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Keith A. Foster *Editor*

Molecular Aspects of Botulinum Neurotoxin

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Editor

Molecular Aspects of Botulinum Neurotoxin

Volume 4

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List of Abbreviations

ACh	Acetylcholine
ACMSF	Advisory Committee on the Microbiological Safety of Foods
ADP	Adenosine diphosphate
AFLP	Amplified fragment length polymorphism
ALISSA	Assay with large immunosorbent surface area
AMP	Adenosine monophosphate
ANS	1-anilinonaphthalene-8-sulfonic acid
ANTPs	Associated non-toxic Proteins
4-AP	4-aminopyridine
AS	Active site
ATCC	American type culture collection
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAT-AB	Bivalent AB antitoxin
BAT-E	Type E antitoxin
BIG-IV	Botulism immune globulin intravenous (Human)
BLAST	Basic Local Alignment Search Tool
BoNT	Botulinum neurotoxin
BoNT/A	BoNT type A
BoNTs	Botulinum neurotoxins
BWSV	Black widow spider venom
CA IBTPP	The California Infant Botulism Treatment and Prevention Program
CBER	Center for Biologics Evaluation and Research
CBMS-JVAP	Chemical Biological Medical Systems-Joint Vaccine Acquisition Program
CDC	Centers for Disease Control
CDC U.S.	Centers for Disease Control and Prevention, United States
CGRP	Calcitonin gene related peptide
CHO	Chinese hamster ovary
C_{MAX}	Maximum plasma concentration
CNT	Clostridial neurotoxin
CNS	Central nervous system
CS	Core streptavidin
CS/BoTIM/B	Innocuos mutant of BoNT/B fused to core streptavidin

CSTE	Council of State and Territory Epidemiologists
DAF	Decay accelerating factor
3,4 DAP	3,4-diaminopyridine
DC	Di-Chain
DCH	2,4-dichloro-cinnamic hydroxomate
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
DVC	DynPort Vaccine Company
ECDC	European Centre for Disease Prevention and Control
ECFF	European Chilled Food Federation
ECL	Electrochemiluminescence
ED	Effective dose
EDB	Extensor digitorum brevis
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
EEA	European Economic Area
EEA1	Early endosome antigen 1
ELCA	Enzyme-linked coagulation assay
ELISA	Enzyme-linked immunosorbent assay
EPP	Endplate potential
EPSC	Excitatory postsynaptic current
ESI-MS	Electrospray ionization spectroscopy-mass spectrometry
EU	European Union
EWRS	Early Warning and Response System
FACS	Fluorescence-activated cytometry
FDA	Food and Drug Administration
FRET	Förster resonance energy transfer
GABA	Gamma amino butyric acid
GBL	Ganglioside binding loop
GD	Sialylglycolipids
GEECs	GPI-anchored protein enriched early endosomal compartments
GEF	Guanyl nucleotide exchange factor
GI	Gastrointestinal
GFP	Green fluorescent protein
GM3	Sialylglycolipids
GPI-APs	Glycosylphosphatidylinositol-anchored proteins
GST	Glutathione-S-transferase
H	Heavy
HA	Hemagglutinin
HBAT	Equine-derived heptavalent antitoxin
HBAT	Heptavalent botulism antitoxin
HC	Heavy chain
HCR	Receptor binding domain
HCT	Translocation domain

HHS U.S.	Department of Health and Human Services
H_C	C-terminal domain of HC
H_C	C-terminal domain of HC
H_C	C-terminal half of HC
H_N	N-terminal domain of HC
H_N	N-terminal half of HC
i.m.	intramuscular
i.n.	Intranasal
i.p	Intraperitoneal
i.v.	Intravenous
IAC	Immunoaffinity column
IAC	Internal amplification control
IC_{50}	Half maximal inhibitory concentration
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IND	Investigational new drug
IU	International units
K_I	Inhibition constant
L	Light
LAP	Lysosomal-autophagy system
LC	Light chain
LCA	Light chain of BoNT/A
LD	Lethal doses
LD	Luminal domain
LD50	Median lethal dose
LOC	Lab-on-a-chip
mAb	monoclonal antibody
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MDCK	Madin Darby canine kidney
MEPP	Miniature endplate potential
MLD	Minimum lethal dose
MLD_{50}	Mouse Intraperitoneal median lethal dose
MPN	Most probable number
mpp	2-mercapto-3-phenylpropionyl
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTR	Multi-toxin resistant
NAPS	Neurotoxin associated proteins
NAPs	Neurotoxin associatied proteins
NBP	Neurotoxin binding protein
NCI	National Cancer Institute
NCTC	National collection of type cultures
NGF	Nerve growth factor
NMR	Nuclear magnetic resonance
NTNH	Non-toxic and non-hemagglutinin
NTNH	Nontoxic non-hemagglutinin

ORF	Open reading Frame
PBT	Pentavalent (A, B, C, D, and E) botulinum toxoid vaccine
PBT	Pentavalent botulinum toxoid
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PFO	Perfringolysin
PIF	Powdered infant formula
PPMP	DL-threo-1-Phenyl-2-palmitoyl-amino-3-morpholino-1-propanol
PSCs	Postsynaptic currents
RASFF	Rapid Alert System for Food and Feed
rBV A/B	serotype A and B recombinant vaccine
RMS	Risk management strategy
RMS	Risk mitigation strategy
ROS	R-Roscovitine
SAM	Self-assembled monolayer
SAR	Structure-activity relationship
SBCAMD	Synthesis-based computer-aided molecular design
SC	Single chain
SDS	Sodium dodecyl sulfate
SMI	Small molecule inhibitor
SNAP-25	Synaptosomal-associated protein of 25 kDa
SNAP-23	Synaptosome-associated protein 23 kDa
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNAREs	Soluble N-ethylmaleimide sensitive factor attachment protein receptor proteins
SNS	Strategic National Stockpile
SV	Synaptic vesicle
SV2	Synaptic vesicle glycoprotein 2
SV2	Synaptic vesicle protein 2
Syt	Synaptotagmin
TEER	Trans electrical epithelial resistance
TeNT	Tetanus neurotoxin
TEOTWAWKI	The end of the world as we know it
Tm	Midpoint temperature
TPEN	*N*, *N*,*N*',*N*'tetrakis(2-Pyridylmethyl)ethylenediamine
TRAF2	Tumor necrosis factor receptor-associated protein 2
TRF	Time-resolved fluorescence
TRPV1	Transient receptor potential vanilloid type 1
TTX	Tetanus toxin
UPS	Ubiquitin-proteasome system
USAMRIID	US Army Medical Research Institute of Infectious Diseases
USDA	US Department of Agriculture
VAMP	Vesicle associated membrane protein
VSV-G	Vesicular stomatitis virus glycoprotein
YEW	A tripeptide Tyr-Glu-Trp

Chapter 1
Overview and History of Botulinum Neurotoxin Research

Keith A. Foster

Abstract Botulinum neurotoxin is a highly successful therapeutic agent used for the treatment of a range of severe, chronic diseases, and is also widely used and recognised as a cosmetic agent for reduction of facial wrinkles. And yet, this blockbuster therapeutic product is also the most lethal toxin known and a Centers for Disease Control and Prevention (CDC) category A bioweapons threat. These apparently conflicting applications of the same agent have their origins in the unique biological properties of this fascinating family of proteins. Unravelling the biology of the neurotoxins has informed significant developments in the understanding of the biology of neurotransmission as well as elucidating the basis of both neurotoxin toxicity and therapeutic activity. Establishing the structural basis of neurotoxin activity has further increased understanding of neurotoxin function together with opening up opportunities to engineer the toxins to create proteins of increased therapeutic effect and potential.

Keywords Botulinum neurotoxin · Botulism · Neurotoxin-associated proteins · Neurotoxin complex

1.1 Introduction

Botulinum neurotoxin (BoNT) holds a unique status in public perception. It is the most lethal acute toxin known, with an estimated human lethal dose of 1.3–2.1 ng/kg intravenously or intramuscularly and 10–13 ng/kg when inhaled [1], and is the only one of the six Centers for Disease Control and Prevention (CDC) category A bioweapons threats (anthrax, smallpox, plague, botulism, tularaemia and the viral haemorrhagic fevers) that is a toxin and not an infectious agent. At the same time, it is a highly successful therapeutic agent that is used to treat a range of severe, chronic medical conditions resulting from hyperactivity of peripheral cholinergic neurons (see Chaps. 3–6 of the companion volume to this book, KA Foster (ed) *Clinical Applications of Botulinum Neurotoxin*, Springer, New York). Indeed, such

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is the safety of BoNT when used as a therapeutic agent, that its use has extended into cosmetic applications for the reduction of facial lines caused by habitual facial muscle contractions, and it has the Food and Drug Administration (FDA) approval for treatment of glabellar lines [2]. In the context of its cosmetic use, BoNT regularly features in lifestyle magazines and the proprietary name of the most widely used product, Botox®, has become widely recognised in general usage, often being inappropriately applied to any BoNT product or even the neurotoxin itself. It is interesting to note that this dichotomy was apparent in the first scientific descriptions of botulism, long before the molecular identity of the causative agent was known.

1.2 Botulism and its Cause

The earliest recorded systemic research into botulism was by the district medical officer of Württemberg in South West Germany, Justinus Andreas Christian Kerner, who, following a number of outbreaks of "sausage poisoning", published a series of papers between 1817 and 1822 that provide the first accurate and complete description of the symptoms of food-borne botulism [3]. Kerner extracted the active substance, which he termed "sausage poison" and believed to be a type of fatty acid, from the contaminated food and studied its effects, both in animals and on himself. Amazingly, in 1822, based upon these studies, he predicted the toxin's potential clinical utility, not only in conditions of muscular hypercontraction but also in autonomic conditions of glandular hypersecretion, such as hyperhidrosis and hypersalivation.

It was to be another 80 years before the source of the "sausage poison" was identified as a bacterium. Following a botulism outbreak after a funeral dinner with smoked ham in the small Belgian village of Ellezelles, the Professor of Bacteriology at the University of Ghent, Emile-Pierre-Marie van Ermengem, identified the causative pathogen, and named it *Bacillus botulinus*. The organism, subsequently reclassified as *Clostridium botulinum*, is an anaerobic, rod-shaped bacterium that produces and releases the potent toxin, now termed BoNT, that causes the symptoms of botulism. At this time, however, the nature of the toxin was still essentially unknown.

Isolation of the causative organisms enabled demonstration of toxigenicity by injection of animals with culture filtrates. Inactivated culture filtrates were used to raise toxin-neutralizing antiserum. Using this approach, Leuchs at the Royal Institute of Infectious Diseases in Berlin reported in 1910 that the bacterial strain responsible for the van Emergen outbreak of botulism differed from that isolated from an outbreak of botulism in Germany due to canned white beans, the Landman strain, and that the toxins were serologically distinct [4]. In 1919, type A and type B designations of toxin types were established, using strains isolated in investigations of botulism outbreaks in the USA [5]. Although the van Ermengem and Landman strains were no longer available, it is believed that the toxin of the former would correspond to type B and that of the latter would correspond to type A. Serotype C

was first reported by Bengston in 1922 [6], followed by D in 1928 [7], E in 1936 [8], type F [9] in 1960 and finally type G in 1970 [10]. To date, of the seven distinct serotypes of BoNT that have been isolated not all have been associated with poisoning of humans. Serotypes A, B, E, and F have been clearly identified in numerous human poisoning episodes (see Chap. 12 of this book). More recently, with the sequencing of the neurotoxin and its gene, multiple subtypes of each of the different serotypes are being identified. As more and more strains are isolated and sequenced, this diversity is expanding. The identification of subtypes and their differences is detailed in Chap. 10 of this book. It is important to note that the different serotypes have distinct differences in their biology, which leads to profound differences in their effect on host physiology, be it as a toxin or a therapeutic. This functional diversity is now also being realized to be a feature of the multiple subtypes that are being described. Thus, the BoNTs should be viewed as a large and growing family of proteins with diverse biological effects.

1.3 Purifying the Neurotoxins

In 1928, Snipe and Sommer at the Hooper Foundation at the University of California purified BoNT type A (BoNT/A) for the first time as a stable acid precipitate [11]. With the advent of World War II, the **USA** began research at a **US** Army facility at Camp Detrick, Maryland (now known as Fort Detrick) on biological warfare agents, including BoNT. Working at Fort Detrick, Lamanna and co-workers reported in 1946 purification of the toxin in crystalline form [12]. Continuing the work at Fort Detrick, Duff and colleagues improved the method of Lamanna further and established the method for purifying crystalline BoNT/A that provided the basis of the purification methods leading to the clinical product [13].

BoNT is produced by the clostridia as part of a high molecular weight complex containing the neurotoxin moiety itself and a set of associated proteins [14] (see Chap. 4 of this book). The non-toxin complex proteins are also called neurotoxin-associated proteins (NAPS) [15]. The BoNT preparation produced by Lamanna and Duff was a purified complex. Lamanna and Lowenthal subsequently separated purified BoNT/A into toxic and non-toxic components by precipitating the non-toxic components with erythrocytes leaving the toxin in solution [16]. Wagman and Bateman showed that the neurotoxin complex moved as a single substance in the ultracentrifuge with a sedimentation coefficient of 19S at pH 5.6, but at pH 7.3 dissociated with the neurotoxin component having a sedimentation coefficient of 7S [17]. Pure neurotoxin is a di-chain protein of 150 kDa. The neurotoxin complex is stable under slightly acidic conditions, pH 3.5–6.8, but under slightly alkaline conditions, above pH 7.1 and in the blood and tissues of animals, dissociates to release the neurotoxin. Neurotoxin was able to be separated from non-toxin complex proteins by column chromatography at alkaline pH [18]. It was not until 1998 that the crystal structure of purified BoNT/A was reported by the group of Ray Stevens using material purified by DasGupta [19] (see Chap. 5 of this book).

