change in secondary structure content. The lower thermostability of mutant Y1290A, compared to mutant Y1290F, can apparently be ascribed to the missing phenyl ring, which presumably causes an expansion of the 3_{10}-helix H5 (S1287 to H1293)[22] involving residues L1294 and K1295, as analyzed by molecular modeling. One consequence is a shift of the H1293 imidazole ring towards W1289, which culminates in the displacement of W1289 from the lactose-binding cavity, and consequently impairs the integrity of the local structure. Thus, the low binding affinity observed, when the phenyl ring in mutant Y1290A was removed, appears to be indirect. Therefore, we conclude in opposition to the earlier report of Sutton *et al.*[44] that the relocation of amino acid residues directly involved in ganglioside binding, for example W1289, causes the phenotype of the mutants Y1290A and Y1290S.

As a further approach to assess the structural integrity of the lactose-binding site upon mutation of the sialic acid binding site, fluorescence spectra for wild-type H_C-fragment and H_C-G1215F/R1226F alone, and in the presence of GT1b were recorded. It was previously shown for BoNT/A that the tryptophan fluorescence signal excited by light of 295 nm wavelength is quenched in the presence of GT1b in low ionic strength buffer.[36] On the basis of our above presented results, according to which W1289 constitutes a central residue in the lactose-binding site, and the fact that this strictly conserved residue is located in analogous positions in TeNT, BoNT/A, and BoNT/B, this tryptophan likely accounts for fluorescence quenching. As presented in Figure 6, GT1b quenched the tryptophan fluorescence of H_C-G1215F/R1226F to the same extent as does wild-type H_C-fragment. As expected, no quench was observed in the case of H_C-N1219I/D1222L (data not shown). Thus, these data confirm results of the thermal denaturing experiments and demonstrate that mutation of the sialic acid binding site left the lactose-binding site unharmed.

Sequence and structural homology of the binding sites throughout CNTs

In a sequence and structural alignment study within the less conserved H_{CC}-domains of TeNT and BoNT/A, B, E, F, and G (23% sequence

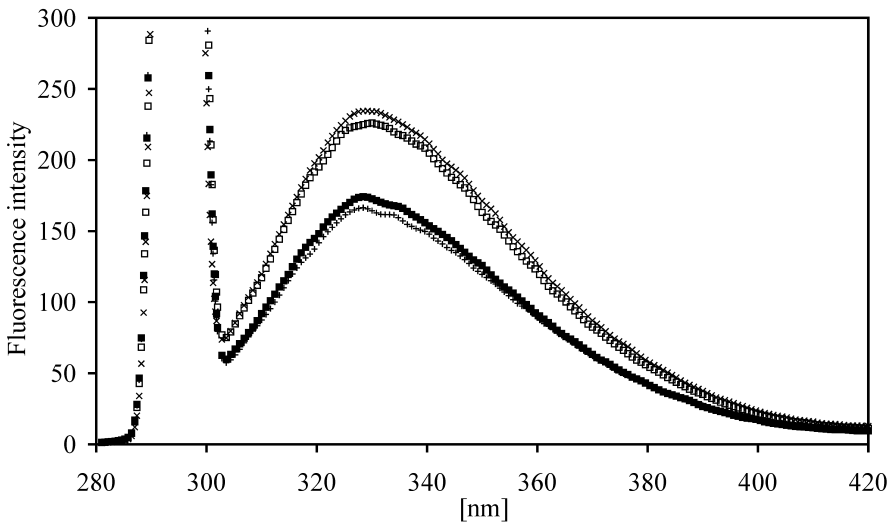

Figure 6. Tryptophan fluorescence quenching in the lactose-binding site of TeNT H_C-fragment upon GT1b binding. Spectra were recorded in the absence or the presence of tenfold molar excess of GT1b: H_C-wild-type (×), H_C-wild-type + GT1b (+), H_C-G1215F/R1226F (□), H_C-G1215F/R1226F + GT1b (■).

similarity on average), 12 structurally conserved regions, each of five residues, were identified as β-trefoil forming segments; 44 of these 60 amino acid residues are highly conserved.[45] This sequence homology was confirmed by the similar secondary structure found in the crystals of BoNT/A and B.[46,47] Moreover, employing the coordinates of BoNT/A, BoNT/B, and TeNT H_C-fragment in electrostatic surface plots, in both BoNT serotypes a cavity of similar size (~9 Å × ~9 Å) in a position analogous to the lactose-binding pocket of TeNT is formed by a homologous set of amino acid residues, i.e. G, H, S, W, Y. The "SXWY…G"-motif is strictly conserved throughout TeNT and BoNT/A, B, E, F, and G, while in BoNT/C serine and glycine switch their positions. Serotype D is exceptional in this respect, because only the central tryptophan of this motif is maintained. Altogether, the conserved lactose-binding pocket in the H_{CC}-domains is presumably essential for ganglioside binding of all serotypes. This hypothesis is supported by the co-crystallization of BoNT/B with sialyllactose, where this trisaccharide occupies the analogous lactose binding site.[47] Although here the carbohydrate binding mode seems to differ to some extent, the lactose-binding site may constitute a target for the development of an inhibitor directed against all CNTs. Knowledge of the precise carbohydrate–protein interactions is the prerequisite for a rational inhibitor design.

A search for an equivalent of the TeNT sialic acid binding site in electrostatic surface plots of BoNT/A and BoNT/B H_C-fragment did not succeed. It remains to be shown whether BoNTs possess only one ganglioside interaction site or structurally different sites adopted that function.

TeNT H_C-fragment binds two GT1b molecules

The results of the mutational analysis advised that both, the lactose and the sialic acid binding pocket of TeNT are directly involved in ganglioside binding, as also supported by co-crystals of the TeNT H_C-fragment and a synthetic GT1b-β sugar portion.[33] Although a bidental binding mode, as present in CT, can largely be ruled out due to the large distance between both binding sites (W1289 ↔ R1226: 23 Å on the surface; Figure 4), mass spectroscopy analyses were performed to definitely distinguish between binding of two ganglioside molecules and simultaneous binding of one molecule to both sites. Masses of H_C-fragment/ganglioside complexes were determined employing the MALDI-TOF principle. The measurements of the wild-type H_C-fragment, H_C-G1215F/R1226F, and H_C-N1219I/D1222L (Figure 7(A), (C) and (E)) provided m/z values closely matching their theoretical masses and were thus used for internal calibration. Analyses following pre-incubation with 20-fold molar excess of GT1b for 30 minutes at 37 °C generated extra peaks at m/z 55,863.9 Da, 55,876.6 Da, and 55,808.4 Da (decorated with * in Figure 7(B), (D) and (F)), representing a mass increase of 2180 Da, 2110 Da, and 2120 Da, respectively. These gains in m/z are in close accord with the molecular mass of GT1b of 2180 Da, indicating binding of one GT1b molecule. Furthermore, an additional peak at m/z 57,794.1 Da was observed for the wild-type H_C-fragment/GT1b mixture (highlighted by * * in Figure 7(B)). This mass shift by ~4100 Da demonstrates the simultaneous binding of two molecules of GT1b to the two carbohydrate binding sites of TeNT.

GT1b does not induce oligomerization of native TeNT

Encouraged by results of the mass spectroscopy analyses and the recent finding that addition of a synthetic GT1-β sugar portion to the mother liquor of the TeNT H_C-fragment seems to link two binding domains within different unit cells,[33] we

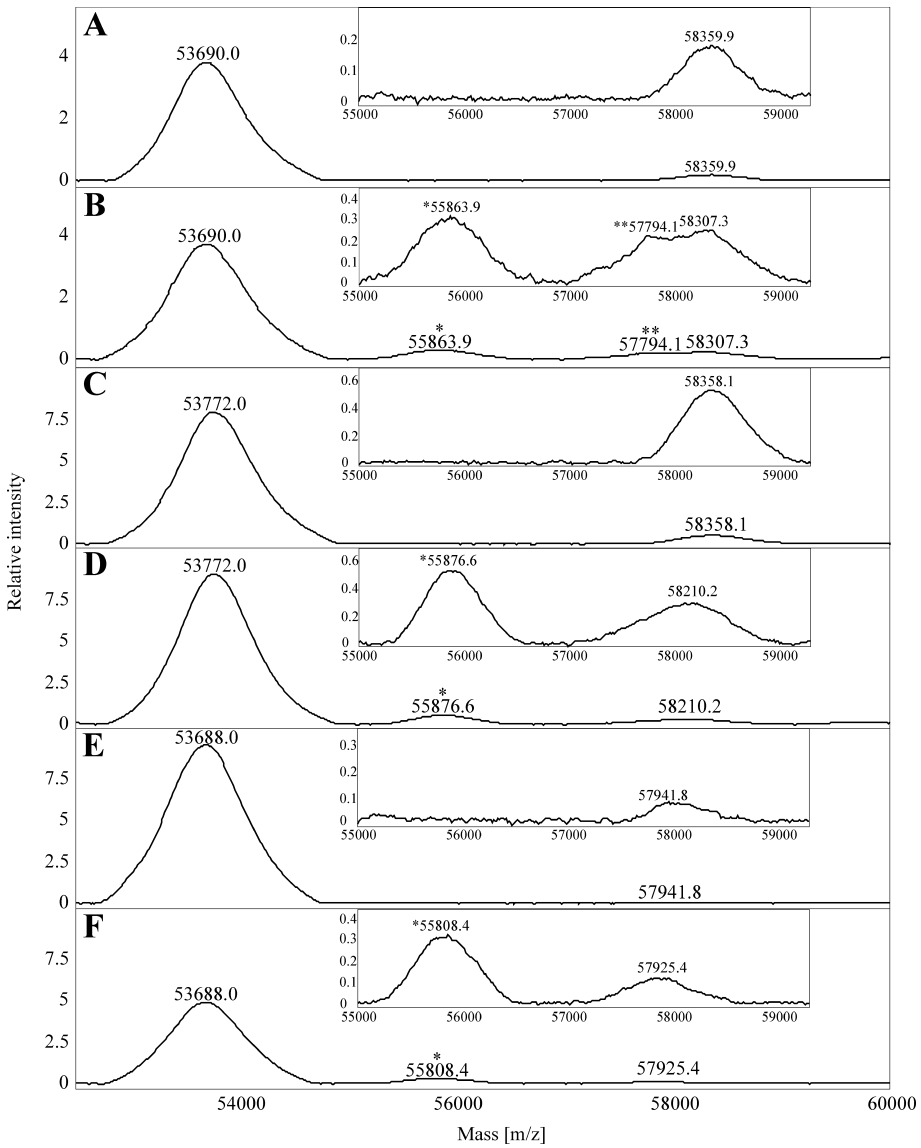

Figure 7. Binding of GT1b to wild-type and mutated H_C-fragments assessed by MALDI-TOF mass spectroscopy. Mass spectra of (A) H_C-wild-type, (C) H_C-G1215F/R1226F, and (E) H_C-N1219I/D1222L. Mass spectra of (B) H_C-wild-type, (D) H_C-G1215F/R1226F, and (F) H_C-N1219I/D1222L after incubation with 20-fold molar excess of GT1b. Insets show the range of 55,000 to 59,000 (m/z) with different scaling of the y-axis. Asterisks mark ganglioside/H_C–fragment complexes. Extra peaks in the range of 57,925 to 58,360 (m/z) represent unidentified impurities of the H_C-fragment preparations.

investigated the putative linking in solution. Native TeNT (100 μg; 1.1 μM) was incubated in the presence of various concentrations of complete GT1b (1.1 μM, 2.2 μM, 5.5 μM, 11.1 μM). Subsequent size exclusion chromatography (SEC) revealed multimer formation only when exceeding the critical micellar concentration (CMC) of GT1b (10(±5) μM;[48] data not shown). The observation of high molecular mass complexes can thus be ascribed to the binding of TeNT to developing GT1b micelles. This is in line with results of earlier ultracentrifugation experiments by Lazarovici *et al.*[49] Therefore, linking of TeNT molecules *via* complex gangliosides does apparently not occur in low ionic strength solutions.

Role of the dual ganglioside binding capacity of TeNT

A cooperation of gangliosides with a proteinaceous receptor in TeNT binding was postulated to explain findings of previous research.[12] As BoNTs conceivably do not exhibit a carbohydrate binding site equivalent to the sialic acid binding site (see above), it is tempting to speculate that this site might subsequently interact with a protein receptor. Recently, a glycosylated, GPI-anchored protein, clustered within lipid rafts, was identified as a supplementary cell surface receptor for TeNT.[20,21] In this scenario, in a first step abundant gangliosides on the neuronal cell surface would

attract TeNT, employing both of the two carbohydrate binding pockets. The association of gangliosides within lipid rafts,[40] and the co-existence of the glycoprotein receptor would then allow a displacement of the bound ganglioside in the sialic acid binding site by the carbohydrate portion of this GPI-anchored glycoprotein. This exchange may be driven by a higher affinity of TeNT for the carbohydrates of the glycoprotein. Protein–protein interactions may contribute as well. Furthermore, the association of TeNT to raft components would include lipid microdomains that are specialized in the internalization of proteins and pathogens,[50,51] allowing endocytosis and subsequent retrograde intra-axonal transport, a special feature of TeNT amongst the CNTs. Therefore, the sialic acid binding pocket may be a prerequisite for the different sorting of TeNT *versus* BoNTs proceeding in peripheral neurons upon endocytosis. Further studies are required to clarify the formation of this glycoprotein–TeNT complex.

Materials and Methods

Molecular modeling

Molecular modeling was done using an Octane workstation (Silicon Graphics, Inc.) and the Insight II 2000 software (Molecular Simulations, Inc.).

Plasmid constructions

The gene portion encoding the H_C-fragment of TeNT was fused to an oligonucleotide coding for the StrepTag and inserted into pQE3 (Qiagen) yielding the expression plasmid pAR11. Mutations in the H_C-fragment were generated by PCR, using pAR11 as template DNA and suitable primers. The PCR products were digested and inserted into the correspondingly cleaved pAR11 and the transcription vector pSP72 (Promega). Nucleotide sequences of all mutants were verified by DNA sequencing. The plasmid encoding full length wild-type TeNT with carboxyl-terminal StrepTag (pAR1) was constructed using pSP64tet97/7, pEJ6/3,[52] and pAR11. Fragments of the mutated pAR11 plasmid were transferred into pAR1, to yield the corresponding full length TeNT mutants.

Purification of recombinant proteins

Wild-type and mutated recombinant H_C-fragments and full length TeNT, fused to a carboxyl-terminal StrepTag, were produced utilizing the *E. coli* strain M15pREP4 (Qiagen) during ten hours of incubation at room temperature, and were purified on StrepTactin-Sepharose beads (IBA GmbH) following to the manufacturer's instructions. The carboxyl-terminal StrepTag was not removed, as tagged H_C-fragments and corresponding H_C-fragments with authentic carboxyl termini exhibited indistinguishable properties in our binding assays. Fractions containing the desired proteins were pooled, frozen in liquid nitrogen, and kept at −70 °C. H_C-fragments designated for CD analyses were further purified to a purity of >96%, by SEC (Superdex 200 column, Äkta purifier, Amersham Pharmacia Biotech) in Lubrol buffer (20 mM Tris–HCl buffer (pH 7.2), containing 100 mM NaCl, 0.01% Lubrol (v/v)).

Mouse phrenic nerve toxicity assay

The MPN toxicity assay was set up as described by Habermann *et al.*[53] Electrical stimulation of the phrenic nerve was continuously performed at a frequency of 1 Hz. Isometric contractions were transformed with a force transducer and recorded with the VitroDat Online software (FMI GmbH). The time required to decrease the amplitude to 50% of the starting value (paralytic halftime) was measured. Full length scTeNT or its mutants were nicked between LC and HC, applying trypsin immobilized on agarose beads (Sigma-Aldrich, 0.002 U/μg TeNT) during one hour of incubation at 30 °C. Proteolytic activation proved to be complete and no degradation of the resulting LC and HC was detectable by Coomassie blue staining for any of the mutants (data not shown). The nicked wild-type TeNT was applied in triplicate at final concentrations of 0.66, 1.97, 6.65, 19.67, and 65.57 nM, to plot a dose-response curve to which the power function $y = 154.18x^{-0.3014}$ ($R^2 = 0.999$) can be ascribed. TeNT mutants were tested in triplicate at either 65.57 nM or 327.87 nM final concentrations. The resulting paralytic halftimes were converted to the corresponding concentrations of wild-type TeNT, using the equation mentioned above. The toxicities were finally expressed as a percentage of wild-type activity.

The catalytic activity of the LC of all mutants was validated in cleavage assays employing ^{35}S-labeled rat synaptobrevin 2 and nicked TeNT at a final concentration of 0.3 μM (data not shown).

In vitro transcription and translation

H_C-fragments were synthesized *in vitro* from the linearized pSP72 derivatives, using the reticulocyte lysate system (Promega) in the presence of L-[^{35}S]methionine (555 KBq, >37 TBq/mmol; Amersham Pharmacia Biotech), in a total volume of 25 μl.

Binding assay using rat brain synaptosomes

Synaptosomes were obtained following the protocol described by Jones & Matus.[54] Brains were dissected from 150 g Wistar rats and the cerebellum, pons and medulla were discarded. The remainder of the brains was homogenized in 10 ml of 0.32 M sucrose, adjusted to pH 7.3 with 5 mM Hepes–NaOH, and the homogenate was centrifuged at 4 °C for ten minutes at 800*g* followed by centrifugation of the supernatant fraction at 12,000*g* for 15 minutes. The resultant pellet was washed once and resuspended in 2 ml of sucrose buffer. This suspension was layered on top of a discontinuous gradient consisting of 10%, 15%, and 23% Percoll and centrifuged for seven minutes at 33,000*g*. Functional synaptosomes were recovered from the 15–23% Percoll interface, carefully diluted in physiological buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 10 mM glucose, 0.5% (w/v) BSA, pH 7.4), and centrifuged at 12,000*g* for 15 minutes. The pellet fraction was resuspended in physiological buffer, with the final synaptosomal protein adjusted to a concentration of 10 mg/ml.

Binding assays were performed in triplicate, in a total volume of 100 μl of physiological buffer for two hours at 0 °C, containing a 5 μl aliquot of the translated

H_C-fragment (reaching a final concentration of ~3 pM) and 30 µl of the synaptosome suspension. For competition assays various amounts of the recombinant wild-type H_C-fragment, native TeNT, sialic acid, NAcGal, or lactose were added as well. Control samples lacking synaptosomes were treated equally. After incubation, the synaptosomes were collected by centrifugation (5000*g*; five minutes) and unbound material in the supernatant fraction was discarded. The pellet fractions were washed twice each with 50 µl of physiological buffer and boiled in SDS sample buffer. The samples were analyzed by SDS-PAGE, together with a 5 µl aliquot of translated H_C-fragment to verify efficiency of translation. Bound ^{35}S-radiolabeled H_C-fragments were visualized using a BAS-1500 phosphor imager (Fuji Photo Film), and quantified applying the Tina 2.09f software (Raytest Isotopenmeßgeräte GmbH). Amounts of bound H_C-fragments were calculated after subtraction of the control value as a percentage of the total H_C-fragment protein in the assay, and finally compared with the binding efficiency of the wild-type H_C-fragment.

Binding to ganglioside GT1b

Ganglioside GT1b (Sigma-Aldrich) was dissolved to 10 µg/ml in methanol and applied to high-affinity 96-well polystyrene microtiter plates (Costar) (100 µl/well). The solvent was evaporated at room temperature and the wells were washed three times with binding buffer (10 mM Tris–HCl, 10 mM Na_2HPO_4, 0.5% BSA, pH 7.2). Binding assays were performed in binding buffer (100 µl/well) containing 250 ng H_C-fragment for two hours at room temperature. Unbound protein was removed by three washing steps, each one with 250 µl of PBS/Tween buffer (140 mM NaCl, 7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% (v/v) Tween 20, pH 7.2). After blocking unspecific binding sites using 3% (w/v) milk powder in PBS buffer (two hours at room temperature), bound H_C-fragments were detected by sequential incubations, each of two hours at room temperature, with a monoclonal mouse anti-TeNT H_C-fragment antibody (ascites liquid, 1:100 diluted in binding buffer) and peroxidase-conjugated goat anti-mouse antibody (Jackson Immuno Research Laboratories, Inc.). 1,2-diaminobenzene (4 mM dissolved in 2% H_2O_2, 32 mM Na-citrate, 68 mM Na_2HPO_4, pH 5.5) was used as the substrate, and the extinction at 490 nm was scanned with a SpectraCount microplate photometer (Packard).

CD spectroscopy

CD data were collected with a Dichrograph III (Jobin-Yvon) spectropolarimeter equipped with a computer-controlled temperature cuvette holder. Far-UV data in the range of 200 to 250 nm were obtained with a 0.1 mm path length cuvette, containing 0.5–1.0 mg/ml protein in Lubrol buffer. CD spectra were recorded at 21 °C, at a speed of 6 nm/minute and with a response time of two seconds. Temperature-induced denaturation was performed in the same buffer at a protein concentration of 75 µg/ml, by monitoring the CD signal at 220 nm in a 4 mm path length cuvette. The temperature increase of 0.9 °C/minute from 25 °C to 80 °C was measured directly in the protein solution with a thermistor.

Tryptophan fluorescence quench measurements

Wild-type TeNT H_C-fragment, the double mutants H_C-G1215F/R1226F, and H_C-N1219I/D1222L and a combined quadruple mutant thereof were purified in binding buffer (minus BSA) by SEC. 3.6 µg of each H_C-fragment (1.1 µM) was incubated with 22 µM GT1b in binding buffer (minus BSA) for 30 minutes at 37 °C. Subsequently, tryptophan fluorescence signals were collected with a RF-2001PC spectrofluorimeter (Shimadzu) equipped with a computer-controlled temperature cuvette holder at 37 °C with an excitation wavelength of 295 nm. The fluorescence spectra were recorded in the range of 280 to 420 nm using a 0.3 mm path length cuvette.

Mass determination of TeNT H_C-fragment/GT1b complexes

Wild-type TeNT H_C-fragment and the double mutants H_C-G1215F/R1226F and H_C-N1219I/D1222L were purified in binding buffer (minus BSA) by SEC. 1.8 µg of each H_C-fragment (1.6 µM) was incubated with or without 32 µM GT1b in binding buffer (minus BSA) for 30 minutes at 37 °C. Aliquots (5 µl) of each mixture were mixed in a 1:1 ratio with either of the matrices of a saturated sinapinic acid solution or EAM-1 (Ciphergen Biosystems) in 50% acetonitrile-water containing 0.5% trifluoroacetic acid. Twice, 2 µl of each mixture were dropped on an eight-spot gold surface chip and air-dried. Data were collected by a surface enhanced laser desorption/ionization time of flight mass spectrometer (SELDI-TOF MS) PBS II (Ciphergen Biosystems) and analyzed with the Ciphergen ProteinChip software 3.0.1.

HPLC analysis of TeNT/GT1b complexes

Native TeNT was purified in binding buffer (minus BSA) by SEC (Superdex 200 column, detection 280 nm, Äkta purifier), and the fractions at a retention volume V_e of 11.8 ml, containing TeNT monomer, were collected. 100 µg of native TeNT monomer and GT1b were incubated in various molar ratios (1:0, 1:1, 1:2, 1:5, and 1:10) for one hour at 0 °C in binding buffer (minus BSA, plus 12% methanol). Again, they were subjected to SEC to check formation of TeNT/GT1b-complexes.

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The H_{CC}-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction

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Summary

Tetanus and botulinum neurotoxins selectively invade neurons following binding to complex gangliosides. Recent biochemical experiments demonstrate that two ganglioside binding sites within the tetanus neurotoxin H_C-fragment, originally identified in crystallographic studies to bind lactose or sialic acid, are required for productive binding to target cells. Here, we determine by mass spectroscopy studies that the H_C-fragment of botulinum neurotoxins A and B bind only one molecule of ganglioside GT1b. Mutations made in the presumed ganglioside binding site of botulinum neurotoxin A and B abolished the formation of these H_C-fragment/ganglioside complexes, and drastically diminished binding to neuronal membranes and isolated GT1b. Furthermore, correspondingly mutated full-length neurotoxins exhibit significantly reduced neurotoxicity, thus identifying a single ganglioside binding site within the carboxyl-terminal half of the H_C-fragment of botulinum neurotoxins A and B. These binding cavities are defined by the conserved peptide motif H…SXWY…G. The roles of tyrosine and histidine in botulinum neurotoxins A and B in ganglioside binding differ from those in the analogous tetanus neurotoxin lactose site. Hence, these findings provide valuable information for the rational design of potent botulinum neurotoxin binding inhibitors.

Introduction

Seven botulinum neurotoxins (BoNTs, serotypes A–G), produced by various strains of *Clostridium botulinum*, *C. barati* and *C. butyricum*, are the causative agents of the disease botulism. These bacterial protein toxins and the closely related tetanus neurotoxin (TeNT) synthesized by *C. tetani* are produced as ~150 kDa single chain (sc) proteins and subsequently cleaved by proteases into a ~100 kDa heavy chain (HC) and a ~50 kDa light chain (LC), which remain associated through a single disulphide bridge and non-covalent interactions. The recent elucidation of the crystal structures of BoNT/A and B (Lacy *et al.*, 1998; Swaminathan and Eswaramoorthy, 2000) illustrates that all clostridial neurotoxins (CNTs) are composed of three functionally independent domains that perform individual tasks in the multistep intoxication process.

Intoxication at the neuromuscular junction starts with specific binding to peripheral nerve endings. It is well established that this adherence involves gangliosides (van Heyningen and Miller, 1961; Simpson and Rapport, 1971; van Heyningen, 1974; Williamson *et al.*, 1999; Yowler *et al.*, 2002), a class of complex glycosphingolipids that are found particularly in membranes of neuronal cells, and the carboxyl-terminal half of the HC, the H_C-fragment (H_C). This H_C-fragment is composed of an amino-terminal (H_{CN}) lectin-like jelly roll domain and a carboxyl-terminal (H_{CC}) β-trefoil domain (Umland *et al.*, 1997; Lacy *et al.*, 1998; Knapp *et al.*, 1998; Swaminathan and Eswaramoorthy, 2000). As an isolated fragment, H_{CC} retains full binding affinity (Halpern and Loftus, 1993), whereas the H_{CN}-domain has no known function. Following cell attachment, internalization via receptor-mediated endocytosis brings the neurotoxins into the endosomal compartment. Here, the acidic environment allows the structural rearrangement and concomitant insertion of the largely α-helical amino-terminal half of the HC, the H_N-domain, into the membrane. At the same time the LC is partially unfolded (Koriazova and Montal, 2003). Upon reduction of the disulphide bond, the LC functions as a zinc dependent endopeptidase in the cytosol. CNT LCs hydrolyse specific SNARE (soluble NSF attachment protein receptor) proteins, thereby abrogating the fusion of synaptic vesicles with the presynaptic membrane (reviewed in Niemann *et al.*, 1994; Bigalke and Shoer, 2000; Schiavo *et al.*, 2000).

Intoxication by BoNTs and TeNT produces two truly opposite symptoms, flaccid paralysis caused by BoNTs

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and spastic paralysis evoked by TeNT. BoNTs act at their site of entry in synapses at the neuromuscular junction, whereas TeNT undertakes a journey within the axon of motorneurons by vesicular transport toward the spinal cord. Here, it is discharged into the synaptic cleft, binds to an inhibitory interneuron, undergoes endocytosis and translocation to the cytosol where the LC acts to finally inhibit neurotransmitter release. The molecular basis for that different sorting in peripheral neurons is so far not established.

Possibly, CNTs associate with different proteinaceous co-receptors. Indeed, a two-receptor model was postulated to account for greatly differing affinities observed for CNTs binding *in vitro* compared with binding *in vivo* as well as for the identified protease-sensitive binding of TeNT to neurons (Lazarovici and Yavin, 1986; Pierce *et al*., 1986). Binding to ganglioside occurs in the high nM range, whereas binding at neuronal tissue is more avid (K_d = 1.2 nM). In the two-receptor model, polysialogangliosides are considered to accumulate CNTs on the plasma membrane surface, and protein receptors are supposed to mediate specific endocytosis (Montecucco, 1986; Niemann, 1991). Later, synaptotagmin II was shown to be involved in the binding of BoNT/B (Nishiki *et al*., 1994, 1996a, b; Kozaki *et al*., 1998; Dong *et al*., 2003), and glycosylphosphatidylinositol-(GPI)-anchored glycoproteins in neuronal rafts were recently identified as specific receptors for the H_C-fragment of TeNT (Herreros *et al*., 2000a, 2001; Munro *et al*., 2001). Their binding site, however, and the relevance of gangliosides and protein receptors for the uptake and sorting process, beyond the fact that the H_{CC}-domain of TeNT interacts with a GPI-anchored glycoprotein (Herreros *et al*., 2000b) and mediates retrograde intra-axonal transport in chicken spinal cord cells (Rummel *et al*., 2001), are not ultimately deciphered. Also, it is unclear at present, whether the recently discovered involvement of two ganglioside binding sites in the intoxication process of TeNT is a peculiarity of this neurotoxin and whether it relates to its retrograde intra-axonal transport.

Both binding sites on TeNT, originally identified by their ability to bind lactose or sialic acid, have recently been localized by co-crystallization approaches and biochemical experiments employing TeNT mutants (Emsley *et al*., 2000; Fotinou *et al*., 2001; Rummel *et al*., 2003). The lactose binding site is characterized by the presence of the peptide motif H…SXWY…G. This motif is conserved among the majority of BoNTs (Fig. 1), and crystallographic data available on BoNT/A and B (Lacy *et al*., 1998; Swaminathan and Eswaramoorthy, 2000) revealed a structural resemblance of these segments with the lactose binding pocket of TeNT (Fig. 2). In addition, results

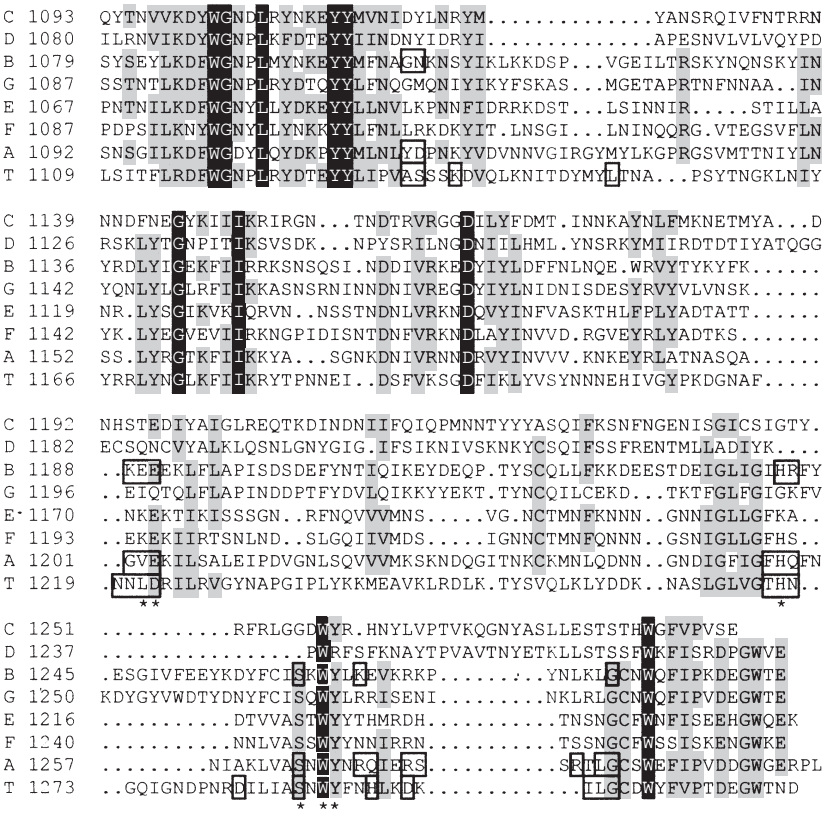

Fig. 1. Amino acid sequence alignment of TeNT (T) and BoNT (A–G) H_{CC}-fragments. Identical amino acids are presented in white letters on black background. Amino acids conserved in the majority of sequences are shaded grey, and those known to build the lactose and sialyllactose binding pockets of TeNT and BoNT/B, respectively, as well as the proposed equivalent amino acids in the structure of BoNT/A are boxed. Positions of amino acids of BoNT/A or B selected for mutational analyses are highlighted by asterisks below the sequence of TeNT.

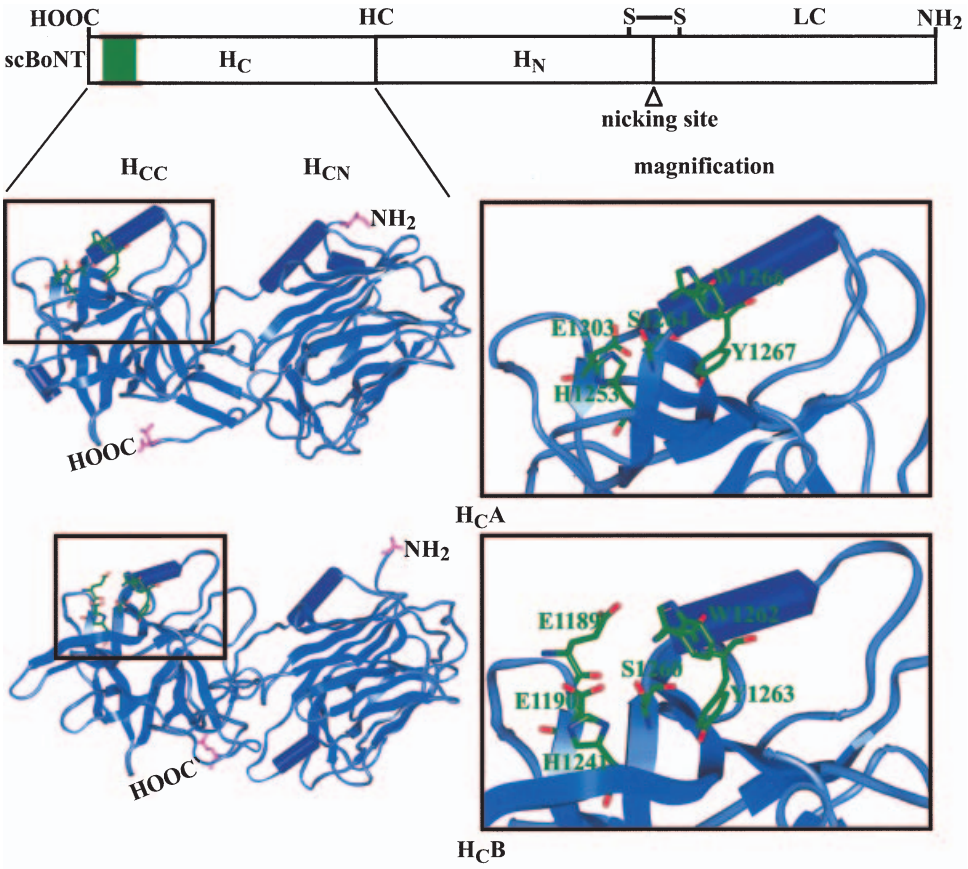

Fig. 2. Upper panel: schematic drawing of BoNT and designation of its domains. The nicking site between LC and HC is depicted. The region forming the putative ganglioside binding site is highlighted by a green bar. Middle left and lower left panels show secondary structure presentations of BoNT/A and B H_C-fragments, respectively. α-Helices are presented as dark blue cylinders, β-strands as light blue arrows, and residues subject of mutational analyses in this study and proposed to be involved in ganglioside binding are depicted in the stick model. Middle and lower right panels: Magnified illustration of the area framed in left panels.

of a co-crystallization study using the trisaccharide sialyllactose suggest this site as ganglioside binding site in BoNT/B (Swaminathan and Eswaramoorthy, 2000). No additional information is available on ganglioside interaction with BoNTs on the molecular level.