1.4 Mechanism of Neurotoxin Action

It was recognised in 1949 that BoNT blocks neuromuscular transmission through decreased acetylcholine release [20], but it was to be more than 40 years before the biochemical mechanism underpinning this activity was elucidated (see Chaps. 2 and 8 of this book). In 1992, it was reported that both tetanus neurotoxin and BoNT/B are zinc endopeptidases specific for synaptobrevin (also known as vesicle-associated membrane protein (VAMP)), an integral membrane protein of synaptic vesicles [21]. Synaptobrevin is a member of a family of proteins called soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that are essential for the docking and fusion of vesicles with their target membrane [22]. Synaptobrevin is a neuronal SNARE protein essential to the docking and fusion of synaptic vesicles at the pre-synaptic membrane. The cleavage of synaptobrevin by BoNT/B prevents the docking and fusion of the synaptic vesicle and thus leads to the inhibition of acetylcholine release by the neurotoxin. Following this initial report, it was rapidly established that the other BoNT serotypes also cleaved SNARE proteins: synaptosomal-associated protein of 25 kDa (SNAP-25) is cleaved by BoNT/A [23–25], BoNT/C [26] and BoNT/E [24, 25], synaptobrevin/VAMP is cleaved by BoNT/D [24], BoNT/F [26] and BoNT/G [27] in addition to BoNT/B. BoNT/C is the only serotype of neurotoxin identified to date that cleaves two SNARE proteins, cleaving syntaxin in addition to SNAP-25 [28, 29].

The other key aspect of neurotoxin function that underpins both the toxicity and clinical application of BoNTs is their potency and selectivity for targeting neuronal synapses, in particular peripheral cholinergic nerve terminals. The mechanism of this potency and selectivity and the identity of the neuronal targets to which the neurotoxins bind have only been fully elucidated in the last decade (see Chap. 6 of this book). A ‘dual receptor’ model for BoNT binding to neurons in which BoNTs initially interact with glycolipids such as gangliosides on the pre-synaptic membrane and then bind a second glycolipid and/or protein co-receptor that triggers receptor-mediated endocytosis was first proposed by Montecucco and colleagues in 2004 [30]. BoNTs bind gangliosides of the G1b series, namely GT1b, GD1b and GQ1b, very specifically. In BoNT/A, /B, /E, /F and /G, binding to ganglioside occurs via a conserved binding pocket whereas BoNT/C and /D display two different ganglioside-binding sites (see Chap. 6 of this book). The identity of the second glycolipid and/or protein co-receptor has proved challenging to establish and is still not known with certainty in respect of all the BoNTs. The first reports identifying a potential protein receptor for BoNTs was from Nishiki and co-workers who reported that BoNT/B bound to the synaptic vesicle protein synaptotagmin [31, 32]. It was not until 2003, however, that it was established by use of gain-of-function and loss-of-function approaches that synaptotagmins I and II can function as protein receptors for BoNT/B [33]. Following the identification of the intraluminal domain of synaptotagmin as the protein receptor for BoNT/B, the protein receptors for the other BoNT serotypes have also been found to be intraluminal domains of synaptic

vesicle proteins. Thus, BoNT/G and the chimeric BoNT/DC also bind via the intraluminal domain of synaptotagmin, whilst BoNT/A, /D, /E and /F all bind via loop 4 of SV2, with variable requirements for glycosylation (see Chap. 6 of this book for more details). BoNT/C is the only serotype for which proteins are not a receptor component—protease treatment or boiling of solubilised rat brain synaptosomes having no effect on binding [34]. BoNT/C displays a minimum of two ganglioside-binding sites and has been reported to bind to phosphoinositide-containing liposomes [35], but the identity of the binding event leading to neuronal uptake has yet to be elucidated. Identification of intraluminal domains of synaptic vesicle proteins as the protein co-receptor that triggers receptor-mediated uptake of BoNTs into the synaptic terminal provides an explanation for an aspect of neurotoxin pharmacology that had been known for many years, namely that BoNTs are more effective against active nerve terminals.

Having bound and internalised into the synaptic terminal via recycling synaptic vesicles, the neurotoxin has to deliver its endopeptidase domain into the neuronal cytosol. The translocation of the endopeptidase through the vesicle membrane remains the least well understood aspect of neurotoxin function, although in recent years progress has been made to dissect this process (see Chap. 7 of this book). In recent years, therefore, significant progress has been made in understanding the biochemical functions underpinning all aspects of BoNT activity. This understanding combined with the detailed structural knowledge of the neurotoxin has informed structure–function understanding of BoNT function at the molecular level. This molecular understanding, combined with recombinant expression technology, has further allowed mutational studies of neurotoxin function and informed rational design of engineered neurotoxins with modified activity. In the future, therefore, one can anticipate the creation of bespoke therapeutic proteins employing the unique pharmacology of these fascinating proteins, a topic that is explored further in Chap. 7 of the companion volume to this book (KA Foster (ed) *Clinical Applications of Botulinum Neurotoxin*, Springer, New York).

1.5 Conclusion

Despite having been known and feared throughout history, it is only in the last century that the identity and unique pharmacology of BoNTs have been understood. This understanding has been driven both by the human health threat presented by this most potent toxin and in the past four decades by its remarkable development as a highly effective therapeutic agent. The resulting detailed molecular characterisation of all aspects of the unique biological properties of this fascinating family of protein toxins has opened up significant new opportunities, in regard of both protection against the threat of intoxication by BoNT and in its therapeutic application. This is the subject of this book and its companion volume (KA Foster (ed) *Clinical Applications of Botulinum Neurotoxin*, Springer, New York).

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Chapter 2
Pharmacology of Botulinum Neurotoxins: Exploitation of Their Multifunctional Activities as Transmitter Release Inhibitors and Neuron-Targeted Delivery Vehicles

J. O. Dolly, V. B. O'Leary, G. W. Lawrence and S. V. Ovsepian

Abstract Quantal transmitter release from nerves is inhibited by all seven serotypes (A–G) of botulinum neurotoxin (BoNT), with some subtle but functional differences. Commonalities and dissimilarities in these proteins, and new recombinant forms, are highlighted in terms of their multiple activities and domains responsible for binding to the neuronal acceptors, subsequent endocytosis, translocation and proteolytic inactivation of intracellular soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) culminating in the blockade of neuro-exocytosis lasting for weeks or months. The neurotoxins bind to dual acceptors, gangliosides and intra-lumenal regions of vesicular proteins, and co-traffic into neurons. Subsequently, their proteases pass to the cytosol via a channel created in the endosomal limiting membrane and cleave distinct bonds in the substrate SNARE(s). Modification of these targets is responsible for their characteristic pharmacological activities. The prolonged duration of type A seems attributable to an identified stabilisation motif that extends the longevity of its protease. BoNTs have proved instrumental in deciphering a molecular basis for regulated exocytosis; now, emerging knowledge is helping to explain why synchronisation of released quanta of transmitter is perturbed by certain serotypes (/B, /D and /F) and not others (/A, /C1 and /E). Novel chimeras created by protein engineering are endowed with advantageous features of two serotypes for targeting sensory neurons and alleviating inflammatory pain (LC/E-BoTIM/A). Likewise, an innocuous mutant of /B (BoTIM/B) fused to core streptavidin (CS-BoTIM/B) has been exploited for guiding molecular cargo and viral vectors into nerve cells. These novel discoveries exemplify the versatility of BoNT in targeting and delivering therapeutics into neurons.

Keywords Botulinum neurotoxin · Chimera · Gene delivery · Neuronal acceptors · SNARE proteins · Quantal transmitter release

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2.1 Introduction

The exquisite competencies of botulinum neurotoxins (BoNTs) for recognising and invading peripheral nerve endings, to inhibit quantal transmitter release at low picomolar concentrations, have been exploited for therapeutic purposes with great success [1–3]. Seven serotypes (A–G) of BoNTs are produced by variants of the anaerobic gram-positive bacterium *Clostridium botulinum,* as single-chain (SC) proteins (Mr ~150 kDa), which are converted into active di-chain (DC) forms following selective cleavage by proteases. A heavy chain (HC, ~100 kDa), capable of high-affinity binding to neuronal acceptors in synaptic vesicles through its C-terminal domain (H_C), is linked via a disulphide bridge and non-covalent interactions to a protease-containing light chain (LC, ~50 kDa) [4–6]. Acceptor binding is followed by endocytosis [7, 8], a process enhanced by nerve stimulation [9], demonstrated functionally at mammalian motor nerve terminals [10]. In trafficking inside vesicles, the toxins pass through an acidic milieu which results in the translocation of their LC proteases into the neuronal cytosol, where these cleave and inactivate soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), proteins essential for vesicle fusion and Ca^{2+}-regulated exocytosis [1, 3, 11]. Exocytotic secretion of all transmitters tested, from small synaptic clear vesicles and large dense-core vesicles, is susceptible to blockade by BoNTs provided they can be administered intracellularly [12–14]. Elucidation of the molecular steps in this multiphasic process and pinpointing subtle differences in the action of the toxin serotypes provide a framework for deciphering exo-/endocytosis, thereby improving and extending their applications as research tools and clinical therapeutics.

On the other hand, recent utilisation of detoxified forms of BoNTs have opened up promising avenues for guided neuronal delivery of therapeutic cargo, and genes for expression [15–18]. This new application for intracellular delivery of potential drugs is of prime importance because the majority of chronic neurological disorders and degenerative conditions respond poorly to conventional medications, which are inefficient in entering nerve terminals and the central nervous system. Despite significant advances in developing potential therapeutics in model systems, their effectiveness has been frustratingly modest in clinical trials for a range of devastating conditions including Alzheimer's, Parkinson's and Huntington's diseases, amyotrophic lateral sclerosis and other progressive motor neuron degenerative disorders, multiple sclerosis, lysosomal storage diseases and brain tumours [19–23]. A lack of efficient methods for adept delivery of remedies to affected neurons constitutes one of the key impediments. Thus, a strong need exists for developing efficient methods for targeted transfer into neurons of intracellularly acting therapeutics to ameliorate such disease processes. Exquisite toxicities of BoNTs have curbed exploitation of their extraordinary neurotropism and ability to enter nerves, so applications have largely been limited to research on the neuronal binding of HC fragments. More recently, this obstacle has been overcome by using the intact neurotoxins in which their metalloprotease activity is abrogated by the mutation of key residues in the enzymatic site [24, 25]. Moreover, the full-length proteins are proving to be much

more efficient than their binding fragments as targeting and delivery vehicles for biotherapeutics.

2.2 Distinct Features of the Inhibition of Neuro-Exocytosis by BoNT Serotypes Extend Their Usefulness for Research and Clinical Purposes

Although all the seven serotypes (plus subtypes [26] and some mosaic neurotoxins [27]) share similar multi-domain structures and are homologous, significant differences in the amino acid sequences and three-dimensional structures (where known) underlie subtle but important dissimilarities in the pharmacological actions of the proteins which are available for testing (Table 2.1).

2.2.1 Resting and Stimulated Release of Acetylcholine Are Inhibited by All BoNT Serotypes but with Some Subtle Differences That Could Yield Insights into the Synchronisation of Quanta

Except for type G which has not been studied in-depth, each of the others has been shown to inhibit both spontaneous and evoked release of acetylcholine from mammalian motor nerves, by their near-abolition of the frequency of miniature endplate potentials and amplitude of endplate potentials, respectively. Evidence for exclusive blockade of vesicular transmitter release has been provided in the case of type A which fails to affect non-quantal release of acetylcholine at mouse endplates [76]. Notably, BoNT/D does not inhibit neuromuscular transmission in human-isolated muscle as it is unable to bind the nerve membrane, whereas /A and /C1 act there (Table 2.1). This exception provides an explanation for why no human case of type D botulism has ever been reported [34]. One of the earliest differences noted between the serotypes was the ability of increased intracellular [Ca^{2+}] (by various means, reviewed in [77]) to reverse the inhibition of quantal transmitter release by /A or /C1 to a far greater extent than that seen with the other four serotypes tested (Table 2.1), though there are some discrepancies with the data for /E. Moreover, the residual release of acetylcholine that could be evoked by high-frequency stimulation of motor nerve endings treated with /A, /E or /C1 was synchronous. In contrast, only asynchronous release could be elicited in this way from the samples blocked with /B, /D or /F. Even prior to identification of the intracellular neuronal targets for these toxins, pharmacological investigators had wisely predicted that these two toxin groups affect different components of the Ca^{2+}-regulated exocytotic process [36, 78]. It was postulated that /A and /E in some way interfere with Ca^{2+} triggering of release, whereas /B, /D and /F perturb a step concerned with synchronisation of quanta released (discussed later).

Table 2.1 Commonalities and dissimilarities in the functional properties of BoNTs Serotypes

		A	B	C1	D	E	F	G
Inhibition of quantal transmitter release from mammalian motor nerves	Miniature endplate potentials frequency diminished	Mouse [28, 29] 95 % Rat [30–33] Human [34]	Rat [32]	Mouse [29] Human [34]	Mouse [35] Rat [30] Human 0 [34]	Rat [31]	Rat [33]	–
	Endplate potential amplitudes dramatically reduced	Mouse [28, 29] Rat [30–33]	Rat [32]	Mouse [29]	Mouse [35] Rat [30] Human 0 [34]	Rat [31]	Rat [33]	–
	Inhibition reversed upon elevation of $[Ca^{2+}]_i$	Rat [30, 32] +	Rat [32] +	Mouse [29] +	Mouse [35] Rat [30] +	Rat [36] + Rat [37, 38] + Mouse [37] +	Rat [33] +	–
	Nature of toxin-resistant-evoked release	Rat Synchronous [30, 32]	Rat Asynchronous [32]	Mouse Synchronous [29]	Mouse [35] Rat Asynchronous [30]	Rat Synchronous [36]	Rat Asynchronous [33]	
	Duration of neuromuscular paralysis (days)	Mouse (35; 28–30) [29, 39, 40] Human ≫ (90; up to 180) [41, 42]	Mouse (7–10) [42] Human (60–90) [42, 43]	Mouse (28) [29] Human (≫ 90; up to 180) [41, 42]	Mouse (25–30) [35]	Mouse (5) [39, 40] Human (28–42) [42]	Mouse (7–8) [39] Rat (7) [33] Human (30–60) [42]	–
	Transient nerve sprouting	Yes [44]	–	Yes [29]	Yes [35]	No [39]	Yes [39]	–
Neuronal ecto-acceptors	Density (/μm²) on unmyelinated motor nerve endings	Mouse 150 [7, 8]	Mouse 630 [7, 8]	ND	ND	ND	ND	ND
	Identity of dual acceptors: Protein Lipid	SV2A, B, C [45] or C [46] Gangliosides [47]	Syt I/II [48, 49] Gangliosides [50]	Unknown	SV2A, B, C [51] Gangliosides [51]	Glycosylated SV2A, B [52] Gangliosides [52, 53]	SV2A, B, C [53, 54] Gangliosides [53, 54]	Syt I/II [55] Gangliosides [50]