The goal of the present study is to identify similarities and distinctions in ganglioside binding among TeNT and BoNTs. We applied mass spectroscopy (MS) combined with the use of site specific mutants to determine the number and position of ganglioside binding sites in BoNT/A and B. In addition, a mutational analysis study was conducted to assess the contribution of various residues within identified binding pockets of BoNTs to ganglioside interaction via binding experiments and toxicity assays.

Results

A single molecule of ganglioside GT1b binds to H_CA or H_CB

Results of recent analyses employing MS and site directed mutagenesis demonstrated that two ganglioside molecules can bind simultaneously to the TeNT H_C-fragment and that both binding sites are required for the establishment of toxicity (Rummel *et al.*, 2003). To examine whether the poisoning process of the closely related BoNTs also involves the binding of more than one ganglioside molecule, we assembled H_C-fragment/GT1b complexes and determined their molecular weight by matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) MS. The trisialoganglioside GT1b was chosen for complex formation, as earlier experiments on BoNT/A and B attributed highest binding affinities to this compound (Kitamura *et al.*, 1980; Nishiki *et al.*, 1996b; Yowler *et al.*, 2002). Measurements of wild-type H_C-fragment of BoNT/A and B provided m/*z*-values closely matching their theoretical masses and were thus used for internal calibration (Fig. 3). Analyses of H_C-fragment/GT1b mixtures resulted in the detection of extra peaks with m/*z*-values of 53 876.6 Da, and 56 269.3 Da (indicated with * in Fig. 3B and D), representing mass increases of 2000 Da and 2129 Da respectively. Gains in m/z are in close accord

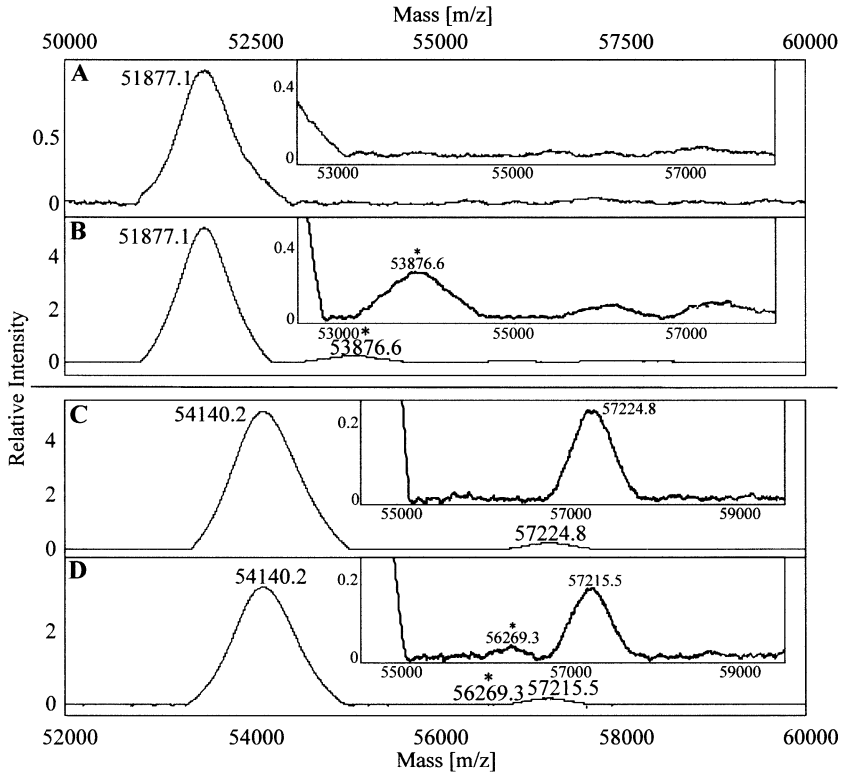

Fig. 3. Binding of GT1b to wild-type H_C-fragments of BoNT/A and B assessed by MALDI-TOF MS. Mass spectra of A and C show wild-type H_CA and H_CB. B and D represent wild-type H_CA and H_CB, respectively, after incubation with 20-fold molar excess of GT1b. Insets show the range of 53 000–57 000 (m/z; A, B) or 55 000–59 000 (m/z; C, D) with different scaling of the *y*-axis. Asterisks mark H_C-fragment/ganglioside complexes. Extra peaks in the range of 57 220 (m/z) represent unidentified impurities of the H_CB preparation.

with the molecular mass of GT1b (2180 Da) and thus provide evidence for the binding of one GT1b molecule to each H_C-fragment.

The sialyllactose interaction site in H_CB and its counterpart in H_CA are the sole sites that mediate binding to GT1b

In order to localize the sites involved in ganglioside binding within the H_C-fragment of BoNT/A and B, we analysed mutated H_C-fragments. It is known from previous experiments on TeNT that replacement of W1289 by leucine disables the lactose binding pocket (Rummel *et al.*, 2003). Alignment of the primary sequences of the CNT H_{CC}-domains indicates that W1266 and W1262 in BoNT/A and B, respectively, are homologous to TeNT W1289 (Fig. 1). We thus constructed mutants with a single amino acid change, H_CA-W1266L and H_CB-W1262L, and determined molecular weights of the mutants alone and after 30 min of incubation with GT1b. Both mutants did not form H_C-fragment/GT1b complexes (Fig. 4). This study demonstrates that only single sites are participating in carbohydrate binding. Amino acids surrounding W1262 in BoNT/B have been previously identified as a binding site for sialyllactose (Swaminathan and Eswaramoorthy, 2000) and we recognized an equivalent cavity in BoNT/A employing molecular modelling. We therefore conclude that BoNT/A and B each possess only one carbohydrate binding site, which is located in their H_{CC}-domain and is apparently homologous to the lactose binding site of TeNT.

Recombinant H_CA or H_CB and the corresponding native full-length neurotoxins exhibit similar binding properties

As it cannot be excluded that recombinant H_C-fragments feature binding characteristics differing from the full-length neurotoxins, we examined, whether they competed with similar efficacy for the binding of radiolabelled BoNTs as the corresponding native full-length neurotoxins. Figure 5 shows that unlabelled recombinant H_CB and native BoNT/B displaced with virtually identical potency the adherence of ^{125}I-BoNT/B to immobilized GT1b. Recombinant H_CA and native BoNT/A exhibit very similar efficacy in competing the binding of ^{125}I-BoNT/A to GT1b (Fig. 5). Both H_CA and H_CB competed with similar efficacy like the corresponding native neurotoxins the attachment of ^{125}I-BoNT to synaptosomal membrane preparations, too (data not shown). In fact, recombinant H_C-fragments obviously reflect the binding capacity of the corresponding native full-length BoNTs.

The affinity of H_CT to immobilized GT1b clearly exceeds that of H_CA and H_CB

In order to determine if the lack of the counterpart to the

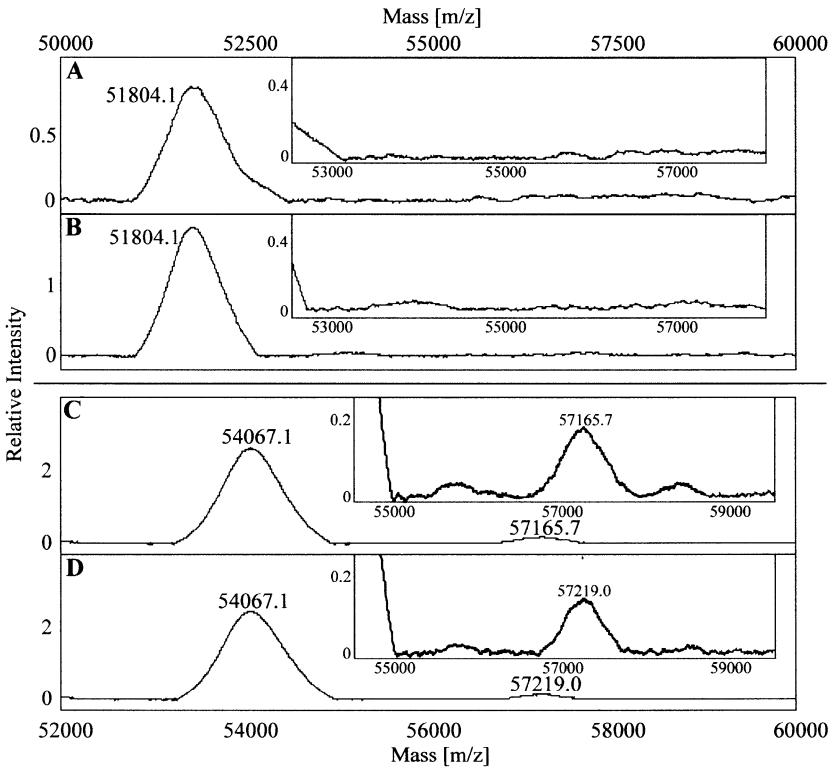

Fig. 4. Binding of GT1b to mutated H_C-fragments of BoNT/A and B assessed by MALDI-TOF MS. Mass spectra of (A) H_CA-W1266L, and (C) H_CB-W1262L. Mass spectra of B and D represent H_CA-W1266L and H_CB-W1262L, respectively, after incubation with 20-fold molar excess of GT1b. Insets show the range of 53000–57000 (m/z; A, B) or 55000–59000 (m/z; C, D) with different scaling of the *y*-axis. The peaks around of 57200 (m/z) represent an unidentified impurity of the H_CB-W1262L preparation. Note that no extra peaks were detectable upon incubation with ganglioside.

TeNT sialic acid binding pocket in BoNTs leads to reduced affinity in ganglioside interactions, we performed a comparative binding study. H_C fusion proteins, carrying carboxyl-terminal StrepTag motifs, were used to directly compare H_C-fragment binding affinities. H_CT exhibited an order of magnitude higher affinity compared to H_CA and H_CB (Fig. 6). The use of H_CT-R1226L, a mutant in which the second ganglioside binding site of H_CT was eliminated without affecting the structural integrity of the molecule (Rummel *et al.*, 2003), allowed us to obtain information on the affinity of the TeNT lactose binding pocket. The affinity of H_CT-R1226L to GT1b was only slightly lower than the affinity of unaltered H_CA or H_CB (Fig. 6). This result was confirmed by experiments employing synaptosomal membrane preparations. Again H_CT exhibited the highest affinity and H_CT-R1226L a slightly lower affinity compared to

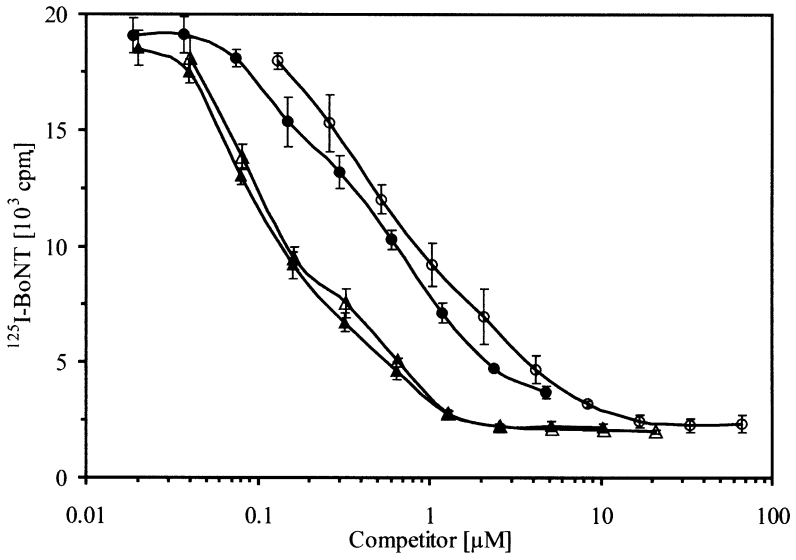

Fig. 5. Characterization of binding properties of recombinant BoNT H_C-fragments. The binding of ^{125}I-radiolabelled native BoNT/A or BoNT/B to ganglioside GT1b immobilized on microtitre plates was studied in the presence of various concentrations of unlabelled native neurotoxin or unlabelled recombinant H_C-fragment. ^{125}I-BoNT/A plus BoNT/A (●), ^{125}I-BoNT/A plus H_CA (○), ^{125}I-BoNT/B plus BoNT/B (▲), ^{125}I-BoNT/B plus H_CB (Δ). Each data point represents the mean ± S.D. of three separate experiments each performed in duplicate. H_CA and H_CB displace ^{125}I-neurotoxins with similar efficiency as the respective native full-length BoNTs.

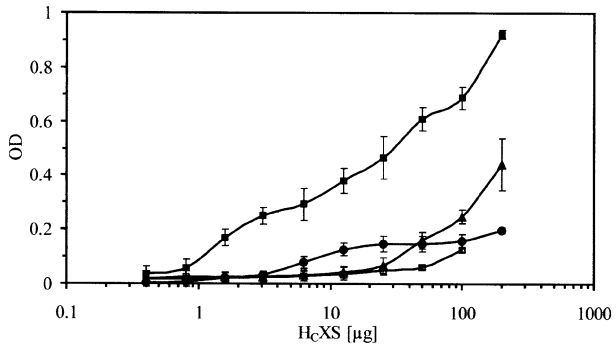

Fig. 6. Comparative analysis of H_CT (■), H_CT-R1226L (□), H_CA (●), and H_CB (▲) binding to GT1b. 1 µg of ganglioside GT1b was immobilized on microtitre plates and binding of H_C-fragments was studied in low ionic strength buffer. Various amounts of bound H_C-fragment were applied and the bound portions were detected using StrepTactin conjugated alkaline phosphatase. This reagent recognizes the StrepTag peptide motif fused in an identical manner to the carboxyl-terminus of each of the H_C-fragments, allowing the comparative assessment of binding.

H_CA and H_CB (data not shown; Rummel *et al*., 2003). Thus, we conclude that the existence of a single ganglioside binding site in BoNTs explains their reduced affinity to immobilized purified GT1b compared to H_CT which has previously been shown to include two binding sites (Rummel *et al*., 2003).

Tyrosine and histidine in the binding pocket of H_CA and H_CB play a different role than the homologous residues in the TeNT H_C-fragment

To assess the role of particular amino acids shaping the binding pockets in interaction with ganglioside, we performed a mutational analysis of the sialyllactose binding site of BoNT/B and the corresponding binding pocket of BoNT/A. Amino acids homologous to those of the lactose binding site of TeNT were mutated. They are depicted in their spatial configuration in Fig. 2. The effect of the various mutations on the properties of BoNT were measured employing three different assays. First, the ability of the mutated full-length BoNTs to inhibit exocytosis at motoneurons was analysed employing the mouse phrenic nerve (MPN) toxicity test. The measured paralytic halftimes and the corresponding toxicities of the various mutants are presented in Table 1. The MPN toxicity assay provides exact data especially for mutants with low affinity. This may presumably relate to the lower availability of receptor molecules at the MPN preparations and was experienced earlier for mutants of TeNT as well (Rummel *et al*., 2003). Second, the competency of mutated H_C-fragments to bind to nerve cell membranes was studied employing rat brain synaptosome preparations (Fig. 7). Binding of mutated radiolabelled H_C-fragments to synaptosomal membranes proved to be specific, as it was saturable, and the micromolar addition of recombinant wild-type H_C-fragment displaced radiolabelled molecules (data not shown). Third, the ability to adhere to isolated ganglioside was measured by a microtitre well assay using immobilized GT1b (Fig. 8). The effects of the individual mutations on GT1b interaction, synaptosomal membrane binding, and in MPN toxicity are strongly correlated.

Mutations designed to sterically block access to the binding pocket, like the replacement of the imidazole side

Table 1. Activity of wild-type and mutated BoNT/A and B in the MPN toxicity assay.

Recombinant scBoNT	Paralytic halftime $t_{1/2}$[a] *min* 661.8 pM	Paralytic halftime $t_{1/2}$[a] *min*	% Toxicity versus wild-type scBoNT[b]
scBoNT/A wild type	35.5 ± 5.0		100[c]
scBoNT/A E1203L	55.0 ± 2.8		17.4 ± 0.9
scBoNT/A S1264A	39.0 ± 1.4		63.3 ± 2.3
scBoNT/A W1266L	126.5 ± 7.8		0.7 ± 0.1
scBoNT/A Y1267F	101.5 ± 12.0		1.7 ± 0.2
scBoNT/A Y1267S	185.5 ± 6.4		0.2 ± 0.1
scBoNT/A H1253A	49.5 ± 6.4		25.9 ± 3.3
scBoNT/A H1253W	n.d.[d]		
	1954 pM	6515 pM	
scBoNT/B wild type	53.5 ± 4.9		100[c]
scBoNT/B E1189L	77.0 ± 1.4		27.5 ± 1.4
scBoNT/B E1190L	>180	160.0 ± 2.8	0.5 ± 0.1
scBoNT/B S1260A	>180	138.0 ± 1.4	0.9 ± 0.1
scBoNT/B W1262L	>180	179.5 ± 3.5	0.3 ± 0.1
scBoNT/B Y1263F	154.5 ± 2.1		2.0 ± 0.1
scBoNT/B Y1263S	>180	174.0 ± 8.5	0.4 ± 0.1
scBoNT/B H1241A	64.0 ± 5.7		28.0 ± 3.3
scBoNT/B H1241W	>180	175.5 ± 15.7	0.4 ± 0.1

a. Data are means ± S.D. (n = 3–4).
b. To a five point concentration-response-curve each of wild-type scBoNT/A and B a power function was fitted (y (A) = $198.94x^{-0.2683}$, R^2 = 0.989; y (B) = $406.03x^{-0.2645}$, R^2 = 0.988). For each mutant the correlative concentration of wild type was calculated and set in ratio.
c. Toxicity of wild type is set to 100%.
d. Not determined.

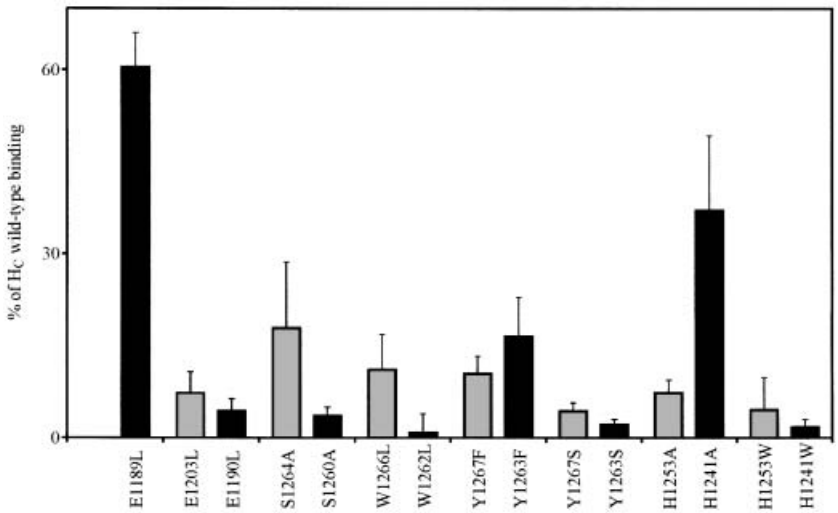

Fig. 7. Binding of *in vitro* translated ^{35}S-labelled H_C-fragment mutants to rat brain synaptosomes. Binding experiments were performed in physiological buffer. Incubation was done for 2 h at 0°C. Washed pellet fractions were subjected to SDS–PAGE, and the amount of bound [^{35}S]-H_C-fragment mutants was quantified by phosphor imaging and depicted as a percentage of wild-type binding. Black and grey columns refer to mutants of H_CB and H_CA respectively. Values for analogous H_C-fragment mutants of the two serotypes are shown side by side. Data represent mean values ± S.D. of three independent experiments each performed in triplicates.

group of H1253 in BoNT/A and H1241 in BoNT/B by the bulky indole of tryptophan, consistently cause drastic drops in binding affinity of H_CA and H_CB and in the toxicity of BoNT/B. Recently it has been shown that the analogous mutant of TeNT also lost binding affinity and neurotoxicity, suggesting a similar overall geometry of the binding pocket in TeNT and BoNTs (Rummel *et al*., 2003).

A strong effect on binding and toxicity is consistently attained for both of the BoNTs by the substitution of the indole ring of W1266 (W1262 in BoNT/B) by 2-methyl-1-propyl and the removal of the phenyl ring of Y1267 (Y1263 in BoNT/B). Aliphatic replacement of the carboxyl group of E1203 (E1190 in BoNT/B) and elimination of the hydroxyl group of S1264 (S1260 in BoNT/B) severely decrease binding affinity to GT1b and neuronal cell membranes. In contrast, the mutation E1189L in BoNT/B has only a minor effect on the functionality of the molecule, as was found for mutation of the amino acid occupying the corresponding position in TeNT, N1220 (Rummel *et al*., 2003).

Interestingly, when binding pocket mutants of the BoNTs and TeNT are compared, there are two remarkable differences regarding interaction with gangliosides. The hydroxyl group of TeNT Y1290 does not significantly contribute to binding (Rummel *et al*., 2003), whereas the mutation of the amino acids in BoNT/A and B diminishes binding to synaptosomal membranes to 10.5% and 16.5% of the wild-type binding, respectively, and toxicity to less than 2%. The second difference occurs at the imidazole ring of BoNT/B H1241 (H1253 in BoNT/A). In case of BoNT/B, adherence to GT1b and synaptosomal membranes was only reduced by ~ 50%, and BoNT/B H1241A also has a high residual toxicity. In contrast, the corresponding mutation of TeNT, H1271A, reduced the binding affinity 10-fold and led to a nearly inactive molecule in MPN toxicity assays. Thus, it appears that some of the

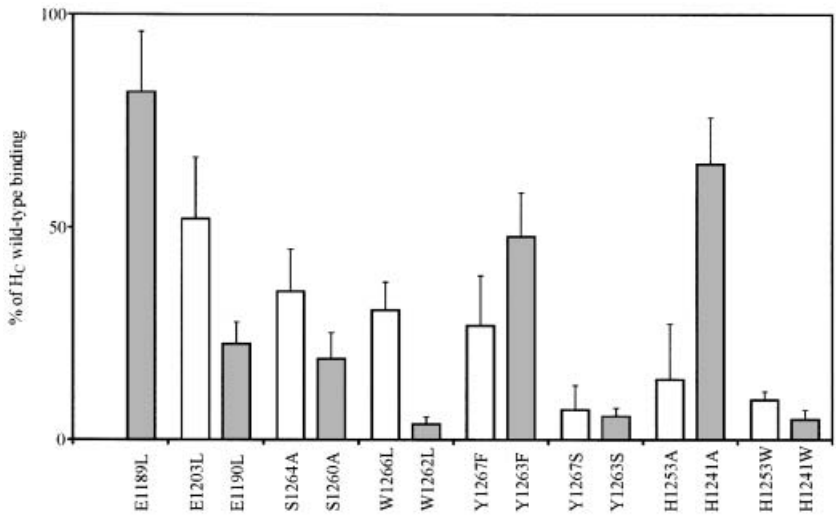

Fig. 8. Binding of BoNT/A and B H_C-fragment mutants to 1 μg ganglioside GT1b immobilized on microtitre plates in low ionic strength buffer. The amount of bound H_C-fragments was determined using a StrepTag specific antibody, and illustrated as percentage of wild-type binding. Filled and open columns refer to mutants of H_CB and H_CA respectively. Values for analogous H_C-fragment mutants of the two serotypes are juxtaposed. Data represent mean values ± S.D. of three to four independent experiments performed in triplicates.

amino acids within the TeNT lactose binding pocket interact with ganglioside differently than the corresponding residues within the BoNT pockets.

Spectroscopic structure analyses of mutated BoNT H_C-fragments

To exclude the possibility that the individual mutations had affected structural elements of the H_C-fragment, temperature-induced denaturing measurements employing circular dichroism (CD) spectroscopy were conducted. Changes in the molar ellipticity at 220 nm were recorded as a function of temperature. The scans of the wild-type H_CA and its mutants yielded steeply sigmoidal curves (data not shown). Their melting temperatures (T_m), defined by the inflection point, were between 48.3°C and 49.2°C, virtually identical with a ±0.5°C accuracy for measurements. Hence, neither of the introduced mutations impaired the structure of H_CA. Corresponding analyses of the structural integrity of mutants of H_CB revealed two-phase melting curves (data not shown). A contingent of the protein melted between 44.5°C and 49.7°C. The remaining structure denatured at 60.9–63.0°C. Stepwise melting was not observed for the H_C-fragment mutants of BoNT/A as described above nor of TeNT (Rummel *et al*., 2003). Moreover, we found some variation in the ratio of secondary structure loss for both phases for the same H_CB mutants among different preparations, whereas their T_m-values kept constant. Because we experienced a dependency on the protein concentration, both effects may perhaps be ascribed to partial aggregation of molecules. If so, the first phase could correspond to melting of H_C-fragment monomers and the second to denaturing of aggregates. Focussing on the first phase, one mutant, H_CB-Y1263S, exhibited a T_m-value of 41.0°C for the first unfolding phase, being more than 5°C lower compared to the average T_m-value of all other mutants, whereas T_m of the second unfolding phase did not diverge significantly. Thus, Y1263 of BoNT/B appears to be critical for the structural integrity of H_CB, as was also found earlier for the corresponding residue of TeNT (Y1290; Rummel *et al*., 2003). The effect of this mutation on the activity of the molecule must in part be attributed to local rearrangements in the sialyllactose binding site. In contrast, the phenyl ring of Y1267 of H_CA likely contributes directly to carbohydrate coordination, as its elimination did not affect maintenance of the structure while drastically diminishing binding and neurotoxicity.

Discussion

Ganglioside binding capacity of BoNTs

In the present study we provide evidence that BoNTs unlike TeNT exhibit only a single ganglioside interaction site in their H_{CC}-domain. Analyses of H_C-fragment/GT1b mixtures by MS revealed that mutation of the sialyllactose binding pocket of H_CB and its counterpart in H_CA abrogated their ability to form complexes with GT1b. Circular dichroism measurements indicate that loss of binding was not caused by structural alterations imposed by the mutation. In contrast, experiments with the corresponding mutant of TeNT, performed under identical experimental conditions, demonstrated a second binding site. The sialic acid binding pocket in TeNT showed a sufficiently high affinity to be identified by MALDI-TOF MS (Rummel *et al*., 2003). It is unlikely that a second carbohydrate binding site was not detectable due to the preferred interaction of BoNT-H_C with a different ganglioside species, because GT1b has been found to be the primary glycosphingolipid receptor for BoNTs (Kitamura *et al*., 1980; Schengrund *et al*., 1991; Lalli *et al*., 1999; Yowler *et al*., 2002).

Further support for the lack of a second ganglioside binding site in BoNTs was provided by comparative binding experiments employing H_CT, H_CA and H_CB. Wild-type H_CT showed approximately one order of magnitude higher affinity to immobilized GT1b, whereas the mutant H_CT-R1226L, carrying a loss of function mutation in the sialic acid binding site, bound via its intact lactose binding site with an affinity slightly lower than that of H_CA and H_CB to GT1b. In keeping with these findings are our results of electrostatic surface plot inspections, which showed that BoNT/A neither features a binding cavity at the position of the TeNT sialic acid binding site nor has the capacity of binding carbohydrate units at any other location within H_C. In case of BoNT/B, mutation of E1191 and K1192, residues located in positions comparable to the sialic acid binding pocket in TeNT, did not cause changes in binding characteristics (data not shown). It is thus unlikely that this area had escaped detection as a ganglioside binding site in MALDI-TOF MS studies as a result of too low affinity.

Contribution of individual amino acids to GT1b binding

Our study provides evidence that the three CNTs investigated so far achieve coordination of the carbohydrate ligand in an unique manner. Mutation of the tryptophan and serine residues and removal of the phenyl group of tyrosines (see Figs 1 and 2) dramatically affected the functionality of all three neurotoxins. This was not surprising, as these amino acids are conserved among the binding pocket shaping residues in CNTs (Fig. 1). Tryptophan is essential, forming strong ring stacking interactions with the hydrophobic side of one sugar ring, in maintaining the function of the ganglioside pocket. D1222 of TeNT represents apparently the counterpart of the glutamates 1203 and 1190 of BoNT/A and B respectively. Isosteric mutation of these amino acids to those with aliphatic side groups,

in all cases, strongly decreases ganglioside binding of H_C. This finding that D1222, E1203 and E1190 are key amino acids in the binding of sugars is consistent with the documented H–bond interactions found in the H_CT/GT1b-β and BoNT/B/sialyllactose co-crystal structures (Swaminathan and Eswaramoorthy, 2000; Fotinou *et al*., 2001). N1219 of TeNT interacts in addition to D1222 with the distal (NAcGal) of the two coordinated sugar units in the lactose binding site (Fotinou *et al*., 2001; Rummel *et al*., 2003). Similarly, E1189 in the BoNT/B structure was reported to shape an H-bond to O-2 of the distal sugar unit (Gal; Swaminathan and Eswaramoorthy, 2000). However, the mutation of E1189 did not interfere much with binding and only moderately with toxicity of the full-length neurotoxin. Thus, we propose that E1189 in BoNT/B rather corresponds to N1220 of TeNT, whose side group according to mutational analyses and the H_CT-GT1b-β co-crystal structure points away from the binding cavity while in contrast the amide side group of the adjacent N1219 in TeNT is directed toward the ligand. It remains to be shown, whether K1188 of BoNT/B can fulfil the role of N1219 in TeNT.

A discrepancy shown in binding experiments among TeNT and BoNTs pertains the role of tyrosine and histidine. Where the hydroxyl group of tyrosine appears to be redundant in H_CT, its removal critically decreases ganglioside affinities of H_CA and H_CB. On the other hand, the impact of the histidine on the functionality of the BoNT binding pocket is less important compared to TeNT. Because 3D-superimposition of C-α atoms of residues, constituting this binding pocket, revealed a very similar arrangement of the carbon backbone as well as of most of the side chains in TeNT, BoNT/A, and B (data not shown), the different roles of tyrosine and histidine have to be ascribed to different interactions with carbohydrate ligands. This conclusion is consistent with the recently attained co-crystal structures of GT1b-β and sialyllactose bound to TeNT-H_C and BoNT/B respectively. The BoNT/B binding pocket and its analogue in TeNT coordinate different portions of the ganglioside carbohydrate structure (Swaminathan and Eswaramoorthy, 2000; Fotinou *et al*., 2001). The terminal Gal-NAcGal disaccharide of the ganglioside sugar backbone was found to occupy the TeNT binding pocket, whereas a NAcNeu-Gal segment binds to BoNT/B. Interestingly, the protruding glyceryl side group of NAcNeu, that is not present in Gal, forms two H–bond interactions with the hydroxyl group of Y1263, which may therefore explain the prominent role of the tyrosine hydroxyl group in BoNTs. In contrast, nitrogen ND-1 of H1241 in BoNT/B keeps only one H–bond interaction with the O-10 of the glyceryl group of NAcNeu whereas H1271 in TeNT forms simultaneously three H-bonds to different oxygen atoms (O-4, O-5 and O-6) of the terminal Gal (Fotinou *et al*., 2001). This explains the moderate influence on ganglioside binding upon removal of the H1241 imidazole ring in BoNT/B.

Hence, only the side groups of the aspartic/glutamic acid and the strictly conserved tryptophan and serine in the ganglioside binding pockets of TeNT, BoNT/A and B similarly interact with common sugar structures of the NAcNeu-Gal and Gal-NAcGal disaccharide portions of GT1b.

The different binding mode does not compensate the lack of a second binding pocket in BoNTs

Although TeNT and BoNTs exhibit similar affinities to nerve cell membranes, different binding affinities to gangliosides were observed (Bakry *et al*., 1991; Nishiki *et al*., 1994). Results obtained in this biochemical study support the recent crystallographic finding that amino acids of the identified single BoNT ganglioside binding pocket interact with different carbohydrate portions compared to the analogous pocket in TeNT. These different binding modes could presumably be ascribed to the limited degree of freedom imposed on TeNT at the membrane surface by its simultaneous binding to two glycosphingolipid molecules derived of the same layer, whereas in case of BoNTs the ganglioside might invade the binding pocket in a steeper angle. The absence of either carbohydrate binding site in BoNTs is only on a minor level compensated by the different nature of ganglioside binding within the extant pocket.

It is well established that both, glycolipid and protein receptors are crucial for high affinity binding to nerve cell membranes (Halpern and Neale, 1995). As BoNTs and TeNT bind with similar affinity (in the low nM range) to those receptors, binding has to be interpreted as the simultaneous occupation of the identified ganglioside binding pocket by GT1b and of a yet to be localized binding site for a proteinaceous ligand. This was approved for the high affinity binding of BoNT/B, that depends on the presence of synaptotagmin II and GT1b (Nishiki *et al*., 1996a; Dong *et al*., 2003). In TeNT, the second adherence site, the sialic acid binding pocket, probably interacts at a later stage of the uptake process with the carbohydrate portion of a glycoprotein co-receptor. A GPI-anchored glycoprotein was indeed recently recognized as a supplementary cell surface receptor for TeNT (Herreros *et al*., 2001; Munro *et al*., 2001).