Table 2.1 (continued)

		A	B	C1	D	E	F	G
Internalisation	Via acceptor-mediated endocytosis	Yes [7, 9]	Yes [7, 9]					
	Antagonised by lysosomotropic agents	Yes [9, 56]	Yes [9, 56]					
	Blocked by v-ATPase inhibitors	Yes [57]	Yes [57]	Yes [57]	Yes [57]	Yes [57, 58]	Yes [57]	
	Enhanced by nerve stimulation	Yes [9]	Yes [9]			Yes [58]		
Translocation	Requires di-chain form with inter-disulphide; via H_N-formed membrane pore requiring pH 7→5 gradient; cytosolic reducing potential activates the protease	Yes [59, 60]				Yes [11]		
Targets	SNAREs	SNAP-25	VAMP 1/2	Syntax. 1A/B; SNAP-25;	VAMP 1/2	SNAP-25	VAMP 1/2	VAMP 1/2
	Protease cleavage sites	$Q^{197}R$ [61, 62]	x/$Q^{76}F$ Rat [63]	$K^{254}A/K^{233}A$ [64, 65]; $R^{198}A$ [66, 67]	$K^{61}L/K^{59}L$ [68, 69]	$R^{180}I$ [61, 70]	$Q^{60}K/Q^{58}K$ [68, 71]	$A^{83}A/A^{81}A$ [72, 73]
LC protease	Half-life in central neurons (days)	≫31 [74] Lifetime 80 [75]	~10 [74]	≫25 [74]	–	<1 [74]	<2 [74]	–

ND not determined, *SNARE* soluble *N*-ethylmaleimide sensitive factor attachment protein receptor proteins, *SNAP* synaptosome-associated protein, v-*ATPase* v-adenosine triphosphatase, *LC* light chain, H_N *N*-terminal domain, *VAMP* vesicle-associated membrane protein, *Syt* synaptotagmin, *SV2* synaptic vesicle protein 2

2.2.2 *Serotypes Provide Means of Arresting Exocytosis for Various Times Useful for Clinical and Research Purposes: A Stabilisation Motif Discovered in BoNT/A Underlies the Exceptional Longevity of its Neuromuscular Paralysis*

In terms of the duration of neuromuscular paralysis in vivo, BoNT/A has a unique and clinically important advantage in displaying the longest action in rodents (~30 days), causing even more persistent relaxation of human muscles for as long as 6 months (Table 2.1). Notably, neurodegeneration has not been reported in any of the many thousands of patients worldwide who have benefited from such an amazingly prolonged response, upon treatment more than 20 years with type A toxin for numerous disorders due to overactive cholinergically innervated muscles or glands [3, 79]. This very impressive and extensive clinical record, especially the absence of any adverse effects, questions the relevance of a claim that a minute amount of type A reaches the central nervous system when injected peripherally into rodents [80]. Accordingly, no detectable migration of 'clinical-like' doses of BoNT/A or /E was seen when applied to compartmentalised cultures of rat superior cervical ganglionic neurons [81]. Furthermore, an observed movement of a trace of /A, when large therapeutically irrelevant amounts were used, failed to affect neurotransmission at distal synapses. Type /C1 caused prolonged weakening of muscles in 15 human volunteers, with a time course similar to /A [41]. However, this neurotoxin has not been evaluated more fully in the clinic probably because it can induce death of cultured central neurons [74]; although gross morphological features of muscle from mice treated with /C1 seem to recover (at least partially), demonstration of near-complete restoration of function and provision of evidence excluding neurodegeneration are needed to warrant embarking on any detailed clinical evaluation of /C1. Despite the fact that the latter cleaves syntaxin 1A/B as well as synaptosome-associated protein 25 kDa (SNAP-25), the sole target of /A (Table 2.1 and described later), similarity in their duration of actions may relate to cleavage by /C1 of the /A substrate; this is reminiscent of the observation that /C1-induced inhibition of exocytosis from rat cerebellar neurons correlates with its truncation of SNAP-25 rather than syntaxin 1A/B [74]. Recently, the remarkable long-lived paralysis by BoNT/A of mouse muscles in vivo has been attributed to the presence of a di-leucine motif in its protease LC [82]. Substitution of alanines for leucine 428 and 429 dramatically shortens the duration of neuromuscular paralysis induced in mice by this mutated recombinant BoNT/A to ~10 days from 30 days for the purified wild-type neurotoxin (Fig. 2.1). Curiously, this motif in /A [83] is absent from /C1 indicating the involvement of another stabilisation domain or mechanism. The abbreviated functional duration of the di-leucine mutant of /A toxin approaches that for the short-acting BoNT EA chimera (Fig. 2.1a, b). Interestingly, adding eight N-terminal residues from /A onto the EA chimera failed to extend its time course of neuromuscular paralysis. Likewise, removal of six amino acids from the N-terminus of BoNT/A did not alter its persistence (Fig. 2.1a, b), validating that the leucine motif near the end of LC is a major contributor to the toxin's remarkable longevity.

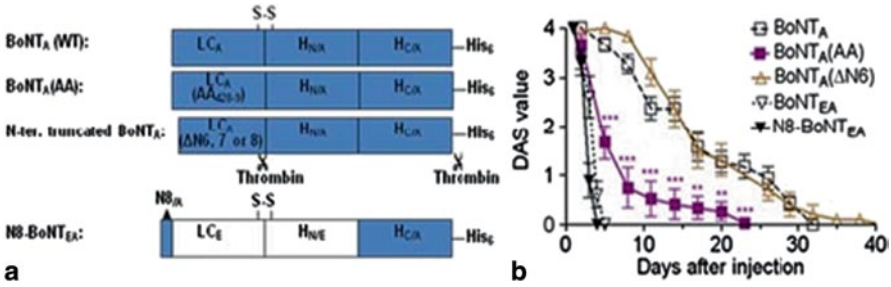

Fig. 2.1 A di-leucine in the LC of BoNT/A is essential for persistent neuromuscular paralysis in vivo, but residues at the N-terminus are dispensable. **a** Schematic representation of recombinant BoNT/A and variants created by site-directed mutagenesis, deletions or a gene fusion with sequences encoding fragments of /E. *LC* indicates the light chain whereas H_N and H_C represent the N- and C-terminal half of the heavy chain, respectively. *S–S* denotes the inter-chain disulfide. Note the two thrombin recognition sites, one in the HC/LC loop (for controlled nicking) and the other between the C-terminus of HC/A and a His_6-tag (added to aid purification by affinity chromatography). **b** The maximum dose of each of the indicated activated variants that could be tolerated (i.e. without systemic paralysis; see [82] for details) was injected into the right gastrocnemius muscle of mice and hindlimb paralysis was measured at 1–3 days interval thereafter, using the digital abduction score [84]. BoNTA(AA), in which L428/L429 were substituted by alanines, showed significantly reduced paralysis compared to wild type (*WT*) by day 5 and at all times measured thereafter (two-way analysis of variance (ANOVA) ($p<0.0001$) and post-hoc Bonferroni tests (***$p<0.001$, **$p<0.01$; $n\geq5$))

All the other serotypes, with the exception of /C1, display much shorter durations than /A for paralysing skeletal muscles (Table 2.1). BoNT/E, which also cleaves SNAP-25, acts transiently and shows the shortest duration (less than 7 days in mice and 4–6 weeks in humans). In this regard, it is pertinent that /E lacks the stabilising motif present in /A, a possible contributory factor to the short lifetime of its protease (Table 2.1). Notably, fusing the LC of /E to the N-terminus of BoNT/A stabilises the /E protease activity [82], indicating that longevity is a dominant, transferrable property of /A; alanine substitution of the di-leucine in /A prevented stabilisation of fused /E, reaffirming the importance of this motif in the durability of /A. The vesicle-associated membrane protein (VAMP)-cleaving /B and /F exhibit relatively short durations of action in motor nerve endings, particularly /F (rodents 1 week; humans 1–2 months); presently, there is no convincing molecular basis for such a substantial difference between the time courses for /B and /F. One of the early human studies [43] highlighted that /B is much shorter acting than /A; despite exceptionally high doses of /B being required to give adequate clinical benefits, the amounts injected have been raised to try and extend its persistence [85]. This approach has the disadvantages of increasing the risks of unwanted 'off-site' effects reported (e.g. on autonomic nerves [86] and induction of immune response to such large quantities of toxin [87]). Curiously, the muscle weakening effect of /D seems exceptional amongst the VAMP-targeted serotypes in lasting up to a month in mice [35], though there is no data for humans.

Although type A toxin is understandably the preferred choice for alleviating the chronic symptoms of overactive skeletal and smooth muscles or secretory glands, a transiently acting serotype such as /E could prove beneficial [3]. For example, short-term immobilisation of muscles after surgery and relief of pain (see later), coupled with an ability to promote healing like /A [88], would likely have a demand in clinical practice. In terms of research applications, it is also desirable to have tools for pseudo-reversibly blocking neuro-exocytosis for short or long periods. During prolonged paralysis of mouse motor nerves by locally applied BoNT/A in vivo, microscopic imaging revealed the appearance of nerve sprouts which continued to grow in length (up to 150 μm) over 9 weeks [44]. Importantly, these nerve outgrowths eventually form functional synapses with the muscle whilst endo- and exocytosis remain blocked at the parental nerve terminals. As activity then begins to recover at the original endings, retraction of the sprouts occurs accompanied by full recovery by 3 months. Notably, similar experiments with shorter-acting BoNTs yielded very different outcomes [39]. With the shortest-lasting type E, no sprouting could be detected and significant recovery occurred by 3 days after toxin injection, whereas in the case of the somewhat more persistent neuroparalysis with BoNT/F, short sprouts appeared and regressed by 3 weeks. A reasonable interpretation of these findings is that inhibition of neuro-exocytosis for more than 3 days is required to elicit sprouting, and the lifespan seems to be related to the duration of neuromuscular paralysis. This deduction [39] is supported by the data on /C1 [29] and /D [35] reporting induction of nerve sprouts in mice for a slightly shorter time than /A, consistent with their fairly similar durations of neuromuscular paralysis (Table 2.1).

2.2.3 BoNTs Bind to Intra-Vesicular Acceptors Exposed by Exocytosis and Enter Neurons via Endocytosis

Ecto-acceptors for types /A and /B, shown to contribute to their neuromuscular paralysis, were first identified and quantified in mouse motor nerves, by labelling with ^{125}I-iodinated derivatives and analysis using electron microscopic autoradiography [7, 8]. Each toxin displayed high-affinity saturable binding to distinct sites on phrenic nerves that occur at densities of 150 and 630/μm^2 of presynaptic membrane [8]. Notably, the greater abundance of binding sites for BoNT/B relative to those for /A accords with the content of synaptotagmin (Syt) in purified synaptic vesicles being higher than for synaptic vesicle protein 2 (SV2) [89], the toxins' respective acceptors (Table 2.1). Our observation that these reside only on the unmyelinated areas of the nerve endings, even in young mice where the myelination is incomplete, was taken to implicate them somehow in transmitter release. Moreover, the fact that the toxins' uptake following binding [8] culminated in their internalisation [9] indicated that these are 'productive' ecto-acceptors, i.e. they partake in the blockade of transmitter release [90], consistent with a hypothesised intra-neuronal action [6]. Further evidence for the functional relevance of the aforementioned acceptor sites for ^{125}I-BoNT has been provided by the lack of ^{125}I-BoNT/D binding

to nerves in human muscle, a tissue non-susceptible to this serotype (Table 2.1). Accordingly, saturable binding sites for ^{125}I-labelled BoNT/A were autoradiographically detected in putative cholinergic neurons in mouse intestine; labelling of peptidergic-like nerves could not be visualised, possibly due to a low content of sites [91]. Rodent brain synaptosomes were also shown to bind ^{125}I-iodinated BoNT/A [92], /B [93] and /F [94] including a low content of high-affinity sites and abundant low-avidity components. In light of much newer data (detailed below), it appears that the high-affinity sites represent the SV2 and Syt acceptors, respectively (Table 2.1 and see later). The less avid binding sites reported for BoNTs are likely to involve gangliosides, later found to contribute to acceptor–toxin interactions (Table 2.1). Recent biochemical identification of protein acceptors for six of the BoNTs (Table 2.1) represents a major advance, particularly those localised at motor nerve endings and/or shown to initiate the multiphasic intoxication process. Isoforms of SV2 act as neuronal acceptors for BoNT/A, /D, /E and /F, whereas Syt I/II serves this role for serotypes /B and /G (see Table 2.1 for the original publications). Only the acceptor for /C1 remains to be discovered. In addition to these protein acceptors, characterised gangliosides act as the second binding components (Table 2.1) in the well-accepted dual receptor model [95]. The latter has been substantiated by elegant structural data on the binding of these neurotoxins to both protein and ganglioside acceptors [96].

Binding of BoNTs to protein ecto-acceptors [7, 8, 90] on motor nerve endings leads to internalisation by a process that fulfils the criteria of acceptor-mediated endocytosis [7, 9]. Monitoring the uptake of ^{125}I-labelled BoNT/A and /B at murine motor nerve endings revealed that this is dependent on saturable binding, temperature and energy; also, radiolabel could be visualised on synaptic/endocytotic vesicles. Furthermore, by perturbation of their trafficking by lysosomotropic agents it was confirmed that these toxins pass through acidic compartment(s) [9, 56], and associated antagonism of neuromuscular paralysis reported for all serotypes [57] (Table 2.1). Stimulation of the phrenic nerve in mouse diaphragms increases uptake of BoNT/A and /B [9], also known to accelerate the toxins' neuroparalysis, consistent with this acceptor-mediated endocytosis being essential for their actions. It is noteworthy that two uptake processes can be distinguished for BoNT/E but not /A (Table 2.1) at mouse phrenic nerves terminals [58]; this is an interesting observation because /E shows a faster onset of neuromuscular paralysis [97] as well as greater susceptibility to antagonism by inhibitors of v-adenosine triphosphatase (v-ATPase) H^+ pump [40, 58].