In conclusion, these results provide valuable information for the development of effective binding inhibitors as antidotes against botulism. Antidotes are urgently needed, because currently hyperimmune sera from horse affords the only chance to treat acute botulism. Serotype specific peculiarities in the ganglioside binding mode of TeNT, BoNT/A, and BoNT/B point out that inhibitors may be unique for individual neurotoxins.

Experimental procedures

Molecular modelling

Molecular modelling was done using an Octane workstation (Silicon Graphics) and the Insight II 2000 software (Molecular Simulations).

Plasmid constructions

Gene portions encoding the H_C-fragments of BoNT/A (strain 62 A) and B (strain Okra) were fused to a double stranded oligonucleotide coding for the StrepTag II (IBA GmbH) and inserted into pQE3 (Qiagen) yielding the expression plasmids pH_CAS and pH_CBS and into the transcription vector pSP72 (Promega). Mutations in the H_C-fragments were generated by PCR using suitable primers and pH_CAS and pH_CBS as template DNAs. Nucleotide sequences of all mutants were verified by DNA sequencing. The plasmid encoding full-length wild-type BoNT/A with carboxyl-terminal StrepTag (pBoNTAS) was constructed using pBN3 (encoding the LC, Binz *et al*., 2002), pUC18bot13, pUC18bot320 (Binz *et al*., 1990) and pH_CAS. The plasmid encoding full-length wild-type BoNT/B fused to a carboxyl-terminal StrepTag (pBoNTBS) was assembled by ligation of fragments derived from LC- and HC-specific plasmids and pH_CBS. Corresponding mutated full-length BoNT expression plasmids were generated by swapping DNA fragments between pBoNTAS or pBoNTBS and mutated pH_CAS or pH_CBS plasmids respectively. The construction of equivalent TeNT-specific plasmids was detailed earlier (Rummel *et al*., 2003).

Purification of recombinant proteins

Wild-type and mutated recombinant H_C-fragments of BoNTs and TeNT and full-length scBoNTs were produced as fusion proteins with an carboxyl-terminal StrepTag utilizing the *E. coli* strain M15pREP4 (Qiagen) during 10 h of incubation at room temperature, and were purified on StrepTactin-Superflow matrix (IBA GmbH) according to the manufacturer's instructions. The carboxyl-terminal StrepTag was not removed, as tagged H_C-fragments and corresponding H_C-fragments with authentic carboxyl-termini exhibited indistinguishable properties in our binding assays. Fractions containing the desired proteins were pooled, frozen in liquid nitrogen, and kept at −70°C.

Mass determination of H_C-fragment/GT1b complexes

Wild-type H_CA, H_CB and the mutants H_CA-W1266L and H_CB-W1262L (1.6 μM) were incubated with or without 32 μM GT1b in binding buffer (minus BSA) for 30 min at 37°C. Aliquots (5 μl) of each mixture were mixed in a 1:1 ratio with either of the matrices of a saturated sinapinic acid solution or EAM-1 (Ciphergen Biosystems) in 50% acetonitrile-water containing 0.5% trifluoroacetic acid. Twice, 2 μl of each mixture were dropped on a 8-spot gold surface chip and air-dried. Data were collected by a surface enhanced laser desorption/ionisation time of flight mass spectrometer (SELDI-TOF MS) PBS II (Ciphergen Biosystems) and analysed with the Ciphergen ProteinChip software 3.0.1.

Iodination procedure

Twenty micrograms of native BoNT/A and BoNT/B (#130 A and #136 A, List Biological Laboratories) were radiolabelled applying the lactoperoxidase method (Pearse and Gallagher, 1989). Labelling reactions proceeded for one hour at room temperature in the presence of 60 MBq of carrier free $Na^{125}I$ (>0.6 TBq/mg, Amersham Pharmacia Biotech), and 5 μg lactoperoxidase (ICN) in a total volume of 200 μl by stepwise addition of a total of 60 μl of 3% H_2O_2. Non-incorporated ^{125}I was removed by ultrafiltration using Vivaspin 2 columns (30000 MWCO, Vivascience) and BSA was then added as stabiliser (0.1% final concentration). More than 85% radioactivity of the radiolabelled BoNTs was precipitable with 10% cold trichloroacetic acid. Specific activities were: BoNT/A, 194.4 MBq/mg; BoNT/B, 195.6 MBq/mg.

Binding to ganglioside GT1b

Ganglioside GT1b [NAcNeuα3Galβ3NAcGalβ4(NAcNeuα8 NAcNeuα3)Galβ4GlcβCer] (Sigma-Aldrich) was dissolved in methanol and applied to high-affinity 96-well polystyrene microtitre plates (Corning; 1 μg GT1b in 100 μl/well) or in case of competition assays with ^{125}I-BoNTs to high-affinity C8 single-break stripe plates (Greiner Bio-one; 0.1 μg GT1b in 100 μl/well). The solvent was evaporated at room temperature and wells were washed three times with binding buffer (10 mM Tris-HCl, 10 mM Na_2HPO_4, 0.5% BSA, pH 7.2). Unspecific binding sites were then blocked by incubating for 2 h in PBS/Tween buffer [140 mM NaCl, 7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% (v/v) Tween 20, pH 7.2] supplemented with 3% (w/v) BSA.

Binding assays were performed in binding buffer (100 μl/well) for 2 h at room temperature containing either increasing amounts of wild-type H_CA, H_CB and H_CT and the mutant H_CT-R1226L (0.2–200 μg) or fixed amounts (2.875 μg) of mutants of H_CA or H_CB. Unbound protein was removed by three washing steps, each one with 250 μl of PBS/Tween buffer. Bound H_C-fragments were detected by incubation with StrepTactin conjugated alkaline phosphatase (ST-AP, IBA GmbH) in binding buffer for 2 h at room temperature according to the manufacturer's instructions. *p*-Nitrophenylphosphate (1 mg ml^{-1} in 100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4) finally served as the substrate for alkaline phosphatase. The dephosphorylation reaction was stopped by adding 3 M NaOH solution, and the extinction at 405 nm was scanned employing a SpectraCount microplate reader (Packard).

Competition assays were conducted for 2 h at room temperature in 100 μl binding buffer containing 700 000 cpm/well of [^{125}I]-BoNT, various amounts of either native BoNT or recombinant H_C-fragment. Subsequent to incubations and removal of supernatants, wells were washed three times with PBS/Tween buffer, dried, and separated. Amounts of bound radiolabelled BoNT were then determined in an automatic γ-counter (Wallac 1480 Wizard 3).

In vitro *transcription and translation*

H_C-fragments were synthesised *in vitro* from pSP72 derivatives that were previously linearized downstream of the neurotoxins' carboxyl-terminal codon using the re-

ticulocyte lysate system (Promega) and L-[^{35}S]-methionine (555 KBq, >37 TBq/mmol; Amersham Pharmacia Biotech), in a total volume of 25 μl.

Binding assay using rat brain synaptosomes

Binding of radiolabelled H_C-fragments to rat brain synaptosomes was studied as detailed in Rummel *et al*. (2003). Briefly, functional synaptosomes were recovered from a Percoll gradient after homogenization of rat brain and various centrifugation steps and finally diluted in physiological buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM Hepes, 10 mM glucose, 0.5% BSA, pH 7.4) with the final synaptosomal protein adjusted to a concentration of 10 mg ml^{-1}.

Binding assays were performed in triplicates, in a total volume of 100 μl of physiological buffer for 2 h at 0°C, containing a 5 μl aliquot of the ^{35}S-labelled H_C-fragment (reaching a final concentration of ~ 3 pM), or 700 000 cpm of [^{125}I]-BoNT and 30 μl of the synaptosome suspension. For competition assays various amounts of the recombinant wild-type H_C-fragment or native BoNT were added. Controls were performed with samples lacking synaptosomes. After incubation, synaptosomes were collected by centrifugation (5000 *g*; 5 min) and unbound material in the supernatant fraction was discarded. The pellet fractions were washed two times each with 50 μl of physiological buffer and incubated for 30 min at 37°C in SDS sample buffer or, in the case of ^{125}I-BoNT samples, directly subjected to γ-counting. [^{35}S]-H_C-BoNT samples were then analysed by SDS–PAGE, together with a 5 μl aliquot of the *in vitro* translation mixture to quantify the yield of radiolabelled protein. Bound ^{35}S-radiolabelled H_C-fragments were visualized using a BAS-1500 phosphor imager (Fuji Photo Film), and quantified applying the Tina 2.09f software (Raytest Isotopenmeßgeräte GmbH). Amounts of bound H_C-fragments were calculated after subtraction of the value obtained for control samples as the percentage of the total H_C-fragment protein in the assay, and finally compared with the binding efficiency of the wild-type H_C-fragment.

Mouse phrenic nerve toxicity assay

The MPN toxicity assay was set up as described by Habermann *et al*. (1980). Electrical stimulation of the phrenic nerve was continuously performed at a frequency of 1 Hz. Isometric contractions were transformed with a force transducer and recorded with the VitroDat Online software (FMI GmbH). The time required to decrease the amplitude to 50% of the starting value (paralytic halftime) was measured. Full-length wild-type scBoNT/A was applied in triplicate at final concentrations of 2.0 pM, 6.6 pM, 19.9 pM, 66.2 pM, 198.5 pM and 661.8 pM. A concentration-response-curve was constructed described by the power function $y = 194.7x^{-0.2663}$ ($R^2 = 0.991$). scBoNT/A mutants were tested in triplicate at 661.8 pM final concentrations. Full-length wild-type scBoNT/B was applied in triplicate at final concentrations of 19.5 pM, 65.2 pM, 195.4 pM, 651.4 pM, 1954 pM. A concentration-response-curve was obtained which may be described by the power function $y = 406.2x^{-0.2646}$ ($R^2 = 0.988$). scBoNT/B mutants were tested in triplicate at 1954 pM or 6515 pM final concentrations. The resulting paralytic halftimes were converted to corresponding concentrations of wild-type BoNTs, using the equations mentioned above. Toxicities were finally expressed as a percentage of wild-type activity.

The catalytic activity of the LC of all mutants was validated in *in vitro* cleavage assays employing the appropriate substrates (data not shown).

CD spectroscopy

Circular dichroism data was collected with a Dichrograph III (Jobin-Yvon) spectropolarimeter equipped with a computer controlled temperature cuvette holder. Temperature-induced denaturation was performed in 100 mM Tris-HCl buffer at a protein concentration of 75 μg ml^{-1}, by monitoring the CD signal at 220 nm in a 4-mm path length cuvette. The temperature increase of 0.9°C min^{-1} from 25°C to 80°C was measured directly in the protein solution with a thermistor.

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Synaptotagmins I and II Act as Nerve Cell Receptors for Botulinum Neurotoxin G*

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Botulinum neurotoxins (BoNTs) induce muscle paralysis by selectively entering cholinergic motoneurons and subsequent specific cleavage of core components of the vesicular fusion machinery. Complex gangliosides are requisite for efficient binding to neuronal cells, but protein receptors are critical for internalization. Recent work evidenced that synaptotagmins I and II can function as protein receptors for BoNT/B (Dong, M., Richards, D. A., Goodnough, M. C., Tepp, W. H., Johnson, E. A., and Chapman, E. R. (2003) *J. Cell Biol.* 162, 1293–1303). Here, we report the protein receptor for a second BoNT serotype. Like BoNT/B, BoNT/G employs synaptotagmins I and II to enter phrenic nerve cells. Using pull-down assays we show that only BoNT/G, but neither the five remaining BoNTs nor tetanus neurotoxin, interacts with synaptotagmins I and II. In contrast to BoNT/B, interactions with both isoforms are independent of the presence of gangliosides. Peptides derived from the luminal domain of synaptotagmin I and II are capable of blocking the neurotoxicity of BoNT/G in phrenic nerve preparations. Pull-down and neutralization assays further established the membrane-juxtaposed 10 luminal amino acids of synaptotagmins I and II as the critical segment for neurotoxin binding. In addition, we show that the carboxyl-terminal domain of the cell binding fragment of BoNT/B and BoNT/G mediates the interaction with their protein receptor.

Clostridial neurotoxins (CNTs)[1] are extremely potent bacterial toxins. Among them, the seven serologically distinct botulinum neurotoxins (BoNTs, serotypes A-G) cause botulism, whereas the tetanus neurotoxin (TeNT) provokes the disease tetanus. Each neurotoxin is composed of four domains. Their light chains (LCs) act as zinc-dependent endopeptidases and specifically hydrolyze certain proteins of the vesicular fusion machinery, whereupon the Ca^{2+}-triggered fusion of synaptic vesicles with the presynaptic membrane is disrupted (reviewed in Refs. 1–3). The heavy chains (HCs) are tethered to the LCs via a single disulfide bond and encompass the three remaining domains. The HC serves as the vehicle that delivers the LC to the cytosol of neuronal cells. Therefore, the extreme toxicity has to be largely ascribed to the specific binding of the molecule to nerve terminals at the neuromuscular junction. The amino-terminal segment of the HC, the H_N domain, is responsible for translocating the LC from the lumen of an acidic intracellular compartment into the cytosol subsequent to cell binding and receptor-mediated endocytosis. The carboxyl-terminal segment of the HC, the so-called H_C-fragment (H_C), comprises two domains, H_{CN} and H_{CC}. The latter was shown in the case of TeNT to suffice for cell binding (4) and internalization (5). TeNT travels retrogradely and eventually arrives at inhibitory interneurons in the spinal cord where it provokes spastic paralysis. So far, the corresponding domain has not been identified for BoNTs, which in contrast act locally at motoneurons and cause flaccid paralysis. No function could yet be attributed to the H_{CN} domain.

It has long been known that polysialogangliosides, *i.e.* glycosphingolipids that are particularly enriched in the outer leaflet of neuronal cell membranes, are crucial for the binding of CNTs to neurons. TeNT and BoNTs exhibit affinities in the high nM range for isolated polysialogangliosides. The binding of CNTs to neuronal tissue, however, exhibits yet much higher affinities (reviewed in Ref. 6). This discrepancy as well as other findings led to the proposal of the now confirmed two-receptor model (7). This model suggests that the abundant polysialogangliosides trap and accumulate CNTs in the plane of the cell membrane. Here, the neurotoxins wait until achieving contact with their sparsely occurring protein receptor(s), which are assumed to mediate the subsequent specific endocytosis. Synaptotagmin (Syt)-I and Syt-II, two homologous membrane-anchored proteins of synaptic vesicles (8, 9) thought to link vesicular fusion to Ca^{2+} entry (10, 11), were demonstrated to interact with BoNT/B in the presence of GT1b (12–14). Recently, it was conclusively shown that their luminal domain, which becomes temporarily exposed at the synaptic cleft upon fusion of synaptic vesicles, interacts with BoNT/B and mediates the entry of BoNT/B into neurons (15).

In the present study, we investigated whether other CNTs than BoNT/B can utilize Syt-I or Syt-II as protein receptors. Our results establish that BoNT/G interacts directly with both Syt-I and Syt-II in a ganglioside-independent fashion. Moreover, neutralization studies employing peptides derived from the luminal domain of Syt-I or Syt-II effectively block the toxicity of BoNT/G in mouse phrenic nerve preparations. Thus, the interaction of BoNT/G with Syt-I or Syt-II is crucial for its entry into motor nerve terminals.

EXPERIMENTAL PROCEDURES

Plasmid Constructions and Recombinant Proteins—Plasmids encoding full-length BoNT/A and BoNT/B as well as the H_C-fragments of TeNT, BoNT/A, and BoNT/B were described previously (16, 17). Corresponding constructs for the expression of BoNT/C, -D, -E, -F, and -G H_C-fragments carrying a carboxyl-terminal StrepTag were generated by using PCR with suitable primers and purified bacteriophage DNA (BoNT/C and -D) or total bacterial DNA (BoNT/E, strain NCTC 11219;

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[1] The abbreviations used are: CNT, clostridial neurotoxin; BoNT, botulinum neurotoxin; HC, heavy chain; H_{CC}, carboxyl-terminal domain of the H_C; H_{CN}, amino-terminal domain of the H_C; H_N, amino terminal half of the heavy chain; LC, light chain; scBoNT, single chain BoNT; Syt, synaptotagmin; TeNT, tetanus neurotoxin; RT, room temperature; GST, glutathione *S*-transferase.