2.2.4 The toxins' H_N Domain Shown to Mediate Membrane Translocation of LC/A and LC/E

In terms of translocation across vesicular/endosomal-limiting membranes, an absolute requirement was found for the inter-chain disulphide in BoNT/A, though free thiols in the toxin are not required for neurotoxicity [59]. The reduced and alkylated

protein retains the ability to bind its neuronal ecto-acceptors in *Aplysia* neurons and blocks neurotransmission if microinjected intracellularly, but modifying the inter-chain disulphide prevents internalisation and subsequent neuroparalysis. A requirement for the inter-chain disulphide in /A was subsequently pinpointed for translocating its LC protease (Table 2.1), by elegant recording of single channels formed in membranes by the HC [60]. Interestingly, presence of the C-terminal acceptor binding half (H_C) of HC/A is not essential for translocating LC across membrane patches of cultured cells, though it influences the pH threshold for channel insertion [98]. The single-channel data indicate that a reducing environment is required on the cytosolic side of the membrane for LC release from the channel (reviewed in [11]). An emphasised feature of the hypothetical scheme for membrane translocation of the toxins entails intimate and very precise interactions of groups in the LC with residues lining the channel, formed by the N-terminal domain (H_N) of their HC [60]. This speculation has now to be reconciled with our recent observation on a novel chimera, LC/E-BoTIM/A, created recombinantly [82] whose lethality in mice approximates to that of native BoNT/E and neuromuscular paralytic activity is only slightly lower than/E. A reasonable interpretation of this insightful observation is that LC/E covalently linked to protease-inactive LC/A must be readily translocated by the H_N of BoTIM/A into motor neurons where LC/E-truncated SNAP-25 is produced [3].

2.2.5 *Characteristic Patterns of Inhibition of Transmitter Release by BoNT Serotypes are Influenced by Their Requisite SNARE Targets and Sites of Cleavage*

Although quantal neurotransmitter release is blocked by all BoNTs, dissimilarities described above in the pharmacological effects of the various serotypes are largely attributable to their different targets or isoforms and cleavage sites, reviewed in [77]. Each toxin cleaves a distinct SNARE (SNAP-25, VAMP 1/2 or syntaxin 1A/B), at a particular peptide bond therein (Table 2.1) with the exception of /C1 that acts on SNAP-25 [66] as well as syntaxin 1A/B, reviewed in [95, 99]. In view of the extensive and successful clinical uses of type A toxin [3], considerable attention was focused initially on demonstrating blockade of neuromuscular transmission as a consequence [100] of its cleavage of SNAP-25 [61]. In-depth structural data [96] are now available on the multi-site interactions (via α and β exosites as well as the catalytic site) of LC/A with SNAP-25 that explains this absolute specificity for one scissile bond therein (Q [197]-R [198]). A consequence of /A only removing nine residues from the C-terminus of SNAP-25, whereas 26 amino acids are deleted by /E (R[180]-I [181]), is that raised $[Ca^{2+}]_i$ overcomes to a significant extent the inhibition of transmitter release from motor nerve terminals by /A but not /E (Table 2.1). Likewise, BoNT/A feebly reduces exocytosis of calcitonin gene-related peptide (CGRP) from sensory neurons elicited by capsaicin activation of transient receptor potential vanilloid type 1 (TRPV1) which causes a prolonged and large

elevation of $[Ca^{2+}]_i$ [101], although it blocks the evoked release of this pain peptide due to a smaller increase in $[Ca^{2+}]_i$ caused by K^+ depolarisation [102]. As in the case of the neuromuscular junction, the more extensive cleavage of SNAP-25 by the LC/E in an EA chimera (of types /A and /E) blocks CGRP exocytosis from the sensory neurons of trigeminal ganglia elicited by K^+-induced depolarisation as well as capsaicin [101]. This important difference between the pharmacological effects of /E deleting 17 residues more than /A from the C-terminus of SNAP-25 seems to be due to the /E-truncated protein being unable to form a stable complex with partnering SNAREs, unlike the SNAP-25 cleaved by /A [101, 103]. It is noteworthy that isoforms of VAMP and syntaxin occur at nerve terminals where they contribute to transmitter release, and each has distinct cleavage sites for BoNT/B, /D, /F and /G or /C1 (Table 2.1). A species difference in rat VAMP-1 renders it resistant to cleavage by BoNT/B due to a mutation at the scissile bond, reviewed in [95]. BoNT/C1 has a distinguishing feature in that it cleaves two different SNAREs: syntaxin 1A/B and SNAP-25. Until recently, this created difficulty in establishing the relative contributions of the toxin's truncation of each target to its inhibition of transmitter release; now, a mutant of BoNT/C1 has been engineered that does not cleave SNAP-25 but still blocks neuro-exocytosis by only truncating syntaxin [104]. Such dual substrate specificity might also underlie the death of cultured central neurons caused by /C1 [74], as mentioned above.

Differences noted earlier in the residual release elicited from motor nerves intoxicated with BoNT/A or /E (synchronous) or /B, /D or /F (asynchronous) accord with the requisite cleavage of their target SNARE (Table 2.1). Recent studies with various knockout mice have given further insight into the molecules that interact with the SNAREs to achieve synchronous release. Key roles are played by complexin and the Ca^{2+} sensor, Syt I; deletion of either selectively ablates synchronous release [105, 106]. Complexins promote SNARE complex formation and priming, and are thought to allow synchronisation of release by preparing SNAREs for activation by Syt [107], which binds directly to the C-terminus of SNAP-25 by Ca^{2+}-dependent and -independent interactions [108]. Complexins bind the post-fusion SNARE complex in a groove between VAMP-2 and syntaxin 1 [109]. More recently, a role has been suggested in their cross-linking of adjacent partially zippered pre-fusion SNARE complexes to prevent full zippering of the C-terminus of VAMP with its SNARE partners, syntaxin 1 and SNAP-25 [110]. In this new model, Ca^{2+}-bound Syt is proposed to disrupt the complexin 'bridges' and trigger a chain reaction of SNARE complex zippering-to-completion that initiates membrane fusion [111]. Notably, the apparent Ca^{2+}-affinity of the apparatus for synchronous release is reduced in mice lacking complexins [106], a phenotype reminiscent of the effects of BoNT/A [106]. Asynchronous release involves an unknown Ca^{2+} sensor that is also clamped by complexins [107]; Doc2 proteins are candidates [112]. Knockout of SNAP-25 removes synchronous and asynchronous release, but not spontaneous neuro-exocytosis [113, 123]. Interestingly, its ubiquitously expressed homologue, SNAP-23, can support asynchronous release, but only SNAP-25 can mediate synchronous secretion [123]. Moreover, *N*-type Ca^{2+} channels are implicated in release synchronisation because inhibiting their binding to SNARE complexes

selectively blocks synchronous release and increases asynchronicity [124]; similar roles for other types of Ca^{2+} channels are not excluded. Thus, a picture is emerging of the formation of SNARE complex array(s) being primed by complexins for synchronised vesicle fusion that is triggered by Ca^{2+}-bound Syt, with the whole assembly being held in proximity to voltage-activated Ca^{2+} channels to minimise the lag between Ca^{2+} influx and vesicle fusion.

A final but extremely important aspect for clinical applications of BoNTs is the widely different half-lives of the toxins' LC proteases (Table 2.1). Type A toxin is the most suitable for therapeutic purposes because of causing beneficial muscle relaxation in humans for amazingly protracted periods, up to 6 months (Table 2.1) or longer in the cases of hyper-secretory disorders [86]. This advantage seems to be attributable to the remarkable longevity of LC/A protease. Although this cannot be readily measured in human nerves, the deduction is based on quantitation of its half-life (>31 days) in cultured central neurons [74] and lifetime of ~3 months [75]. Although /C1 protease is also long lived, the possibility of it causing neuronal death poses a concern, as mentioned above. Proteases of the other serotypes investigated show far shorter longevities, including BoNT/B which is employed to a limited extent for a small minority of patients resistant to type A.

2.2.6 Combining the Targeting, Membrane Translocating and Longevity Features of BoNT/A with the Advantageous Protease of /E Creates a Chimera Optimised for Blocking the Release of Pain Mediators from Sensory Neurons

A beneficial additional effect of the use of BoNT/A as a muscle relaxant has been its ability to alleviate certain types of pain, e.g. headaches, back pain and chronic neuropathic pain (reviewed in [3]). Though the mechanism of action in treating these conditions remains somewhat unclear, evidence is accumulating to support a direct interaction with nociceptive nerve fibres. Intrigued by the report that BoNT/A therapy gives benefit to a subset of migrainers, we sought to exploit expertise in protein engineering to design novel BoNT variants that would be more effective in blocking the exocytosis of pain mediators from sensory nerves. As noted above, capsaicin-evoked neuropeptide release from sensory neurons is blocked by BoNT/E protease, but only feebly by /A. Unfortunately, these cells are insensitive to BoNT/E due to sparse expression of the appropriate high-affinity acceptors. Though this lack of cell penetration could be overcome by swapping its H_C-binding domain for its counterpart from /A (Fig. 2.1a), the resultant chimera (EA) retained the short lifetime of BoNT/E (Fig. 2.1b) rather than the advantageous longevity of /A. However, this limitation is overcome by LC/E-BoTIM/A that can bind to sensory neurons. Its double LC translocates into the cytosol and cleaves SNAP-25 like /E toxin but retains the persistence of /A in motor nerve endings. Furthermore, it blocks Ca^{2+}-dependent exocytosis of pain peptides [3, 82] elicited by all stimuli, including activation of TRPV1 channels with capsaicin. Accordingly, injection of LC/E-BoTIM/A into the

foot pad of rats proved effective in alleviating pain behavioural symptoms, upon inducing inflammatory pain by locally administering capsaicin [82]. A great attraction of this 'composite' neurotoxin is that the anti-nociceptive effect of a single low dose lasted longer than 18 days, the latest time point measured. As described earlier for BoNT/A, a di-leucine motif in the LC of BoTIM/A stabilises the protease activity of the delivered LC/E. This again exemplifies the great power of protein engineering in that the most advantageous therapeutic characteristics of two BoNTs can be harnessed into one synergistically acting composite. In this case, LC/E acts as the most robust inhibitor of CGRP release elicited by the activation of TRPV1 on sensory nerves whilst BoTIM/A contributes the domains to mediate binding to SV2C on sensory neurons of trigeminal ganglia, to translocate the /E protease into the cytoplasm and extend its normal transient longevity.

2.3 Full-Length Protease-Inactive Mutant of BoNT (BoTIM) as a Versatile Neuron Targeting and Gene Delivery Vehicle with Therapeutic Potential: A Lesson Learned from Experimentation with CS-BoTIM/B

While the understanding of the pharmacological characteristics of BoNTs has dramatically extended applications of their therapeutic proteases for alleviating pathologies caused by overactive nerves and hyper-secreting glands, the enormous potential of these natural neuron assailants for drug and gene targeting have only emerged more recently. The clinical failure of potential therapeutics against numerous neurological conditions is often not due to the lack of their effectiveness but rather due to shortcomings in the drug delivery methods. To this end, we have recently explored the applicability of a full-length, enzymically inactive BoNT/B (BoTIM/B) fused to core streptavidin (CS) for targeting therapeutic genes to neurons and peripheral nerves. BoNT/B was chosen in light of two important considerations: firstly, its utilisation of Syt I/II, the most abundant synaptic protein acceptor of BoNTs [8, 89] and secondly, its relatively lower toxicity in humans [114].

Through genetic fusion of BoTIM with CS, the resultant CS-BoTIM/B combines the uncompromised neurotropism of a full-length yet atoxic BoNT/B, with CS acting as a versatile adaptor for convenient and tight linkage of biotinylated cargo and viral vectors [115]. The DNA of BoNT/B modified by site-directed mutagenesis (E231A and H234Y) was ligated to the gene encoding CS, which has improved stability and accessibility for biotin [116]. Expression of this construct (pET-29a-CS-BoTIM/B) in *Escherichia coli* yielded a putative neuron targeting/delivery vehicle, CS-BoTIM/B-His6 (Fig. 2.2a–c) as a SC protein, which after purification by immobilised metal-ion affinity chromatography and ion-exchange separation gave one major protein band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) with a relative molecular mass ~163 kDa [115]. The latter is the expected size for SC 150 kDa BoTIM/B plus the 13 kDa CS. This fusion protein