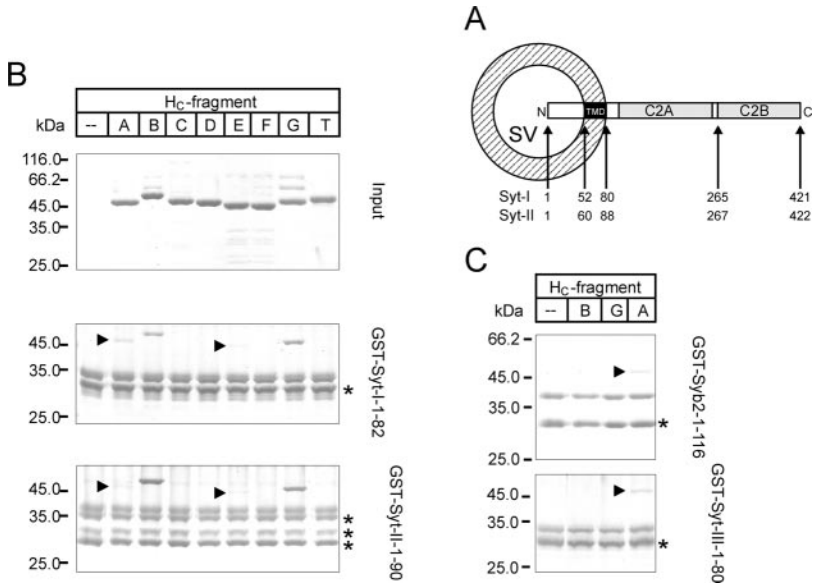


FIG. 1. **The H_C-fragment of BoNT/G binds to Syt-I and Syt-II.** *A*, schematic drawing of Syt. Syts are comprised of an intraluminal, a transmembrane (*TMD*), and two C2 domains (*C2A*, *C2B*). Syt constructs used in GST pull-down experiments lacked the entire cytoplasmic part. *Numbers below* specify amino acid positions of Syt-I and Syt-II. *SV*, synaptic vesicle. *B* and *C*, GST fusion proteins (5 μg each/reaction) immobilized on glutathione-Sepharose beads were incubated for 90 min at 4 °C with CNT H_C-fragments (3 μg each) in the presence of gangliosides (25 μg/ml). Pellet fractions were washed three times, and 20% of the material as well as 2 μg of each H_C-fragment was subjected to SDS-PAGE. Protein was subsequently visualized by Coomassie Blue staining. *Asterisks* denote breakdown products of GST fusion proteins. *Arrowheads* point toward unspecifically bound H_CA and H_CE.

BoNT/F, strain NCTC 10281; BoNT/G (*Clostridium argentinense*)) as template DNA. Plasmids for *Escherichia coli* expression of carboxyl-terminal His_6-tagged H_{CN} and H_{CC} domains of BoNT/A, -B, and -G were constructed by PCR using suitable primers and pQE3 (Qiagen, Hilden, Germany) as parental vector. A plasmid encoding full-length BoNT/G with carboxyl-terminal StrepTag (pBoNTG-wt) was assembled from LC and H_N domain-encoding DNA pieces obtained by PCR in the H_C-encoding vector. An expression plasmid (pBoNTG-thro) for full-length BoNT/G comprising a thrombin cleavage site between L and H_N was constructed in the same manner. Truncated variants of rat Syt-I (1–82, 1–53, 1–43), rat Syt-II (1–90, 1–61, 1–51), and rat Syt-III (1–80) were cloned in pGEX-2T. Nucleotide sequences of all plasmids were verified by DNA sequencing.

Recombinant full-length neurotoxins and the various H_C-derived fragments were produced utilizing the *E. coli* strain M15[pREP4] (Qiagen) during 10 h of induction at RT and purified on *Strep*-Tactin (IBA GmbH, Göttingen, Germany) or nickel-nitrilotriacetic acid beads (Qiagen) according to the manufacturers' instructions. GST fusion proteins obtained from *E. coli* TG1 were purified employing glutathione-Sepharose beads. Fractions containing the desired proteins were pooled and dialyzed against Tris/NaCl buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.2) or, in the case of neurotoxins, directly frozen in liquid nitrogen and kept at −70 °C. GST fusion proteins used for pull-down experiments were prepared in Tris/NaCl buffer supplemented with 0.5% Triton X-100 (Tris/NaCl/Triton buffer).

Syt-I-1–53 and Syt-II-1–61 were also extracted from the respective GST fusion proteins using the thrombin cleavage site present between the GST and Syt sections by applying thrombin (Roche Applied Science) in a 1:100 mass ratio for 2 h at RT. Thrombin was subsequently inactivated using phenylmethylsulphonylfluoride.

GST Pull-down Assays—GST fusion proteins (0.14 nmol each) immobilized to 10 μl of GT-Sepharose beads were incubated with full-length neurotoxins, H_C-fragments, H_{CN} or H_{CC} domains (0.06 nmol each) in the absence or presence of a bovine brain ganglioside mixture (18% GM_1, 55% GD_{1a}, 10% GT_{1b}, and 2% other gangliosides; 5 μg each; Calbiochem) in a total volume of 200 μl of Tris/NaCl/Triton buffer for 90 min at 4 °C. Beads were collected by centrifugation and washed three times each with 35 bed volumes of the same buffer. Washed pellet fractions were boiled in SDS sample buffer and analyzed together with supernatant fractions by SDS-PAGE and Coomassie Blue staining.

Mouse Phrenic Nerve Assays—The mouse phrenic nerve assay was set up as described by Habermann *et al.* (18). Electrical stimulation of the phrenic nerve was performed continuously at a frequency of 1 Hz. Isometric contractions were recorded with a force transducer and analyzed with VitroDat Online software (FMI GmbH, Seeheim, Germany). The time required to reduce the amplitude to 50% of the starting value (paralytic half-time) was determined. Recombinant BoNT/G-thro was applied in triplicate at final concentrations of 2.0, 6.0, 20.0, and 60.0 nM. A concentration-response curve could be described by the power function $y = 128.13x^{-0.2646}$ ($R^2 = 0.9838$). A concentration-response curve described by the power function $y = 69.11x^{-0.2662}$ ($R^2 = 0.9855$) was obtained in the same manner for recombinant single chain BoNT/B (scBoNT/B) applying final concentrations of 195.4, 651.4, and 1954 pM.

For inhibition studies scBoNT/A, scBoNT/B, and BoNT/G-thro were mixed with various concentrations of Syt-I- or Syt-II-derived peptides, incubated for 15 min at RT, and subsequently added to the hemidiaphragm preparation. Paralytic half-times measured in the presence of peptides were converted to corresponding concentrations of the respective isolated BoNTs using the equations mentioned above. Resulting toxicities were finally expressed as percentage of inhibition of toxicity. Individual inhibition experiments were conducted three to six times.

RESULTS

BoNT/G Directly Interacts with the Luminal Domain of Syt-I and Syt-II—Previous studies demonstrated that Syt-I and Syt-II interact with BoNT/B (12–14) and mediate its internalization (15). In contrast, the entry of BoNT/A and BoNT/E does not depend on the presence of Syt-I and Syt-II (15). To screen whether any of the remaining CNTs utilizes interaction with Syts to enter neuronal cells, we analyzed binding of their H_C-fragments to Syt-I and Syt-II *in vitro*. We conducted GST pull-down experiments employing fusion proteins in which the entire luminal and transmembrane domains of rat Syt-I and Syt-II was fused to GST. As demonstrated in Fig. 1*B*, GST·Syt-I-1–82 and GST·Syt-II-1–90 pull down the H_C-fragments of BoNT/B and BoNT/G. The finding with BoNT/B agrees with results of Dong and colleagues (15); the finding with BoNT/G constitutes a novel result. Binding of H_CB and H_CG to GST·Syt-II-1–90 is saturable and reaches a 1:1 stoichiometry at saturation (3-fold molar excess of H_C; data not shown). We also detected traces of H_C-fragments of BoNT/A and BoNT/E pulled down with GST·Syt-I-1–82 and GST·Syt-II-1–90 (Fig. 1*B*, *arrowheads*). This interaction appears to be unspecific, as H_CA is pulled down to a similar extent by GST·Syb2 and GST·Syt-III-1–80 (Fig. 1*C*), and probably explains data of a previous report about the binding of BoNT/A and BoNT/E to Syt-I (19).

To substantiate the newly discovered BoNT/G-Syt interaction, we studied the binding of the full-length neurotoxin as well. For this, we constructed expression plasmids producing BoNT/G fused to a carboxyl-terminal StrepTag. One variant (scBoNT/G-wt) comprised the native loop region between the two cystein residues that form the interchain disulfide bridge. In a second variant (BoNT/G-thro), a recognition site for thrombin was inserted in this region at the expense of four of the original amino acids (Fig. 2*A*). Both neurotoxin variants could be isolated from *E. coli* lysates via their affinity tag and exhib-

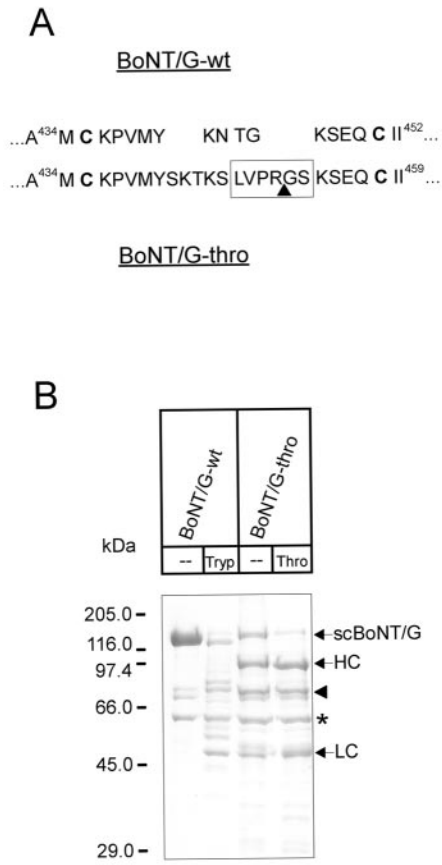


FIG. 2. **Generation of full-length BoNT/G.** *A*, expression plasmids encoding BoNT/G carrying a carboxyl-terminal StrepTag comprised either the native loop region between the LC and HC connecting cystein residues (scBoNT/G-wt) or a thrombin cleavage site that was inserted at the expense of four original amino acids (BoNT/G-thro). The recognition site for thrombin is *boxed*, the scissile peptide bond indicated by an *arrowhead*. *B*, SDS-PAGE analysis of purified recombinant BoNT/G-wt and BoNT/G-thro before and subsequent to protease digestion by trypsin (*Tryp*) or thrombin (*Thro*), respectively. Electrophoresis was conducted under reducing conditions. *Arrows* point at the positions of scBoNT/G, HC, and LC. The *asterisk* marks an uncharacterized neurotoxin byproduct, the *arrowhead* carboxyl-terminal breakdown products of BoNT/G.

ited >85% purity (Fig. 2*B*). BoNT/G-wt was used for *in vitro* binding studies as single chain protein. Full-length scBoNT/G-wt also interacts with Syt-I and Syt-II, whereas no binding was observed for scBoNT/A, underscoring that the pull down of H_CA is indeed unspecific (Fig. 3, *middle panel*).

It was recently shown that the interaction of BoNT/B with Syt-I depends on the presence of gangliosides. Therefore, we checked next whether BoNT/G displays the same binding mode. However, BoNT/G·Syt-I complexes already form in the absence of gangliosides and exhibit a similar affinity as compared with conditions with micelle-incorporated gangliosides (Fig. 3, *right panel*).

BoNT/G Binds to the Membrane Proximal Region of Syt-I and Syt-II—Because the mode of binding differs between BoNT/B and BoNT/G with respect to the requirement of gangliosides, it was interesting to investigate whether BoNT/B and -G associate with the same segment of Syt. Dong *et al.* (15) showed that the membrane proximal region of Syt-II, *i.e.* residues 40–60, comprises the binding site for BoNT/B. We therefore mapped the binding site for BoNT/G by truncating Syt-I and Syt-II. Results presented in Fig. 4*B* show that H_CG binds to GST·Syt-II-1–61. No binding is detected, however, to GST·Syt-II-1–51, suggesting that BoNT/G shares the interaction site with BoNT/B. The binding site for BoNT/B in Syt-I could not be determined via carboxyl-terminal-truncated Syt-I, because this interaction only occurs when Syt-I and gangliosides are concomitantly present in micellar structures (Fig. 4*A*) (15). On the other hand, we were able to establish that the corresponding membrane proximal segment of Syt-I, *i.e.* residues 43–53, is crucial for the binding of BoNT/G because H_CG is pulled down by GST·Syt-I-1–53 but not by GST·Syt-I-1–43. This interaction occurs as with full-length BoNT/G-wt (Fig. 3) whether gangliosides are present or not.

The Isolated Luminal Domain of Syt-I and Syt-II Blocks the Toxicity of BoNT/G at the Mouse Phrenic Nerve—To provide evidence for the physiological relevance of the BoNT/G-Syt interaction, we investigated whether the neurotoxicity of BoNT/G could be blocked by preincubating the neurotoxin with peptides derived from the luminal domain of Syt-I and Syt-II. As an assay system we chose the established mouse phrenic nerve toxicity test (18). In the first set of experiments, we inspected whether it succeeded in blocking the effect of BoNT/B. scBoNT/B was applied in a 2-nM concentration, which results in a 50% reduction of the hemidiaphragm muscle contractile force within 59.2 ± 3.6 min (paralytic half-time). Preincubation of scBoNT/B with a 1000-fold molar excess of GST·Syt-II-1–61 leads to a 61% inhibition of neurotoxicity (paralytic half-time: 76.0 ± 5.7 min), whereas a 10,000-fold molar excess results in a 95.1% inhibition (paralytic half-time: 132.3 ± 13.2 min; Fig. 5). Removal of the GST portion increases the potency of Syt-II-1–61 10-fold because a peptide concentration of 1.95 μM raises the paralytic half-time to 138.5 ± 10.6 min (corresponding to a 95.9% inhibition). In line with results of the binding experiments, GST·Syt-II-1–51, a variant devoid of the complete interaction site, has no influence on neurotoxicity. Secondly, GST·Syt-I-1–53, as well as the corresponding peptide Syt-I-1–53, does not significantly prolong the paralytic half-time of scBoNT/B because preincubation occurred in the absence of micellar gangliosides (Fig. 5).

BoNT/G proved to be far less active at the mouse phrenic nerve than BoNT/B and BoNT/A, probably because of a lower affinity to synaptosomal membranes[2] as well as to a lower catalytic activity of its LC *versus* other synaptobrevin-hydrolyzing CNTs (20). A final concentration of 105 nM scBoNT/G-wt resulted in a paralytic half-time of 120 min (data not shown). Bath concentrations of up to 1 mM GST·Syt would have been required for inhibition studies. Attempts were undertaken to acquire nicked, *i.e.* proteolytically activated, BoNT/G because nicked CNTs are generally far more potent. However, activation of recombinant scBoNT/G-wt with trypsin leads to an inadvertent hydrolysis of peptide bonds within the HC (Fig. 2*B*). To circumvent this problem, we utilized BoNT/G-thro, which is to a great extent specifically activated by *E. coli* proteases during the purification procedure. BoNT/G-thro proves to be about 80-fold more active than scBoNT/G-wt. A final bath concentration of 20 nM results in a paralytic half-time of 55.8 ± 3.8 min and was consequently used for neutralization studies. In contrast to BoNT/B, both GST·Syt-II-1–61 and GST·Syt-I-1–53 are able to efficiently diminish the neurotoxicity of BoNT/G-thro on preincubation with a 1000-fold molar excess by 66.2% and 75.1% (according to 74.4 ± 6.5 min, 80.7 ± 2.5 min paralytic half-time), respectively. Syt-II-1–61 and Syt-I-1–53 applied at 100-fold molar excess decrease the neurotoxicity by 37.9% (63.3 ± 5.9 min) and 66.4% (74.5 ± 4.9 min), respectively, and thus nearly approximate the efficacy of their 10-fold higher concentrated GST-tagged variants. In addition, preincubation of BoNT/G-thro with an equimolar mix-

[2] A. Rummel and T. Binz, unpublished results.

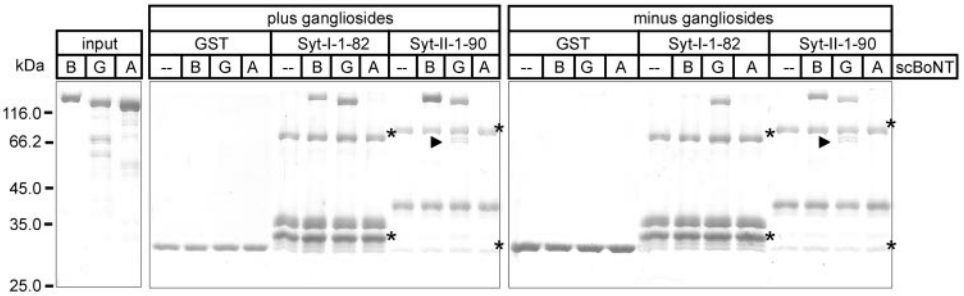
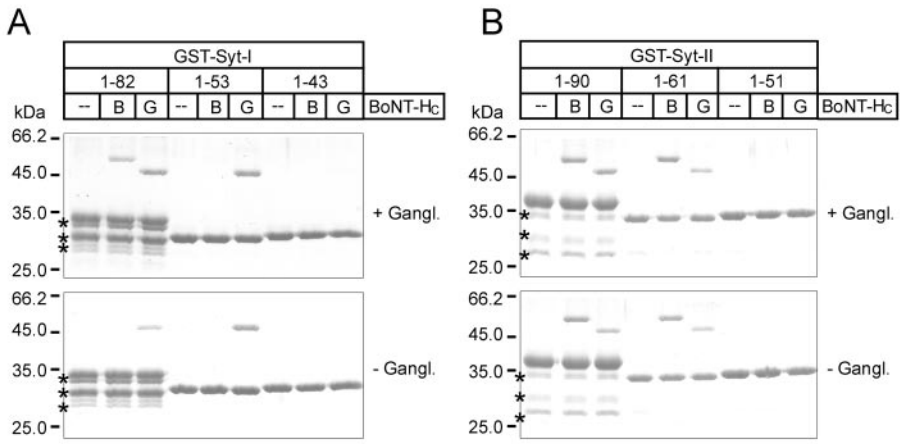


FIG. 3. **The binding of full-length BoNT/G to Syt-I does not depend upon the presence of gangliosides.** Binding assays were conducted as outlined in the legend to Fig. 1. However, binding assays were conducted partly in the absence of gangliosides. *Asterisks* denote breakdown and byproducts of GST fusion proteins. *Arrowheads* indicate truncated Strep-tagged BoNT/G fragments that also bound specifically to GST·Syt-II-1–90. These fragments are difficult to detect in GST·Syt-I-1–82, because they co-migrate with a byproduct of this fusion protein. Notably, BoNT/B does not, but BoNT/G does interact with Syt-I in the absence of gangliosides.