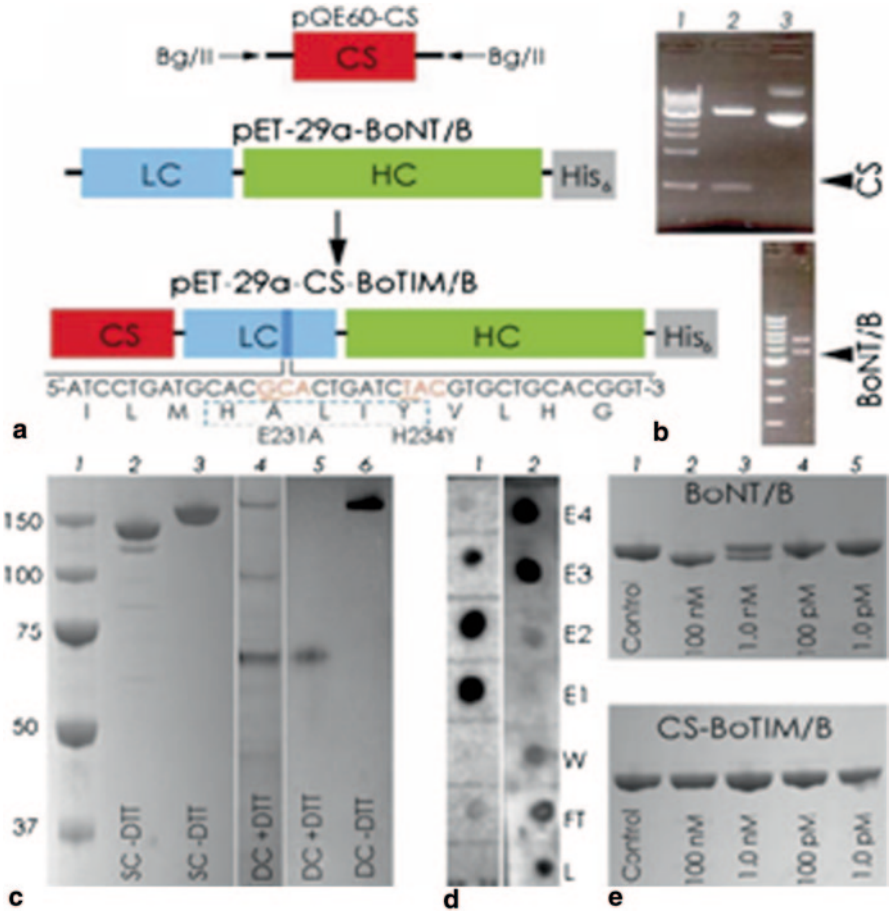

Fig. 2.2 Engineering and characterisation of CS-BoTIM/B. **a** Schematic of CS-BoTIM/B construction: CS digested from pQE60-CS by *Bgl*II (*top*) cloned into pET-29aBoNT/B (*LC* light chain; *HC* heavy chain; *His6* histidine tag) (*middle*). Site-directed mutagenesis produced E231A and H234Y (nucleotide sequence in *red, underlined*) in /B metalloprotease domain (*lower,* in *blue box*). **b** *Upper*: 1.2% agarose gel; *lane 1:* 1 kb DNA ladder; *lane 2:* CS (*arrowhead*) digested from pQE60; *lane 3:* uncut pQE60. *Lower:* BoNT/B (*arrow*) digested from pET-29a. **c** Coomassie-stained BoNT/B and CS-BoTIM/B; *lane1:* protein standards; *lane 2:* BoNT/B (150k) (non-reduced, SC); *lane 3:* CS-BoTIM/B (163k) (non-reduced, SC); *lane 4:* CS-BoTIM/B (DC), reduced (1mM DTT), *lowest band* (63k) CS-LC, *middle band* (100k) HC and *upper band* (163k) remaining un-nicked SC. Western blot using anti-streptavidin; *lane5:* CS-BoTIM/B (DC, reduced), *band* (63k) represents CS-LC; *lane 6:* CS-BoTIM/B, band represents 163k non-reduced DC. **d** Binding of CS-His6 (*1*) and SC CS-BoTIM/B-His6 (*2*) to biotin-HRP; immobilized metal affinity chromatography (IMAC)-purified samples (*dot blot*) probed with biotin-HRP; *L*: loaded fraction, *FT:* flow through, *W:* wash, *E1–E4:* eluted fractions. **e** In vitro verification of the absence of proteolytic inactivity in CS-BoTIM/B. Coomassie stained (12% Bis-Tris SDS) gel showing cleavage of glutathione S-transferase (GST)-VAMP-2–GFP by BoNT/B (*upper*) and the lack of activity towards the same substrate by CS-BoTIM/B (*lower*). Note ~50% cleavage of substrate by BoNT/B (1nM) and complete lack of cleavage by CS-BoTIM/B (1 nM)

displayed the ability to bind biotin-horseradish peroxidase (HRP) (Fig. 2.2d) and treatment with trypsin converted most of the SC into a DC form. Upon reduction (with dithiothreitol, DTT), the latter was decreased in size to HC and CS-LC respectively, the identity of the latter being confirmed by its visualisation with an anti-streptavidin antibody (Fig. 2.2c, d). In a protease assay, CS-BoTIM/B, unlike parental BoNT/B, failed to cleave its substrate, VAMP-2, attributed to the mutation of two residues (E231A and H234Y) essential for protease activity (Fig. 2.2e). Accordingly, the specific neurotoxicity of CS-BoTIM/B in mice was diminished more than 10^6-fold [117]. Overall, these properties advocate CS-BoTIM/B as a suitable candidate for targeting to peripheral nerves and neurons.

2.3.1 Evaluation of the Utility of CS-BoTIM/B for Neuronal Targeting and Gene Delivery in Cultured Neurons and Peripheral Autonomic Ganglion Cells In Vivo

The suitability of CS-BoTIM/B for transferring molecular cargo into neurons was first examined in cultures of rat spinal cord neurons through the analysis of cytosolic translocation of CS or d-biotin linked to CS, under basal and stimulation conditions. Confocal microscopy of neurons labelled with the DC form of CS-BoTIM/B followed by counting the cells revealed robust, activity-dependent intracellular transfer of CS (detected with anti-streptavidin antibody). The presence of a CS signal in the cytosol could be detected after exposing the cells to concentrations as low as 2 nM, with > 50 % cells being CS-positive after exposure to 200 nM CS-BoTIM/B, consistent with CS-LC being internalised and translocated (Fig. 2.3a–d). Importantly, similar to that reported for the parental BoNT/B [7, 48], CS-BoTIM/B uptake by spinal cord neurons exhibited activity dependence, with stimulants of synaptic activity notably increasing the fraction of labelled cells (Fig. 2.3a, b, e). As illustrated, a high concentration of KCl (60 mM) or ionomycin (1 μM) enhanced the intracellular signal in cultured neurons, whereas blockers of synaptic activity such as tetrodotoxin (0.5 μM) or BoNT/B (10 pM) exerted the opposite effect. These data contrast with the observations made on cultured skeletal muscle cells exposed to similar amounts of CS-BoTIM/B, which revealed an absence of internalisation under control or stimulation conditions, establishing its selective recognition of neurons and intracellular delivery. Similar experiments with a small molecular cargo d-biotin, bound to CS-BoTIM/B (200 nM) revealed cellular uptake in dissociated spinal cord cultures under basal and stimulation conditions (KCl, 60 mM), with apparent cytosolic distribution of d-biotin similar to that of the CS [115]. Such highly attractive features of CS-BoTIM/B could potentially be harnessed for targeted transfer of therapeutics via both non-viral and viral systems into neurons and nerve terminals within a mixed cellular population.

To ascertain if the exquisite neurotropism of CS-BoTIM/B could be endowed on viral vectors which typically display broad tropism for numerous cell types, a green fluorescent protein (GFP)-encoding lentivirus—pseudotyped with the vesicular

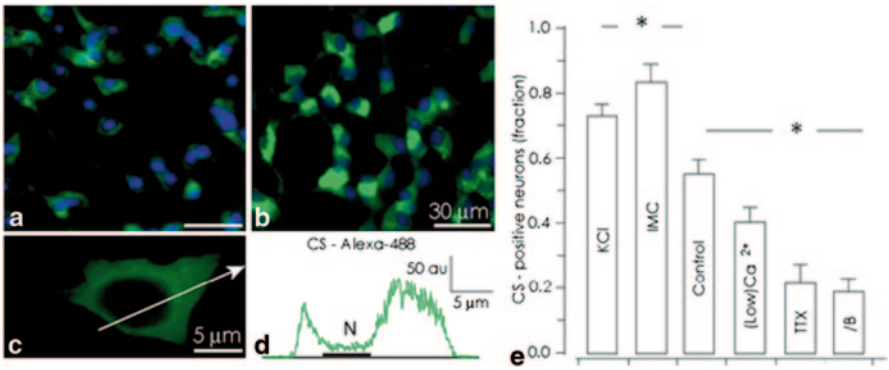

Fig. 2.3 CS-BoTIM/B uptake by cultured spinal cord neurons. Labelling of rat spinal cord neurons exposed to 200 nM CS-BoTIM/B under basal (**a**) and stimulating (**b**) (KCl, 60 mM) conditions, with anti-streptavidin antibody (*green*) and 4',6-diamidino-2-phenylindole (DAPI, *blue*) nuclear staining. **c** Confocal image of neuronal soma illustrating intracellular translocation of CS. **d** Fluorescence intensity profile; green fluorescence visualised inside the cell with a trough over the nuclear area (*N*) is consistent with a cytoplasmic localisation of CS. **e** Summary plots of the proportion of CS-positive cells under various conditions. *From left to right:* KCl–60 mM; IMC–ionomycin, 1 µM; control-[Ca^{2+}] 2 mM; low [Ca^{2+}]–0.5 mM; tetrodotoxin-TTX, 0.5 µM; BoNT/B, 100 pM. *Asterisks* highlight significant differences ($p < 0.05$ Student's *t*-test) between the fraction (±SEM (standard error of mean)) of CS-positive cells in test compared with controls

stomatitis virus glycoprotein (VSV-G) [118] and biotinylated—was conjugated to CS-BoTIM/B and added to the spinal cord neuronal cultures. Analysis of reporter expression 48 h after infection revealed enhanced green fluorescence signal in the stimulated neurons exposed to the targeted vector, as reflected in the significantly increased number of GFP-positive neurons (Fig. 2.4a–c). Importantly, such lentiviral transduction displayed activity dependence, as treatment of sister cultures with CS-BoTIM/B-lentiGFP in the presence of tetanus toxin (TTX) significantly reduced the fraction of GFP-positive neurons [115] (Fig. 2.4c). These findings are consistent with the synaptic vesicle lumenal domain of Syt I/II being utilised by targeted lentiviral vectors for the neuron recognition and gene transduction. Importantly, linking of CS-BoTIM/B to lentiGFP endowed considerable neuron selectivity to the vector, as demonstrated by the preferential expression of the reporter in spinal cord neurons co-cultured with skeletal muscle cells, as reflected by a drop in the fluorescence in myoblasts [115]. This result contrasted with the indiscriminate expression of GFP in both neurons and muscle cells exposed to non-targeted lentiGFP, and accords with the known broad tropism of non-targeted VSV-G pseudotyped viral particles [118].

To determine directly whether the selective neurotropism endowed by CS-BoTIM/B could be exploited for guiding gene transduction into neurons in vivo, advantage was taken of the readily accessible intramural nervous system of the trachea in which ganglionic neurons are amalgamated with several other non-neuronal cell types [119, 120]. Along with being a potential target for therapeutic gene delivery in a range of peripheral ganglionopathies, this model system affords a platform for testing the gene-guiding efficiency of CS-BoTIM/B in vivo. Following

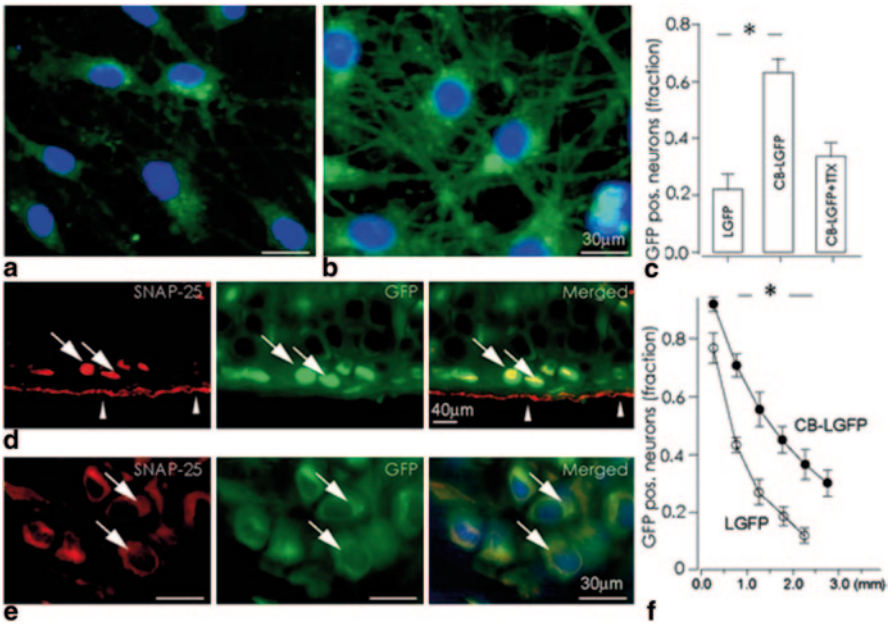

Fig. 2.4 Targeting viral vectors to neurons by CS-BoTIM/B**. a, b** Epi-fluorescence images of cultured rat spinal cord neurons exposed to lentiGFP (**a**) and CS-BoTIM/B–lentiGFP (CB-LGFP) (**b**) under control conditions counterstained with DAPI. Increase in the GFP signal reflects the enhanced infectivity of targeted lentiGFP. **c** A summary plot of the proportion of GFP-expressing neurons in the cultures exposed to non-targeted and targeted lentiGFP in the absence and presence of TTX. *From left to right*: LGFP, CB-LGFP and CS-LGFP + TTX. Note the increase in the fraction of GFP-positive cells with CS-BoTIM/B conjugated virus (CB-LGFP) and its reduction by TTX (0.5 μM). **d–f** CS-BoTIM/B promotes selective transduction of lentiviral GFP vector into tracheal ganglionic neurons in vivo. **d** Representative micrographs of tracheal coronal sections (0.5–1.0 mm from the site of injection) from a rat injected with CS-BoTIM/B–lentiGFP. Tissue stained for SNAP-25 (*left, red*) highlights the nerve plexus (*arrowheads*), and a cluster of ganglionic neurons (*above, arrows*) exhibiting strong and localised GFP expression (*middle, white arrows, green*). Merged images (*right, yellow*) show co-localisation of SNAP-25 and GFP. **e** Confocal micrograph of triple-stained ganglionic neurons (*arrows*): SNAP-25 (*left*), GFP (*middle*) and DAPI (*right*) merged. **f** Plot summarising the fraction of GFP-positive neurons from lentiGFP (*LGFP*) and CS-BoTIM/B–lentiGFP (*CB-LGFP*) injected samples: Data were pooled from each 0.5 mm consecutive section within the distance range 0–3.5 mm from the site of injection. Note the higher fraction of GFP-positive neurons from the CS-BoTIM/B-lentiGFP-injected animal. Asterisks highlight significant differences ($p < 0.05$ Student's *t*-test) between samples pooled from rats injected with non-targeted and targeted lentiviruses

direct injection of lentiGFP or CS-BoTIM/B-lentiGFP into the tracheal wall, expression of the reporter was detected after 6 days in both the epithelial and the smooth muscle layers as well as in neuronal clusters within the inter-cartilaginous space up to 3.5–4.0 mm from the site of injection. Based on the immunoreactivity to anti-SNAP-25 antibody, the latter were confirmed to be intramural ganglionic neurons (Fig. 2.4d, e). Consecutive coronal sections of the trachea injected with non-targeted or targeted viruses followed by quantification of GFP revealed

a steep decline in the reporter intensity in both neuronal and non-neuronal cells with increase in the distance from the site of injection. Noteworthy, the number of GFP-positive neurons was considerably higher in the tissue from rats injected with targeted lentivirus compared to those given lentiGFP alone (Fig. 2.4f). These differences in reporter expression became more pronounced with increasing distance from the injection site. In keeping with in vitro data, an enhanced GFP signal in neuronal agglomerates following exposure to targeted vectors is presumably reflective of the increased neurotropism of lentivirus endowed by CS-BoTIM/B. Unlike the non-targeted vectors, prone to rapid dissipation after injection through systemic clearance, the CS-BoTIM/B-linked lentivirus apparently is retained for longer at the injection locus because of its improved binding to neurons, thereby enhancing reporter expression.