FIG. 4. **The membrane proximal segment of Syt-I and Syt-II serves as an interaction site for BoNT/G.** Binding assays were performed as detailed in the legend to Fig. 1, using the indicated truncated versions of Syt-I (*A*) and Syt-II (*B*). *Upper panels* represent experiments done in the presence of 25 μg/ml of gangliosides (+*Gangl.*); *lower panels* show the results of corresponding experiments without gangliosides (−*Gangl.*). *Asterisks* denote breakdown products of GST fusion proteins.



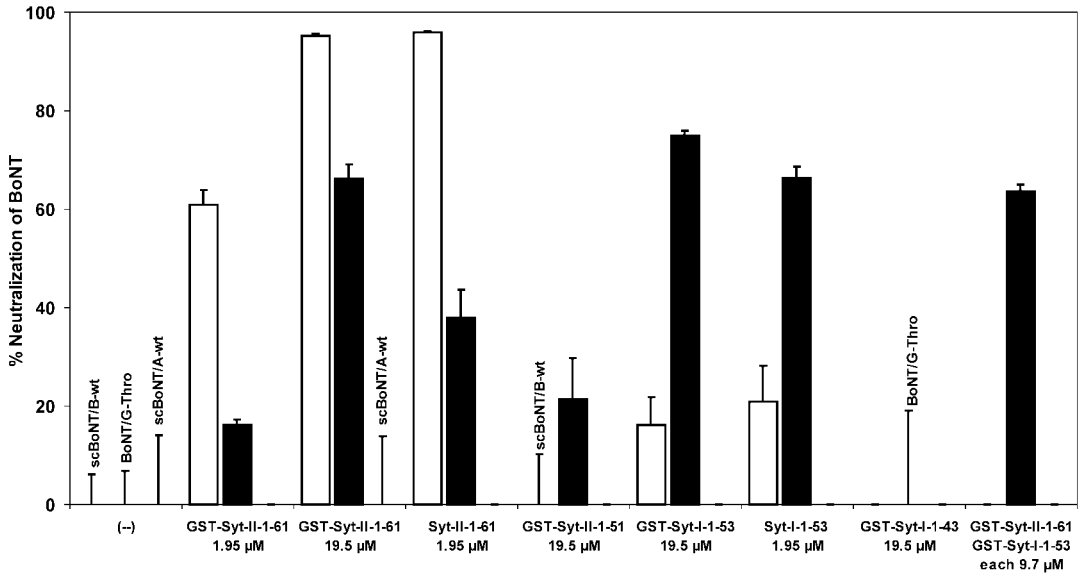


FIG. 5. **Preincubation of BoNT/G with luminal domain-derived Syt peptides blocks its neurotoxicity at the mouse phrenic nerve.** scBoNT/A, scBoNT/B (*open columns*), and BoNT/G-thro (*filled columns*) were preincubated for 15 min at RT with various amounts of truncated GST·Syt-I, GST·Syt-II, or isolated Syt peptides as indicated. Mixtures were then added to electrically stimulated mice hemidiaphragm preparations, and isometric contractions were recorded and analyzed. The time required to decrease the amplitude to 50% of the starting value (paralytic half-time) was determined as well as percent inhibition *versus* the respective untreated neurotoxin (for details see "Experimental Procedures").

ture of GST·Syt-II-1–61 and GST·Syt-I-1–53 (~10 μM each) inhibits phrenic nerve paralysis to a similar extent as 19.5-μM concentrations of the individual fusion proteins (63.7%; 73.0 ± 2.8 min; Fig. 5). The shortened constructs GST·Syt-II-1–51 and GST·Syt-I-1–43 do not affect the toxicity of BoNT/G-thro, as indicated by paralytic half-times of 59.5 ± 6.4 and 46.0 ± 4.2 min, respectively, that do not significantly differ from the untreated control. The results with the latter truncated Syt variants also demonstrate that inhibition through GST·Syts is specific and does not interfere with any other physiological process in the assay system. As a further control experiment, we assessed whether the toxicity of scBoNT/A is effected by preincu-

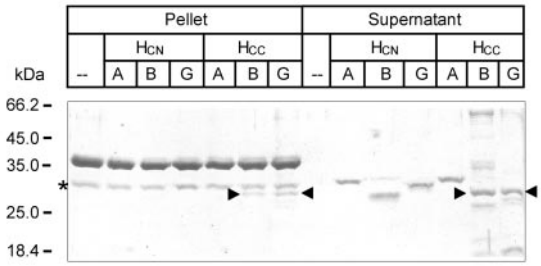


FIG. 6. **The H_{CC} domains of BoNT/B and BoNT/G harbor the binding site for Syt-II.** Binding assays were performed as outlined in the legend to Fig. 1. The *asterisk* specifies a breakdown product of GST·Syt-II-1–61. *Arrowheads* highlight H_{CC}B and H_{CC}G.

bation with GST·Syt, because entry of this neurotoxin has previously been shown to be independent of an interaction with either Syt (15). As presented in Fig. 5, even a 19.5-μM final concentration of GST·Syt-II-1–61 has no detectable effect on the toxicity of 0.22 nM scBoNT/A. This finding further validates the specificity of the assay system. Together, these results prove that Syt-I and Syt-II indeed do mediate the entry of BoNT/G into peripheral nerve cells.

The H_{CC} Domain of BoNT/B and BoNT/G Mediates the Interaction with Syt—It is known that the H_{CC} domain of the TeNT and BoNT H_C-fragments is responsible for ganglioside binding (17, 21, 22). No function could so far be allocated to H_{CN}. To assign the Syt binding site to either of the two domains, we expressed each as His_6-tagged proteins in *E. coli* and conducted pull-down experiments. H_{CN}A and H_{CC}A served as control peptides in this assay. Fig. 6 clearly demonstrates that the H_{CC}, but not the H_{CN}, domain of BoNT/B and -G interacts with GST·Syt-II-1–61. So, in addition to ganglioside binding, the H_{CC} domain of BoNT/B and BoNT/G mediates the interaction with the protein receptor as well.

DISCUSSION

BoNTs, the causative agents of botulism, disrupt the neurotransmission of cholinergic nerves at the neuromuscular junction. More than one decade ago, the underlying molecular basis for the inhibition of neurotransmitter release was deciphered for all BoNTs and TeNT, which turned out to be the proteolysis of one of three intracellular soluble NSF attachment protein receptor proteins by their catalytic domains. On the other hand, receptors that mediate the productive uptake of CNTs into nerve terminals have so far only been unequivocally identified for BoNT/B (12, 15).

In this study, we have identified the cellular receptor for the second of the eight CNTs, BoNT/G, which like BoNT/B is guided into neurons through its specific interaction with Syt-I or Syt-II. Two lines of evidence support this suggestion. First, by means of GST pull-down experiments we were able to show that BoNT/G interacts directly with the luminal domain of Syt-I and Syt-II. Second, peptides derived from the luminal domain of Syt-I and Syt-II are capable of blocking the entry of BoNT/G into motoneurons that innervate the mouse diaphragm. This only occurs when these peptides contain the membrane anchor-juxtaposed luminal 10 amino acids of Syt. The identified segment of Syt-I and Syt-II becomes transiently exposed on the membrane surface only when synaptic vesicles fuse with the presynaptic membrane at synapses. This is in line with the well documented finding that nerve stimulation generally accelerates the uptake of BoNTs and concomitant poisoning of nerve terminals (23). In a similar manner, TeNT was previously also shown to be taken up via recycling of synaptic vesicles (24). Together, these data suggest that CNTs in general enter nerve terminals via this route and may associate with segments of resident synaptic vesicle proteins that are exposed to the luminal side.

In contrast to what was reported for BoNT/B (15), the binding of BoNT/G to Syt-I is apparently independent of the presence of gangliosides. The most obvious explanation for this discrepancy is that a low affinity of BoNT/B for Syt-I does not allow detection by GST pull-down experiments. Here, only the simultaneous interaction with Syt-I and a ganglioside molecule ultimately guarantees high affinity binding. In BoNT/G, sequence variation in its Syt binding fold may account for stronger binding to Syt-I. Alternative explanations like the generation of a high affinity binding site for Syt-I because of ganglioside-induced structural changes is unlikely to occur, because significant structural changes were not observed in several crystals of BoNT/B and its complexes with sialyllactose (21) or doxorubicin (25). Furthermore, gangliosides incorporated in Triton micelles did not support binding of BoNT/B to a truncated Syt-I variant that lacked the membrane anchor domain, indicating strict requirement of both Syt-I and ganglioside within the context of membranes.

Our study illustrates that the H_{CC} domains of BoNT/B and BoNT/G are responsible for the binding to their protein receptor. This interaction is requisite for productive uptake into neurons. Indirect evidence for an employment of H_{CC} in this process has recently been suggested for TeNT as well (5). Thus, the H_{CC} domains likely function as a closed cell entry module in all CNTs, whereas the function of the H_{CN} domain still awaits elucidation.

The novel finding of the present study that BoNT/G, just like BoNT/B, utilizes Syt-I and Syt-II for its entry into nerve terminals is consistent with their degree of sequence conservation. The similarity score for aligning their H_C-fragments is 42.4% and is only exceeded by the score of 58.0% for H_CE (strain NCTC11219)/H_CF (*Clostridium baratii*) (ClustalW software). Almost all other pairwise alignments result in less than 30% similarity. We have further shown that the H_{CC} domain of BoNT/B and -G mediates the binding to Syt-I and Syt-II. With respect to sequence alignments of H_{CC}, H_{CC}B/H_{CC}G actually reaches by far the highest similarity score (39.5%), whereas those for many other pairs drop down to less than 20%. This agrees with the notion that none of the remaining six CNTs binds to Syt-I or Syt-II and the observation that BoNT/B and -G are incapable of competing binding of BoNT/A and E to synaptosomal membranes. [2] These data add further support to the premise that all other CNTs associate with different protein receptors to become endocytosed. Interestingly, TeNT, which is sorted into the retrograde axonal transport route upon endocytosis, was demonstrated to bind simultaneously to two separate carbohydrate structures (16, 26), one of which could be part of the recently discovered glycosylated 15-kDa protein receptor (27). Therefore, the use of a separate protein receptor that ensures exit from the lysosomal transport route of BoNTs is plausible explicitly for TeNT.

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Summary and discussion

The isolation of H_C-fragments from CNTs or their recombinant expression retains their full binding affinities. It is well established that CNT H_C-fragments bind gangliosides, a class of glycosphingolipids that are found particularly in membranes of neuronal cells (van Heyningen and Miller, 1961; Simpson and Rapport, 1971b; 1971c). Deletion mutagenesis studies revealed that the TeNT H_{CC}-domain binds to gangliosides and neuronal cells even more efficiently than the complete H_C-fragment (Halpern and Loftus, 1993), whereas the H_{CN}-domain does not bind at all (Figueiredo *et al.*, 1995). These results were amended in synaptosomal binding experiments employing ^{35}S-labeled full length TeNT derivatives lacking either H_C-fragment (LH_NT), H_{CN}-domain ($LH_{NCC}T$) or H_{CC}-domain ($LH_{NCN}T$). Only $LH_{NCC}T$ bound to the rat brain synaptosomes as efficiently as isolated HC, H_C-fragment and H_{CC}-domain of TeNT (Rummel, data not shown). Moreover, the H_{CC}-domain of TeNT constitutes the section that mediates retrograde intraaxonal transport in chicken spinal cord cells (Rummel *et al.*, 2001) and, as demonstrated by cross-linking experiments, interacts with a GPI-anchored glycoprotein receptor (Herreros *et al.*, 2000a). The H_C-fragments of BoNT/A and B also consist of H_{CN}- and H_{CC}-domains folded similarly to the ones of TeNT (Lacy *et al.*, 1998; Swaminathan and Eswaramoorthy, 2000), but not until recently the first isolated expression of the H_{CN}- and H_{CC}-domains of BoNT/A, B and G was achieved (Rummel *et al.*, 2004b).

Thus, these studies suggest that the receptor binding sites of CNTs are localised within the H_{CC}-domain. Although the H_{CN}-domain displays a lectin-like fold, no carbohydrate binding to this segment is observed. Currently, it is unclear what role the H_{CN}-domain plays in the intoxication mechanism. Hypotheses propose a function as a rigid, complex spacer between H_N- and H_{CC}-domain as well as an involvement in the translocation process.

Characterisation of the ganglioside binding site in TeNT

An early crosslinking experiment employing ^{125}I-azido-GD1b and the TeNT H_C-fragment led to radiolabeling of H1293 in the proximity of a large cavity within the H_{CC}-domain (Shapiro *et al.*, 1997). The neighbourhood 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 the binding to synaptosomal membranes (J.M. Sutton *et al.*, 2001). In computer-aided docking studies employing small molecules for inhibition of

TeNT ganglioside binding, this cavity was chosen out of 52 other pockets possibly serving as ganglioside binding site. Into this pocket the anticancer drug doxorubicin, an anthracycline antibiotic, was docked thereby competitively inhibiting the binding of the TeNT H_C-fragment to liposome-integrated GT1b (Lightstone *et al.*, 2000). A cocrystallization of the TeNT H_C-fragment and four carbohydrate subunits of GT1b exhibited four distinct binding sites, including the one in the proximity of H1293, where lactose 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 *N*-acetyl-galactosamine (NAcGal) or sialic acid (NAcNeu). Two additional sites were identified in cocrystals with galactose (Gal) or NAcGal (Emsley *et al.*, 2000). However, the latter two sites are unlikely to function as binding pockets for polysialo gangliosides, due to insufficient space (Gal) or a high flexibility of the carbon backbone (NAcGal). Isaacs and coworkers refined their cocrystallisation approach by using a synthetic GT1b-*β* analogue lacking the ceramide portion. Indeed, the terminal disaccharide Gal*β*3GalNAc*β* bound to the lactose binding site next to H1293 while the disialic acid branch of another GT1b-*β* molecule interacted with the sialic acid binding site comprising R1226 (Fotinou *et al.*, 2001).

A detailed analysis of the lactose binding pocket of TeNT employing site directed mutagenesis revealed that binding of mutated H_C-fragments to isolated GT1b as well as to synaptosomes was drastically reduced for each of the single mutations N1219I, D1222L, H1271A/W, S1287A/G, W1289G/L/V and Y1290A/F/S (Rummel *et al.*, 2003). These data is in line with reduced binding of TeNT H_C-fragment mutants D1222A, H1270A and W1289A to GT1b in surface plasmon resonance experiments and NGF differentiated PC12 cells (Louch *et al.*, 2002). The physiological importance of the lactose binding site in TeNT was demonstrated by the application of corresponding, recombinant full length TeNT mutants at the mouse phrenic nerve assay leading *e.g.* to a 350 fold reduction in neurotoxicity in case of the single amino acid mutation W1289L (Rummel *et al.*, 2003).

Furthermore, the sialic acid binding site is essential for TeNT action as well. The TeNT H_C-fragment mutants G1215F and R1226F/R showed weak binding to GT1b and to synaptosomes, and the full length mutant TeNT R1226F only retained 1.4 % of TeNT wildtype activity in the mouse phrenic nerve (MPN) assay (Rummel *et al.*, 2003). These results are lately supported by independent cocrystallisations of TeNT H_C-fragment with disialyllactose (NAcNeuα8NAcNeuα3Galβ4Glcβ), a GD3 derivative deficient of the ceramide portion, and with the tripeptide YEW which showed each of the ligands bonded in the sialic acid binding site (Jayaraman *et al.*, 2005).

The presence of two neighbouring carbohydrate binding sites in the TeNT H_{CC}-domain provoked the question whether a single molecule of GT1b is able to bind in a bidentate manner with its two sugar termini into the two ganglioside pocket. Such a binding mode can be observed in cholera toxin (CT) which coordinates five GM1 molecules in its five B-subunits via two interaction sites (Merritt *et al.*, 1994). But according to the cocrystal of TeNT H_C-fragment/GT1b-*β* (Fotinou *et al.*, 2001), the surface distance between both pockets is too long for the length of the disialic acid branch of GD1b and GT1b (Rummel; computational analysis). MALDI-TOF mass spectroscopy (MS) allows the detection of ligands bound to the H_C-fragment due to an increase of the molecular weight by 2.2 kDa. Indeed, the experiments indicated the simultaneous binding of two molecules GT1b to the TeNT H_C-fragment. If binding of GT1b to either sites is blocked by a single aa substitution, only one bound molecule of GT1b was detected. Thus, two gangliosides can independently occupy the two ganglioside binding sites in TeNT (Rummel *et al.*, 2003). Although binding of a ganglioside to the sialic acid binding pocket was shown, its subsequent substitution by or an initial interaction with the reported GPI anchored glycoproteins is conceivable. The binding of the tripeptide YEW to the sialic acid site supports this assumption (Jayaraman *et al.*, 2005). 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 intraaxonal transport.

Characterisation of the ganglioside binding site in BoNT/A and B

An alignment of the aa sequences of the H_{CC}-domains (Tab. 2) reveals an identity of only 24%. Nevertheless, according to sequence alignment the lactose binding site characterised by the presence of the peptide motif H.....SXWY.....G is conserved among the majority of CNTs. This cavity displays the typical features necessary for carbohydrate interaction found also in other protein toxins like ricin and cholera toxin. An aromatic residue, preferable tryptophane or tyrosine, supplies the surface for the hydrophobic face of the sugar ring. Polar residues like glutamate, serine or asparagine are oppositely located to interact with the sugar hydroxyl groups (Hirst *et al.*, 2002). Also, the results of cocrystallisation studies with BoNT/B and either sialyllactose (NAcNeuα3Galβ4Glcβ), which mimicks the sugar portion of GM3, or doxorubicin, respectively, suggest that the homologous lactose site in BoNT/B acts as ganglioside binding pocket (Swaminathan and Eswaramoorthy, 2000; Eswaramoorthy *et al.*, 2001).

Table 2: The aa sequence identities of 18 CNT H_C and H_{CC} (http://align.genome.jp).

H_C	H_{CC}	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	**BoNT/A Hall 62A**	**100**	90,7	24,5	24,0	25,0	18,7	15,8	19,3	16,3	31,9	31,5	31,0	37,3	34,8	35,4	39,6	19,1	22,1
2	**BoNT/A Kyoto-F inf.**	87,2	**100**	24,5	24,0	26,0	20,2	15,3	20,8	15,8	32,4	32,1	32,1	37,3	34,2	35,9	39,6	21,6	21,6
3	**BoNT/B**	32,6	31,2	**100**	98,6	90,6	19,2	17,9	19,3	18,4	24,3	24,5	23,4	22,8	22,0	24,0	25,0	42,1	25,2
4	**BoNT/B Isol. 1436**	33,3	31,4	95,2	**100**	91,0	19,7	17,9	20,3	18,4	24,3	24,5	23,4	23,3	21,9	24,5	25,0	41,6	25,2
5	**BoNT/B inf./111**	34,4	34,2	92,0	91,3	**100**	19,2	18,4	19,8	18,4	24,9	25,0	23,9	24,4	21,4	24,5	26,0	43,1	25,2
6	**BoNT/C1 Phage**	22,8	23,7	21,5	21,9	25,6	**100**	24,0	57,4	23,0	17,8	17,4	17,4	17,1	15,5	18,8	18,8	19,2	15,7
7	**BoNT/C Isol. 6813**	24,2	24,6	25,8	26,2	29,4	33,6	**100**	23,0	93,4	18,4	19,0	19,0	19,2	19,3	19,8	16,1	19,9	16,3
8	**BoNT/C/D**	22,6	23,7	22,0	21,8	25,3	74,9	33,6	**100**	21,9	18,4	17,4	17,9	13,5	14,4	14,6	16,1	19,8	17,3
9	**BoNT/D Phage**	24,6	25,6	26,1	26,1	29,6	32,9	94,5	33,4	**100**	18,9	19,0	19,0	19,2	18,2	19,3	15,6	19,9	16,3
10	**BoNT/E Beluga**	37,7	36,4	24,0	24,5	28,3	21,5	21,3	20,5	20,8	**100**	98,4	95,1	45,9	41,6	44,3	48,6	18,4	17,3
11	**BoNT/E NCTC11219**	39,0	38,0	23,7	24,6	26,9	22,0	21,0	20,7	20,2	98,0	**100**	96,7	44,0	39,7	42,4	47,3	19,6	17,4
12	**BoNT/E *C Butyricum***	37,7	36,4	23,7	24,0	26,7	22,0	21,8	20,8	21,3	97,3	96,6	**100**	44,0	39,7	42,4	47,3	19,0	14,6
13	**BoNT/F NCTC10281**	42,6	41,6	25,4	25,6	30,6	21,9	22,0	17,6	21,6	54,8	56,8	55,3	**100**	78,6	82,3	75,0	21,2	14,5
14	**BoNT/F CDC3281**	35,8	35,4	24,1	24,4	28,7	18,7	20,4	18,7	20,4	50,6	52,2	51,1	81,4	**100**	87,7	63,1	19,8	15,8
15	**BoNT/F 202F**	36,3	33,2	25,1	25,1	28,8	21,3	18,7	18,7	19,0	49,9	51,5	50,4	78,0	91,0	**100**	66,1	20,8	17,7
16	**BoNT/F *C.Baratii***	43,6	42,2	27,1	26,9	30,9	22,2	22,0	19,6	21,6	56,5	58,0	56,7	78,5	70,0	67,1	**100**	21,9	18,2
17	**BoNT/G**	32,6	32,1	42,4	42,7	46,6	23,3	26,1	23,7	26,8	26,7	27,1	26,7	29,6	26,7	26,1	29,2	**100**	22,3
18	**TeNT**	24,2	24,0	23,4	23,4	26,6	18,0	18,7	18,1	18,7	21,8	22,2	22,0	18,8	19,7	20,1	21,7	25,1	**100**

The putative ganglioside binding sites of BoNT/A and B, the most important serotypes with respect to human botulism and use as pharmaceuticals, were in depth analysed to define the individual contribution of various residues within the homologous lactose binding pocket (Rummel *et al.*, 2004a). The H_C-fragment single mutants E1203L (E1190L), H1253W (H1241W), S1264A (S1260A), W1266L (W1262L) and Y1267F/S (Y1263F/S) of BoNT/A (BoNT/B) showed a drastic reduction in binding to GT1b as well as to synaptosomes. Despite of a similar pocket geometry, the residues histidine and tyrosine of BoNT/A and B display reverse effects compared with results obtained from TeNT indicating a different binding mode of the ganglioside (Rummel *et al.*, 2004a). This idea is supported by comparison of the cocrystallisations of TeNT H_C/GT1b-β and BoNT/B/sialyllactose (Swaminathan and Eswaramoorthy, 2000; Fotinou *et al.*, 2001). In the former the terminal Gal interacts with the tryptophane and the NAcNeu sticks out of the pocket while in the latter the terminal NAcNeu binds directly to the tryptophane. However, the mutations of the aromatic key residues, W1266L and W1262L in BoNT/A and B, respectively, lead to a drastic reduction of neurotoxicity in the MPN assay as in case of TeNT (Rummel *et al.*, 2004a).

In contrast to TeNT, MS data revealed the binding of only a single GT1b molecule to the wildtype H_C-fragment of BoNT/A and B. Upon deletion of the characterised ganglioside binding pocket via single mutations, ganglioside binding was no longer detectable in MS (Rummel *et al.*, 2004a). Thus BoNT/A and B only possess one ganglioside binding pocket.

Until now the crystal structures of the remaining BoNT serotypes are unknown. These structures can be predicted subsequent to a correct sequence alignment with an experimentally solved crystal structure used as template. If the template shares a sequence identity of >35% with the target protein, the root mean standard deviation of the prediction will be < 1.5 Å which is in the range of experimentally solved structures (http://swissmodel.expasy.org). According to Table 2 the aa sequences of the H_{CC}-domains of BoNT/E and F are 32% and

36% identical to the known BoNT/A H_{CC}-domain as well as the BoNT/G H_{CC}-domain identity is 43% compared with H_{CC} BoNT/B thus allowing a structure prediction. The aa sequences of BoNT/C1 and D deviate too much from the other serotypes to allow an accurate tertiary structure prediction. In general, the knowledge of the tertiary structure *e.g.* derived from computational predictions allows a rational approach of analysing the binding sites of the remaining BoNTs

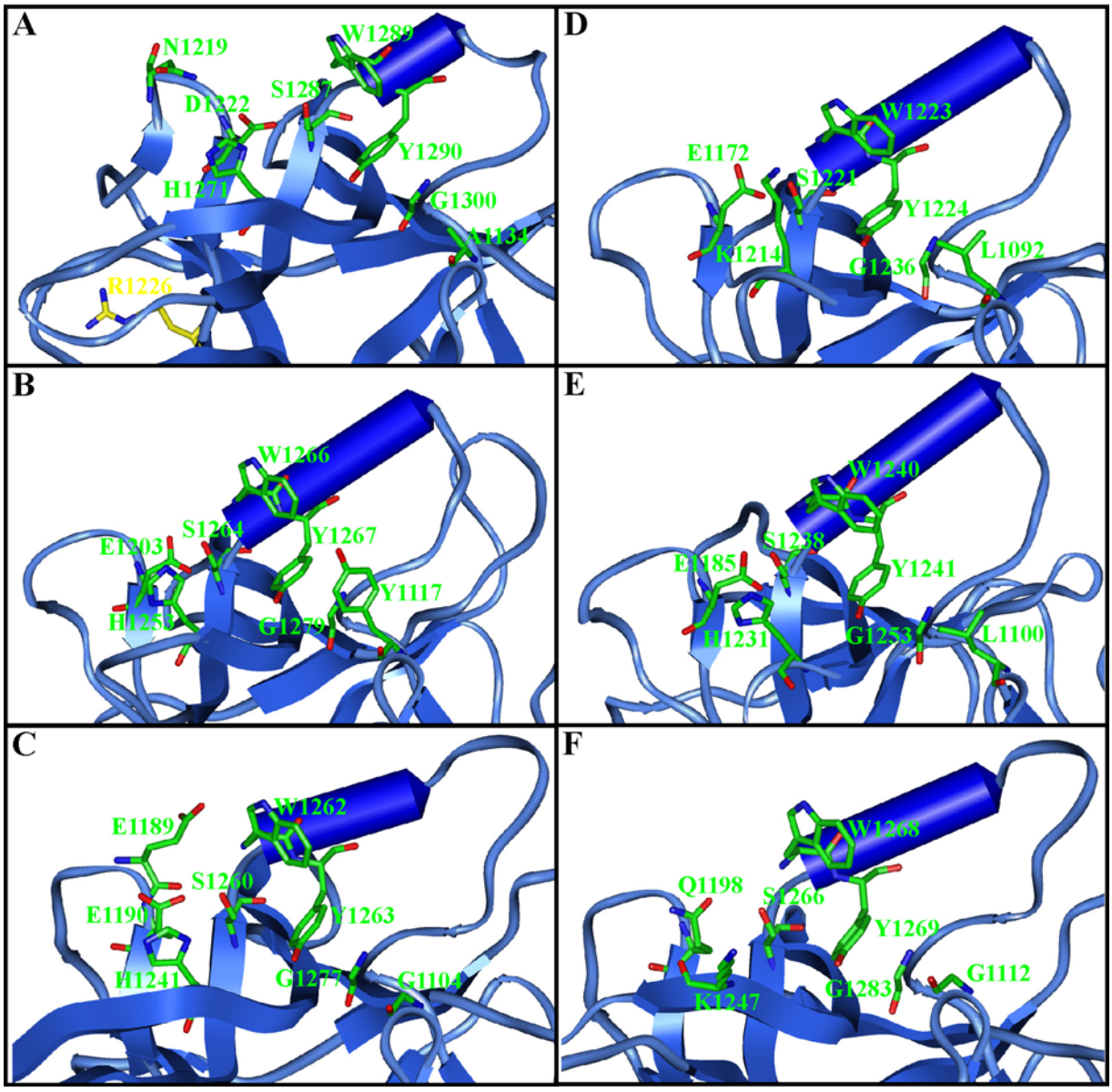

Fig. 9: Analysed residues forming the ganglioside binding sites in the known crystal structures of TeNT (A), BoNT/A (B), BoNT/B (C) and correspondingly the predicted positions of residues forming the putative ganglioside binding pockets in BoNT/E (D), BoNT/F (E) and BoNT/G (F).

The predicted positions of residues forming the putative ganglioside binding pockets in BoNT/E, F and G are highly similar to the corresponding aa in BoNT/A and B, respectively (Fig. 9). *E.g.*, the key residue tryptophane is found in all six serotypes at similar positions.

Hence, a homologous role of this cavity in BoNT/E, F and G as a ganglioside binding site is very likely. This is exemplified by the drastically reduced neurotoxicity (2.5% *versus* wildtype) of the full length BoNT/G mutant W1268L in the MPN assay (Rummel; data not shown). Thus, a single design of a potent inhibitor of ganglioside binding of CNTs might cover the requirements of all serotypes apart from BoNT/C1 and D. Their low degree of similarity hampers a successful prognosis, but a binding of a somewhat carbohydrate related structure in the H_{CC}-domain is very likely since the H_C-fragment of BoNT/C1 shows the highest binding to synaptosomal membranes (Rummel; data not shown).

Characterisation of the protein receptor of BoNT/G

As mentioned earlier (Tab. 2), the H_C-fragment of BoNT/G shares 43% sequence identity with that of BoNT/B. Thus, BoNT/G might employ a protein receptor similar to that of BoNT/B which is Syt-I and Syt-II (Dong *et al.*, 2003). GST-fusion-proteins of the intravesicular domain of Syt-I and Syt-II were generated to allow the analysis of BoNT/G-Syt-I/II interactions in GST-pull-down assays. Indeed, of all CNT H_C-fragments tested, only those of BoNT/B and G showed binding to GST-Syt-I 1-82 and GST-Syt-II 1-90 in the presence of micelle incorporated gangliosides. In the absence of gangliosides the BoNT/B interaction to Syt-I 1-82 was undetectable while the binding of BoNT/B to Syt-II 1-90 and BoNT/G to Syt-I 1-82 and Syt-II 1-90 was reduced, respectively. The interacting domains of Syt-I and Syt-II could be ascribed to the membrane juxtaposed 20 aa. This interaction was physiologically verified by the inhibition of neurotoxicity of scBoNT/B and BoNT/G in the MPN assay upon addition of the intravesicular domain peptides of Syt-I and Syt-II (Rummel *et al.*, 2004b). Thus, it is likely that the remaining BoNTs similarly employ a protein receptor localised in synaptic vesicles and presenting an intravesicular domain.

Dual receptor mechanism of BoNTs

If BoNTs do not possess a second carbohydrate binding site unlike TeNT, the question arises, whether the protein receptor like Syt-II for BoNT/B binds in the pocket homologous to the sialic acid site of TeNT. The interaction of the H_{CC}-domain of BoNT/B and G with Syt-II supports this idea (Rummel *et al.*, 2004b). Although both binding sites would be in proximate distance they could function independently and do not require preformation of a ganglioside/protein receptor complex. By confirming this assumption the dual receptor hypothesis by Montecucco would be proven (Montecucco, 1986).

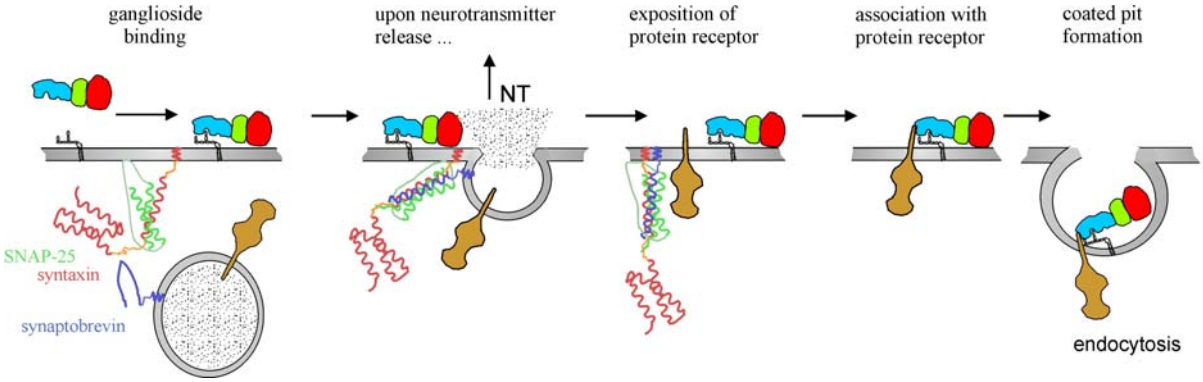

Fig. 10: Mechanism of binding and uptake of BoNTs (Courtesy of T. Binz).

The current understanding of the mechanism of BoNT uptake into motoneurons is displayed in figure 10. The widely distributed polysialo gangliosides accumulate the BoNT molecule via a low affinity interaction on the surface of motoneurons. Upon exocytosis of neurotransmitters the intravesicular domain of a synaptic vesicle protein receptor is exposed extracellularly. The approach of the sparsely occurring protein receptor by the BoNT is facilitated due to the two dimensional movement of the ganglioside/BoNT complex within the plasma membrane. Following formation of a high affinity protein receptor/BoNT complex uptake is mediated. With the knowledge of both receptors types as well as the characterisation of binding sites within the H_{CC}-domain of BoNTs the design of very potent bidentate antagonist is feasible and would allow the therapeutic treatment of acute BoNT intoxications.

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Der Botulinus Neurotoxin A Protein Rezeptor und seine Anwendungen.
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09/1999 - 12/2002	Scientist at Institut für Physiologische Chemie, MHH
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