2.3.2 Transduction of CS-BoTIM/B-Targeted Lentiviral Vectors into Spinal Cord Neurons Yields Expression of a Functionally Competent Protein with Neuro-Protective Effects

The versatility of CS-BoTIM/B in targeting replication-deficient lentivirus capable of accommodating large vectors (up to 8 kb) was explored through the transfer of a gene encoding the SNAP-25 protein, which was rendered resistant to BoNT/A, /E or /C1 proteolysis by a triple mutation (D179K, M182T, R198T) [121, 122]. Unlike control neurons, which showed a dose-dependent truncation of SNAP-25 by BoNT/A, /E or /C1, in sister cultures pre-exposed to targeted virus encoding multi-toxin resistant (MTR) SNAP-25 before treatment with BoNTs, a significantly higher toxin dose was required to produce visible cleavage (Fig. 2.5d, f). The total amount of SNAP-25 in cultures expressing MTR SNAP-25 matched that of naïve controls when normalised against syntaxin (for BoNT/A and /E) or VAMP-2 (BoNT/C1), reflective of the significant fraction of toxin-resistant copies of this SNARE in the neurons.

To evaluate the functional effects of toxin-insensitive SNAP-25 on synaptic neurotransmission, electrophysiological measurements were undertaken, using whole-cell patch-clamp recordings of spontaneous postsynaptic currents (PSCs) from naïve and CS-BoTIM/B-lentiMTR-S25-treated neurons. Inclusion of healthy neurons in the current analysis was assured by the distinctly non-linear voltage–current relationship revealed by slow-voltage ramps, stable recordings of synaptic currents at least for 5 min and by discarding cells with $C_m > 35$ pF and $R_{inp} > 150$ MΩ (Fig. 2.5a, b, c). Notably, the majority of neurons showed occasional spontaneous spiking (Fig. 2.5c, inset). Under symmetrical Cl^- conditions, both excitatory and inhibitory currents clamped at −65 mV holding potential were detected as inward PSCs, which were almost completely abolished by the broad-spectrum antagonists of glutamate and gamma aminobutyric acid (GABA)/glycine receptors (Fig 2.5g). As expected, PSCs were almost abolished in naïve cultures pre-exposed to BoNT/A

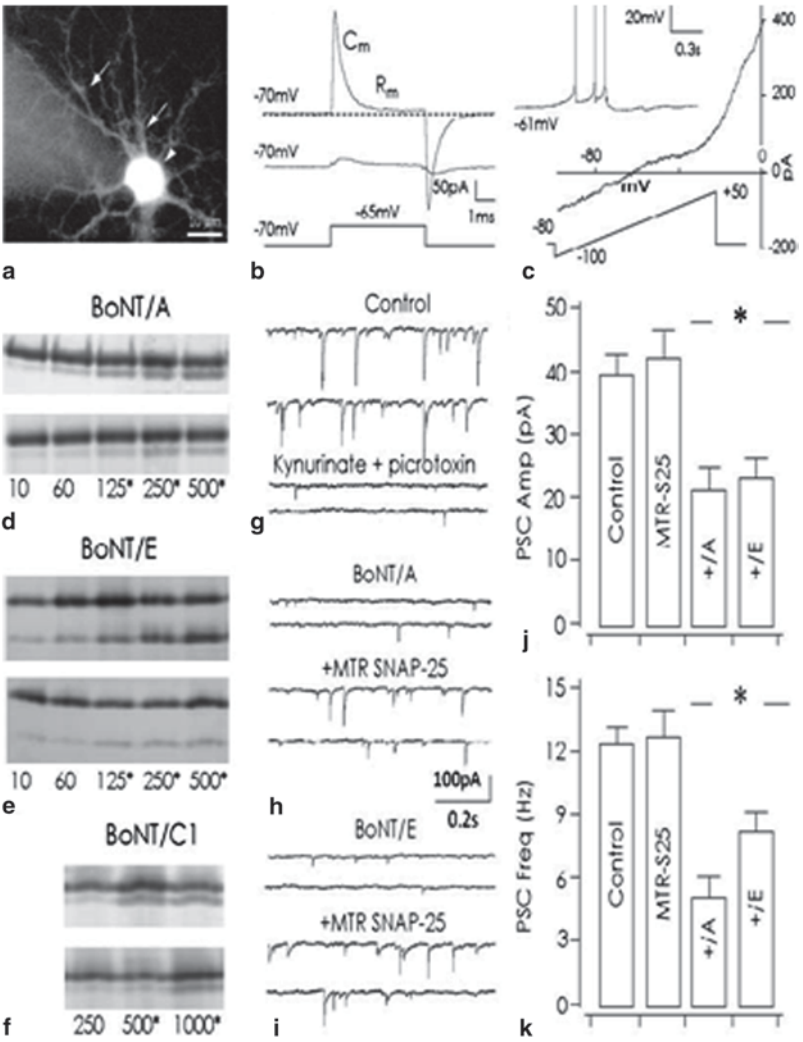

Fig. 2.5 Transduction of MTR SNAP-25 with CS-BoTIM/B-targeted vectors mitigates BoNT-induced SNAP-25 cleavage and protects synaptic transmission from BoNT challenge. **a** A typical spinal cord neuron in culture filled with Alexa 543, through the patch electrode. Cm cancellation (**b**) and a nonlinear voltage-current relation revealed by voltage ramp (**c**). Inset shows a spontaneous spike burst recorded from the same cell. **d–f** Western blots of SNAP-25 from naive and CS-BoTIM/B-lentiMTR-SNAP-25-exposed neuronal cultures (*upper* and *lower*, respectively), treated with increasing picomolar concentrations of BoNT/A, /E or /C1 (*from top to bottom*). The total amount of cleaved SNAP-25 was quantified and compared between the two groups; significant differences from controls are indicated by asterisks. **g** Postsynaptic currents (*PSCs*) recorded from spinal cord neurons before and after blockade of glutamate and glycine/GABA receptors with kynurenic acid and picrotoxin. **h, i** Recordings from naive and MTR SNAP-25-expressing cultured neurons treated with BoNT/A or /E (*upper and lower traces*, respectively); neurons expressing recombinant SNAP-25 show higher levels of synaptic activity. **j, k** Summary plots of PSC amplitude and frequency. *Asterisks* (*, $p<0.05$) highlight significant differences (Student's *t*-test)

(200 pM) or /E (600 pM) but with significant synaptic activity retained in MTR SNAP-25-expressing neurons (Fig. 2.5h–k). The latter indicates successful transduction of a physiologically relevant gene by CS-BoTIM/B and demonstrates its expression in functional form. The fact that MTR SNAP-25 protected synaptic activity from BoNT/A or /E confirmed the capability of an exogenous SNAP-25 in forming competent SNARE complexes with its molecular partners during vesicular fusion. Noteworthy, no protection of synaptic functions was observed in MTR SNAP-25-expressing cultures exposed to BoNT/C1 despite the apparent reduction of the amount of truncated SNAP-25 (Fig. 2.5f), an outcome attributable to the additional SNARE cleaved by this BoNT serotype [66, 67].

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Chapter 3
Absorption and Transport of Botulinum Neurotoxins

Michel R. Popoff and Chloé Connan

Abstract Botulinum neurotoxin (BoNT) is a potent toxin, which blocks the neurotransmitter release at neuromuscular junctions. BoNT can be acquired from the digestive tract (food-borne botulism, *Clostridium botulinum* intestinal colonization), respiratory tract (inhalational botulism), or wound (wound botulism). BoNT associates to nontoxic proteins (ANTPs), which have a main role in toxin protection against acidic pH and proteases, especially in the gastrointestinal tract. BoNT, which enters through the digestive or respiratory tract, has to first cross the epithelial barrier. This is achieved by a receptor-mediated transcytosis, which delivers the whole and active toxin at the basolateral side of epithelial cells. ANTPs containing hemagglutinins (HAs) may have an additional role in altering the intercellular junctions and facilitating toxin passage through the paracellular way. Then, BoNT disseminates locally and at distance via the blood/lymph circulation and possibly via a retrograde axonal transport to the target motor neuron endings, where the toxin uses an endocytic pathway permitting the release of the light (L)-chain into the cytosol and its subsequent proteolytic activity towards the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins involved in the neurotransmitter exocytosis.

Keywords Botulinum neurotoxin · Receptor-mediated transcytosis · Epithelial barrier · Botulinum complex · Toxin absorption · Hemagglutinins · Intercellular junctions · Paracellular passage · Cadherin

3.1 Introduction

Botulism is a severe and often fatal disease in man and animals, which is characterized by a descending flaccid paralysis. The disease is due to a neurotoxin (botulinum neurotoxin, (BoNT)), which is produced by an environmental bacterium, *Clostridium botulinum*. BoNT is the most poisonous substance known and it is responsible for all the symptoms of botulism. For this reason, BoNT is considered

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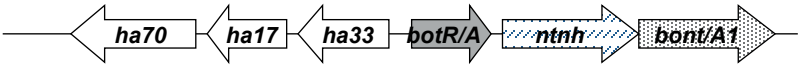
to be a potential powerful bioweapon. But, since 1980, BoNT is also a therapeutic agent with multiple increasing indications. In the natural conditions, botulism is most often acquired by ingesting preformed BoNT in contaminated food or after intestinal colonization by *C. botulinum* under certain circumstances, such as infant botulism and subsequent toxin production in the intestine. Regarding the biological threat, BoNT can be delivered not only by contamination of water or food but also by aerosol. In these circumstances, the first step of the disease consists in the passage of the BoNT through an epithelial barrier of the gastrointestinal or respiratory tract. In the medical application, the toxin is directly injected into the diseased muscle or into other target tissues, but it can disseminate and trigger distant effects. BoNT passage through epithelial/endothelial barriers and dissemination in the organism are still poorly understood. In this chapter, we discuss what we know about these important aspects of BoNT, absorption and dissemination.

3.2 BoNT Versus Botulinum Complexes

BoNTs are divided into eight toxinotypes (A–H) according to their main immunological properties and are subdivided into several subtypes according to amino acid sequence variations (see Chap. 10 of this book). BoNTs are synthesized as a precursor protein (about 150 kDa), which is inactive or only weakly active. The precursor protein, which does not contain signal peptide, is released from the bacteria possibly by a yet poorly understood cell-wall exfoliation mechanism [24]. The precursor is proteolytically activated in the extra-bacterial medium either by *Clostridium* proteases or by exogenous proteases, such as digestive proteases in the intestinal content. The active neurotoxin consists of a light-chain (L-chain, about 50 kDa) and a heavy-chain (H-chain, about 100 kDa), which remain linked by a disulfide bridge. The structure of BoNTs shows three distinct domains (see Chap. 5 of this book): L-chain containing α-helices and β-strands and including the catalytic zinc-binding motif, the N-terminal part of the H-chain forming two unusually long and twisted α-helices, and the C-terminal part of the H-chain consisting of two distinct subdomains (H_{CN} and H_{CC}) involved in the recognition of the receptor. The 100 N-terminal amino acids of the H-chain constitute a belt that wraps the L-chain and might facilitate the unfolding of the L-chain during the translocation process. While the three domains are arranged in a linear manner in BoNT/A and BoNT/B, both the catalytic domain and the binding domain are on the same side of the translocation domain in BoNT/E. This domain organization in BoNT/E might facilitate a rapid translocation process [20, 47, 50, 52, 94–96, 168, 176, 180]. Indeed, BoNT/E translocates more rapidly than BoNT/B into the cytosol of neurons, and in contrast to BoNT/B, BoNT/E requires only GT1b and no low pH in the translocation process [175]. The three structural domains have defined roles but act in concert, including the interdomain chaperone function, to achieve an efficient translocation and intracellular activity [49, 120].

locus HA-BoNT/A1 (strain Hall)

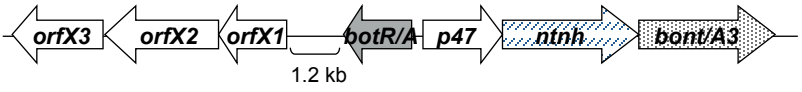


locus OrfX-BoNT/A2 (strain KyotoF)

locus OrfX-BoNT/A1 (strain NCTC2916)

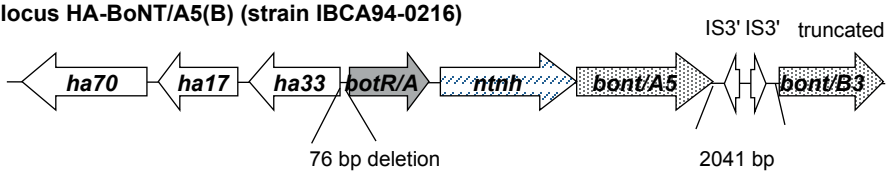
locus OrfX-BoNT/A2 (strain Mascarpone)

locus OrfX-BoNT/A3 (strain Loch Maree)

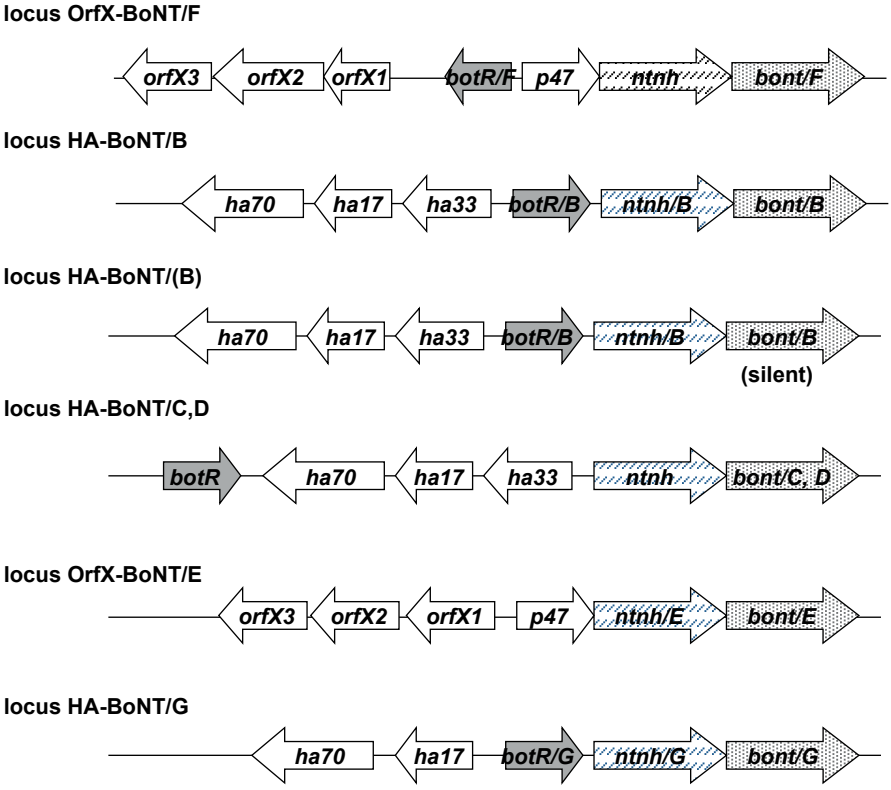


locus OrfX-BoNT/A4 (strain 657Ba4)

locus HA-BoNT/A5(B) (strain IBCA94-0216)



a



b

The overall sequence identity at the amino acid level between BoNTs ranges from 34 to 97%. Several domains are highly conserved which account for the common mode of action of these toxins. Thereby, the central domains of L-chains are related in all the clostridial neurotoxins and contain the consensus sequence (His-Glu-X-X-His) characteristic of the zinc-metalloprotease-active site. The half N-terminal domain of the H-chains is also highly conserved, and it is involved in the translocation of the L-chain into the cytosol. Thus, a similar mechanism of internalization of the intracellular active domain into target cells is shared by all the clostridial neurotoxins. In contrast, the C-terminal half of the H-chain, mainly the H_{cc} subdomains, is the most divergent [137, 138]. This accounts for the different receptors recognized by the clostridial neurotoxins.

BoNTs are complexed with Associated Non-Toxic Proteins (ANTPs) to form large complexes (botulinum complex), also known as progenitor toxins. ANTPs encompass a nontoxic and non-hemagglutinin (NTNH) component, as well as several hemagglutinin (HA) components or other non-hemaggltuinin proteins called OrfX1, OrfX2, OrfX3 or P47 [128, 137, 140, 162] (Fig. 3.1; see Chap. 4 of this book for more details). Botulinum complexes, in which proteins are not covalently linked, are formed in cultures and naturally contaminated food; they are stable at acidic pH and dissociate at alkaline pH (≥pH 7.5). The genes encoding the neu-

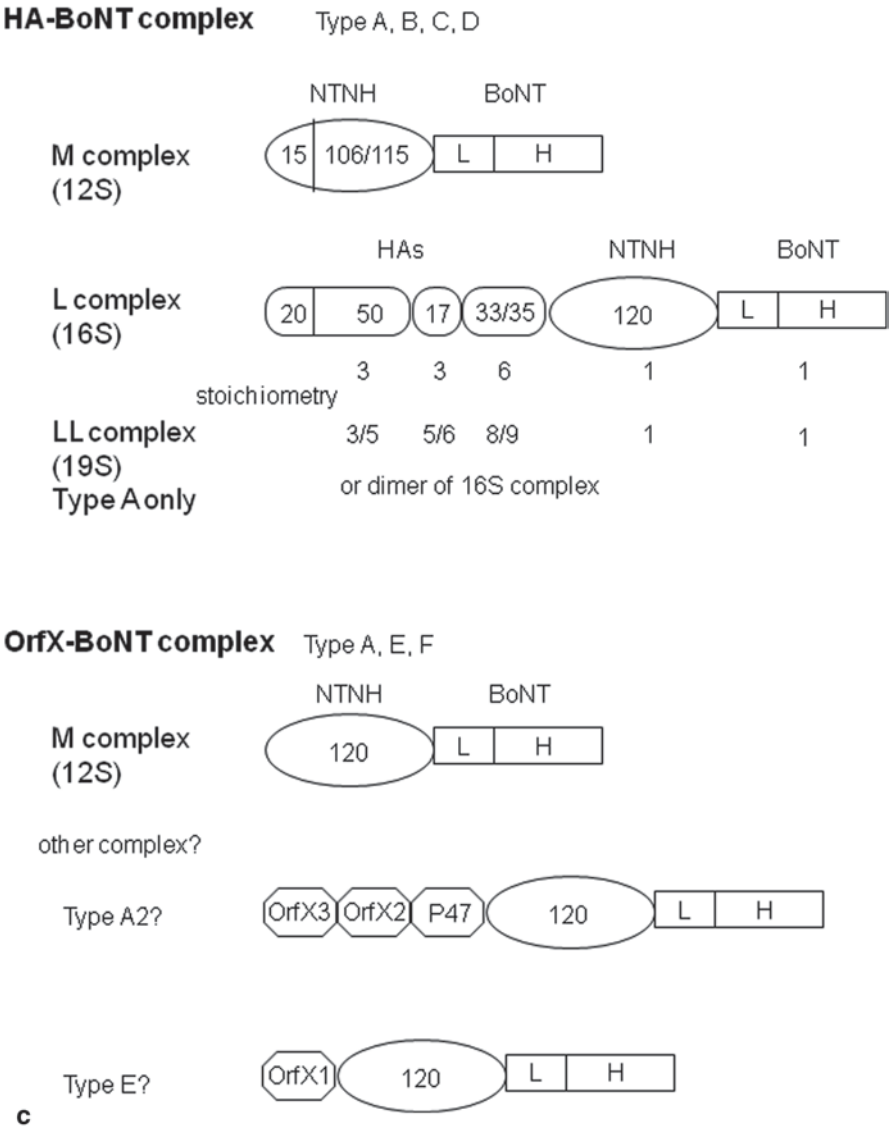


Fig. 3.1 Genome organization of botulinum loci and botulinum toxin organization. **a** Genome organization of botulinum loci in *Clostridium botulinum* type A and subtypes strains**. b** Genome organization of botulinum loci in *C. botulinum* type B to G strains. **c** Composition of the main botulinum complex forms. Numbers inside the boxes indicate protein sizes in kDa. L, light-chain; H, heavy-chain; NTNH, nontoxic non-hemagglutinin; HA, hemagglutinin

rotoxins and ANTPs have been cloned and sequenced in representative clostridial strains of each BoNT type. The neurotoxin and ANTP genes are clustered in close vicinity and constitute the botulinum locus. The organization of the botulinum locus is conserved in the 3′ part, but differs slightly in the 5′ part between the different

types of BoNT-producing clostridia. The *bont* genes are at the 3′ part of the locus and are preceded by the genes of the NTNH components. *ntnh* and *bont* genes are transcribed in the same orientation and form an operon, whereas the HA genes (*ha*33, *ha*17, and *ha*70), which are upstream to the *ntnh–bont* genes, are organized in a second operon transcribed in the opposite orientation. The *ha* genes are missing in the non-hemagglutinating toxinotypes A2, E, and F. The *ha* genes of *C. botulinum* G only comprise *ha*17 and *ha*70. In the toxinotype A2, E, and F, a gene (*p*47) encoding a 47-kDa protein is immediately upstream of the *ntnh* gene, and both genes are transcribed in the same orientation. In addition, two genes (*orfX1* and *orfX2*), which are not related to *ha* genes, lie upstream of *p47*, in the opposite orientation. An additional gene, *orfX3*, was characterized in *C. botulinum* A2 strain kyotoF, downstream the gene *orfX2* and in the same orientation [40]. *p47* and the *orfX* operon are also associated with *bont* subtypes A1, A3, or A4 [81]. Indeed, two main types of botulinum locus can be distinguished, the HA locus containing *ntnh* and *ha* genes and the OrfX locus containing *orfX*, *p47,*and *ntnh* genes, in addition to the *bont* gene (Fig. 3.1). Interestingly, the same *bont* gene can be inserted in a HA or OrfX locus. However, *bont/A1* is the only gene, which has been found in either of the two types of botulinum locus. Therefore, BoNT/A1 can form two different types of complex by association with Ha or OrfX proteins, which can influence its stability, as well as its absorption and dissemination in the organism.

C. botulinum A produces three types of botulinum complex designated LL (19S, 900 kDa), L (16S, 500 kDa), and M (12S, 300 kDa). The 12S complex results from a BoNT/A plus NTNH association and does not possess any hemagglutinating activity. NTNH has a cleavage site within the N-terminus and is separated into 13- and 106-kDa fragments as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The 16S complex consists of BoNT/A, NTNH, and HAs (HA35, HA17, and HA70) at a presumed 1:1:2 ratio. HA70 is proteolytically cleaved into HA50 and HA20. The 19S complex has the same protein composition as that of the 12S complex, but the ratio of HA35 is about twice that found in the 12S complex. The 19S complex is likely a dimer of the 16S complex cross-linked by HA35 [128, 162]. The composition and organization of botulinum complex types A2 and A1, which contain different proteins like P47, OrfX1, OrfX2, and OrfX3, remain undetermined [39]. *C. botulinum* A2, E, and F only produce M complexes devoid of hemagglutinating activity, and *C. argentinense* produces only L complex [128]. M botulinum complex type A2 only contains BoNT/A2 and NTNH; although P47, Orfx2, and OrfX3 are produced in the culture supernatant, OrfX1 is not produced or is produced in very low amounts [102]. OrfX botulinum complexes are possibly unstable and can easily dissociate. OrfX1 has been detected in botulinum complex type E but not in type F, whereas OrfX2 and P47 have not been evidenced in either toxinotype [74].

C. botulinum B, C, and D yield two complex types, L and M, the composition of which is equivalent to those of *C. botulinum* A [6, 128]. The complete subunit structure of *C. botulinum* D strain 4947 L complex (650 kDa) was determined as one BoNT/D, two HA70, four HA33, and four HA17 molecules, with intermediate complexes (540 and 610 kDa) constituted of various HA subunits. In addition, an intermediate M complex (410 kDa) contains BoNT/D, NTNH, and two molecules of HA70 [92, 123].

Overall, the 12S complex results from association of a BoNT together with a NTNH at a 1:1 ratio. The 16S complex consists of a BoNT, a NTNH, and several HAs (HA70, HA17, and HA33) at a presumed 2:4:4 ratio, thus forming a dodecamer. However, transmission electron microscopy studies suggest an ellipsoidal-shaped structure with three arms at a 1:1:3:3:6 ratio [72]. An elegant negative stain electron microscopy and a single particle averaging study showed that BoNT/A and BoNT/B complexes share a similar structure. BoNT/A and BoNT/B 16S complexes adopt an ovoid structure with three flexible appendages, whereas the 12S BoNT/E complex is devoid of these arms. It is deduced that the 16S BoNT/A and BoNT/B complexes are a result of the assembly of BoNT/A or BoNT/B, NTNH, HA33, HA17, HA70 at a presumed 1:1:6:3:3 ratio [14]. The HA33 are likely to be at the periphery of the complex. The 19S complex produced only by serotype A looks like a dimer of the 16S complex; importantly, it contains two molecules of BoNT/A. However, a more refined stoichiometry of the 19S complex (925 ± 45 kDa) indicates 1 BoNT/A, 1 NTNH/A, 5–6 HA17, 4–5 HA23, 3–4 HA48, and 8–9 HA34 [101].

NTNHs from different *C. botulinum* types possess a high identity level (76–83.5 %) and are the most conserved proteins in various botulinum complexes [137]. NTNH/A, NTNH/C, and NTNH/D contain a cleavage site within their N-terminus, yielding 15-kDa N-terminal and 115-kDa C-terminal fragments. NTNH/A is split into 13- and 106-kDa fragments by cleavage between Pro144/Phe145 [62]. NTNH/C and NTNH/D are cleaved at Lys127 by a trypsin-like protease with 7–13 amino acids removed from the N-terminus of the 115-kDa fragment that subsequently results in three proteins starting at Leu135, Val139, or Ser141 [153]. NTNH is only cleaved in the 12S (M) complexes from *C. botulinum* types A, C, and D, but not in the L (16S) or LL (19S) complexes. The cleaved NTNH molecules constitute a nicked structure since the two fragments still remain together after NTNH purification [153]. In contrast, NTNH/E and NTNH/F show an identical deletion of 33 residues in the corresponding regions of NTNH/A, NTNH/C, and NTNH/D encompassing the cutting site, and NTNH/G possesses a slightly different sequence in this region. It is presumed that the processing and additional sequence of NTNH in *C. botulinum* A, C, and D are responsible for forming 12S-, 16S-, and 19S-sized complexes. The inability of *C. botulinum* E and F to form L complexes may result from the absence of HA or other related proteins that bind to NTNH, and from the absence of a putative binding site in NTNH/E and NTNH/F [128, 137].

HA33–35 is the most abundant HA component of the botulinum complex. Type A HA35 binds to oligosaccharides containing galactose-β1–4glucose-*N*-acetyl-D-neuraminic acid (Galβ1–4GlcNAc) [77]. Thereby, hemagglutination induced by 16S and 19S type A botulinum complex is mainly mediated through HA35 binding to erythrocyte membrane glycolipids and glycoproteins containing Galβ1–4GlcNAc, such as paragloboside and glycophorin A [76, 77]. Similarly, HA33 from types C and D botulinum complex binds to paragloboside on Galβ1–4GlcNAc and also sialylglycolipids (GM3), as well as sialoglycoproteins (sialosylparagloboside) on the *N*-acetyl-D-neuraminic acid-α2–3-galactose-β1 motif [58]. The importance of HA33–35 in hemagglutination is also supported by monoclonal antibody studies. Type C-specific monoclonal antibodies against HA33 inhibit hemagglutination, contrary to those against HA50 and HA17 [105]. However, type C HA70 and

its derivative HA50 recognize sialosylparagloboside and GM3 at the *N*-acetyl-D-neuraminic acid-α2–3-galactose-β1motif in erythrocyte membranes, like the corresponding 16S botulinum complex. Thus, HA50 could also be involved in hemagglutination [58]. HA35 purified from *C. botulinum* A is predominantly a dimeric, β-sheet protein in aqueous solutions. In *C. botulinum* A, five N-terminal amino acids are removed from HA35, but similar posttranslational modification has not been observed in HA33 from *C. botulinum* C. The significance of HA35 processing on its biological activity is not known [161]. It was first discovered that the 31 C-terminal amino acids, which contain a predicted carbohydrate recognition site, play an essential role in hemagglutination [154]. The structure of type C HA33 shows two β-trefoil domains consisting of a six-stranded, antiparallel β-barrel capped on one side by three β-hairpins. Related β-trefoil structures bind to oligosaccharides and are found in other proteins, including various lectins like the ricin B-chain, cytokines, trypsin inhibitor, xylanase, as well as the C-terminal part of BoNTs. Type A HA35 retains a similar structure related to the carbohydrate-binding site of ricin, a plant toxin. It is noteworthy that Asp263 and Asn285 of HA35, which are conserved in the lactose-binding site for ricin B-chain, are critical for carbohydrate binding [8, 78].

3.3 Naturally Acquired Botulism

Three main forms and two occasional forms of human botulism are recognized according to the mode of acquisition.

3.3.1 Botulism by Intoxination or Food-Borne Botulism

Food-borne botulism results from ingestion of preformed BoNT in food contaminated by *C. botulinum*. Most botulism outbreaks are due to home-canned, home-fermented, or home-processed low-acid vegetables. Commercial products like preserved food with insufficient heat treatment and storage at temperature permitting *C. botulinum* growth are, however, also responsible of some botulism outbreaks.

C. botulinum spores are heat resistant and can develop in food in anaerobic conditions like canned or vacuum-sealed food. Proteolytic group I *C. botulinum* strains (type A, B, and F) grow between 10 and 40 °C with an optimum temperature around 37 °C, whereas non-proteolytic group II *C. botulinum* strains (type E and non-proteolytic B and F) can develop and produce toxin at low temperature (minimum temperature 3 °C), implying that botulism can occur with refrigerated food. BoNTs are synthesized during the late exponential growth phase and the beginning of the stationary phase [33]. Even a limited multiplication of *C. botulinum* in food can release a sufficient amount of BoNT able to cause the disease.

3.3.2 Botulism by Intestinal Colonization

Ingested *C. botulinum* spores can lead, under certain circumstances, to the development of *C. botulinum* and toxin production in the intestine. This is the case in infant botulism, which is an important problem in infants from birth to 6 months in some countries, such as in the USA, and also in certain adults [167]. *C. botulinum* is not an invasive bacterium and its proliferation in the digestive tract is impaired by the resident bacterial microflora or microbiota. In young infants, however, the microbiota is not yet fully developed or not yet functional, and it is estimated that the ingestion of a low number of *C. botulinum* spores (10–100) is enough to permit an intestinal colonization and to cause the disease [9]. In adults, predisposing factors which induce a perturbation of the microbiota equilibrium, including intestinal surgery, antibiotherapy, chronic inflammation, necrotic lesions of the intestinal mucosa, and anatomical or functional abnormalities of the intestine, can promote *C. botulinum* intestinal colonization from ingested spores.

3.3.3 Wound Botulism

Wounds can be colonized by *C. botulinum* resulting in the onset of botulism. Profound wounds with low exposure to air and the presence of necrotic tissues can support the germination of clostridial spores, such as those of *C. botulinum* or *C. tetani,* and can result in the subsequent multiplication in anaerobic conditions. The occurrence of wound botulism is, however, much lower than that of tetanus. BoNT is released locally and diffuses to surrounding neuronal endings.

3.3.4 Inhalational Botulism

In addition, botulism can be acquired by inhalation. Very few cases of botulism by inhalation have been reported. Three cases of laboratory intoxications, subsequent to the inhalation of dust containing *C. botulinum* type A, have been described [75]. This indicates that the respiratory system constitutes a portal for BoNT entry into the organism, and aerosol dissemination represents a likely way for the use of BoNT as a biological weapon [11]. Indeed, BoNT is considered by the Center of Disease Control as a serious potential threat and has been listed in the category A of the highest risk threat agent for bioterrorism [11, 166].

3.3.5 Iatrogenic Botulism

Iatrogenic botulism is a rare complication of therapeutic use of BoNT/A and BoNT/B. BoNT is commonly used for the treatment of focal dystonia and localized

muscle spasticity. Although BoNT therapeutic doses are relatively safe and effective, some patients develop localized, or more rarely generalized, symptoms of botulism, including dysphagia, dysphonia, muscle weakness, dyspnea, and/or respiratory distress. Indeed, 187 cases of iatrogenic botulism have been reported to the Food and Drug Administration; among them 87 people have been hospitalized and 16 died [13]. After BoNT injection, the symptoms occur several days until 3 weeks. Toxin overdoses have been responsible for some fatal generalized botulism, but adverse effects might be induced by a wide range of BoNT doses including the lowest recommended doses [31]. Various factors might mediate the onset of adverse effects, notably those that influence the toxin diffusion, such as toxin preparation characteristics (pure BoNT or botulinum complexes, heterogeneity, and size of botulinum complexes), dosing and injection technique, properties of the formulation (protein load or other additive), and muscle injected. Host susceptibility might also be involved. Thus, women rather than men develop complications following BoNT injection [30, 82].

3.3.6 Clinical Manifestations of Botulism

The clinical manifestations of botulism are characterized by a symmetric, descending, flaccid paralysis of autonomic nerves followed by that of motor neurons, beginning with the cranial nerves. The initial symptoms usually concern dysautonomic disorders including blurred vision, diplopia, followed by a dry mouth, swallowing difficulty, and then constipation. Additional dysautonomic dysfunctions include loss of vagal cardiac control, hypothermia, and urinary retention. Dysautonomic symptoms can sometimes be the only manifestation of botulism and correspond to benign forms of the disease. They also support that BoNTs act upon both cholinergic and catecholaminergic neurotransmission. In the more severe forms of the disease, the descending paralysis affects first striated muscles innervated by cranial nerves producing drooping eyelid or ptosis, followed by a depressed gag reflex, dysphagia, dysarthria, facial paralysis, tongue weakness, and several neuro-ophthalmological signs. Then, general paralysis extends from proximal to distal muscles of the neck and limbs. When the diaphragm is attacked, respiration is depressed requiring mechanical ventilation [104, 138, 177]. The gastrointestinal symptoms like nausea, vomiting, and diarrhea, which are reported in about half of the cases of naturally acquired botulism, are not due to BoNTs, but due to other enterotoxins or food contaminants. Indeed, these clinical manifestations are observed neither in wound botulism nor in experimental animals with BoNT administration.

3.3.7 BoNT Dissemination in Naturally Acquired Botulism

Except for iatrogenic botulism, which results from parenteral injection of BoNT, the other forms of botulism require a prerequisite step consisting in toxin passage through an epithelial barrier, the epithelium of digestive or respiratory system. In

food-borne botulism, BoNT could be absorbed at one or more sites of the digestive tract from mouth to colon, whereas in botulism by intestinal colonization, the intestinal epithelium is likely the main entry portal. In most patients with food-borne botulism (about 70–80 %), BoNT is demonstrable in the serum from the second day after the onset of symptoms to several days or weeks [103, 159]. In contrast, toxemia is infrequent in infant botulism, whereas BoNT and *C. botulinum* are usually found in feces [10, 21, 38, 73, 133]. In addition, constipation is a major sign of infant botulism and this symptom is more rarely observed in food-borne botulism. Similar findings have been reported in botulism by intestinal colonization in older infants or adults. For example, a 12-year-old girl developed an intestinal colonization by *C. botulinum* type Ab and suffered from obstinate constipation for more than half a year. *C. botulinum* and toxin were detected in stools more than 122 days after the beginning of the disease [90]. This suggests that BoNT uses a different trafficking and possibly distinct absorption sites, in food-borne botulism compared to botulism by intestinal colonization. BoNT absorbed from the small intestine during *C. botulinum* intestinal colonization probably targets neurons directly from the enteric nervous system leading to intestinal muscle paralysis.

Interestingly, whatever the origin of botulism, food-borne botulism, intestinal colonization or wound botulism, the clinical signs develop in the same order with attack of cranial nerves and then spinal motor neurons. BoNT dissemination through the blood circulation would suppose that BoNT would affect all the nerve endings, yielding a generalized paralysis and not a descending paralysis. Possibly, some neurons like cranial nerves are more sensitive to BoNTs than others are, or BoNTs use a more specific transport route to the target neurons. In iatrogenic botulism, BoNT/A used at therapeutic doses can affect neuromuscular junctions at distant sites to the local injection. One explanation could be that BoNT is locally trapped and transported in an axonal retrograde manner to spinal motor neurons [16, 63, 90]. Injection of BoNT/A into cat extraocular muscles changed the activity of motor neurons innervating both lateral and contro-lateral eye muscles [121]. A more recent study in rats shows unambiguously that BoNT/A is retrogradely transported. BoNT/A catalytic activity was observed in synaptic terminals within the retina after toxin injection into the optic tectum or in the facial nucleus following a toxin injection in whisker muscles. This demonstrates that the toxin cannot be transported by simple diffusion, but only by an axonal retrograde migration in motor neurons and central neurons and then transcytosis to afferent synapses [5].

The lethal dose (LD) of BoNT/A in human is estimated to be 1 μg/kg by the oral route, 10–13 ng/kg by inhalation and 1–2 ng/kg by intravenous or intramuscular injection [12, 116, 166]. Thus, passage through an epithelial barrier is less efficient than direct toxin delivery to target neurons by blood circulation or dissemination in muscle tissue. This suggests that only a fraction of BoNT (0.1–1 % when parenteral inoculation is compared to oral or respiratory route) is transported through an epithelium. The epithelial barrier of the respiratory tract is seemingly more permissive than that of the digestive tract, unless BoNT is partially degraded in the digestive tract depending of the toxin type (complex or dissociated BoNT; size of complexes), since no information on toxin conformation is available in these reports.

3.4 Experimental Animal Botulism

Many attempts have been made using experimental animals to understand the absorption and dissemination of the toxin into the organism. Given the extreme potency of BoNT, the major difficulty is to monitor minute amounts of toxin, equivalent to those involved in natural conditions.

3.4.1 Experimental Botulism by Gastrointestinal Route

Oral toxicity of BoNTs was assayed in several animal species, and the activity levels were found to vary according to the molecular form of toxin. Thereby, BoNT/A in LL complex form, orally administrated in mice, was 358-fold more toxic than pure BoNT/A, whereas pure BoNT/A was 43-fold more potent by intravenous route. BoNT/A L complex form was about 20-fold more and M complex form about 12-fold more active than pure BoNT/A by oral route [130]. Overall, the highest oral toxicities correlate with the larger molecular sizes of toxin complexes. The same findings were observed with BoNT/B, BoNT/E, BoNT/C, and BoNT/D [88, 129, 130, 171]. BoNT/B L complex showed the highest oral toxicity compared to the pure toxin (about 16,000-fold more), even more than the largest LL complex form of BoNT/A. Other factor(s) than the complex molecular size might be involved in oral toxicity. Stoichiometry and/or configuration of BoNT complexes could also play an important role (see below). The higher oral toxicity of BoNT complexes versus pure BoNT is mostly attributed to a higher toxin stability. BoNT/A, BoNT/B, and their complex forms of various sizes have been tested for resistance to peptic digestion at pH 2, gastric juice at pH 1.4, pancreatic digestion at pH 6.5 and intestinal juice at pH 7. The largest complex forms (LL type A and L type B complex) showed the highest stability and retained 60–80 % of activity after 120 min exposure to the proteolytic treatments, whereas pure BoNT/A and BoNT/B were almost or completely inactivated after 10 min [171]. Dissociated botulinum complexes type A or E are much more unstable than the corresponding undissociated forms, particularly below pH 4 [88, 171]. Therefore, not only the presence of all ANTPs accompanying the BoNT but also a strict organization or conformation of botulinum complexes seem to be required to yield the most stable and active molecules. Are all ANTPs equipotent to confer the stability of botulinum complexes or which ones play the most important role? HA34 and HA52 from botulinum complex type B seem to encase HA17, which is sensitive to protease digestion, and likely to have an important protective function [7]. HA35 and HA52 from botulinum complex type A are resistant to protease digestion, whereas NTNH/A and BoNT/A are rapidly degraded [28, 33, 51, 161], and yet NTNH, which is structurally related to BoNT, associates with BoNT in a pH-dependent manner to form a medium-sized botulinum complex highly resistant to acidic pH and protease degradation [68, 69]. Similarly, although BoNT/D and NTNH/D are individually easily degraded by pepsin or trypsin treatment, the complexes resulting from association of BoNT/D and NTNH/D are highly

resistant [117]. Thus, NTNH probably also has a crucial role in the stability of botulinum complexes. This further supports the importance of botulinum complex conformation in the protection of BoNT against low pH and protease degradation.

The toxin absorption sites from the digestive tract have been investigated in rabbits and rats. Albeit BoNT can be absorbed from different digestive segments including stomach, ileum, and colon, the most efficient absorption site was identified in the upper small intestine [19]. In mice, however, the uptake of pure BoNT/A or botulinum complex with or without HAs was equivalent from the stomach and intestine. Interestingly, the protective effect of ANTPs in botulinum complex was found to be dose dependent. Indeed, the low BoNT/A concentration (equivalent to 25 ng of pure BoNT/A/mouse) was completely inactivated when administered in the stomach, whereas the same BoNT/A dose in the complex form with HAs was fully active. In contrast, higher BoNT/A dose (1 μg/mouse) as pure toxin or in complex form yielded the same toxicity when injected into the stomach or intestine. In addition, the stability of pure BoNT/A was found to be greater in the intestine versus the stomach [109]. Therefore, the stomach represents the most offensive environment for the stability of BoNT versus the intestine, and the association of BoNT with ANTPs in an optimal stoichiometry and conformation affords toxin protection mainly at low BoNT concentration.

BoNT administered in rat duodenum is found in the lymph and then in the blood, but to a lower extent [19, 172]. When L botulinum complex type B was injected into the duodenum, which was tested as the most efficient digestive segment from the stomach to the rectum for toxin uptake, a maximum level of active toxin was recovered 6 h later in the lymph. The rate of toxin absorption in the lymph ranged from 0.01 to 0.1 % of the intraduodenally administered dose and was about 100-fold less in the blood. Injection of an equivalent dose of M botulinum complex type B or pure BoNT/B in rat duodenum yielded a much lower toxin recovery in the lymph (about 50–100-fold less) [172]. Comparable absorption rate was found with LL botulinum complex type A [98, 111], whereas another report mentions a recovery of 1.5 % or more in the blood of rabbits orally inoculated with M botulinum complex type E (cited in [172]). Experimental differences regarding laboratory animals, toxin preparations, and methods of titration greatly influence the results and make the comparison between the different reports difficult. It can be concluded, however, that BoNT recovery in the lymph and blood, following oral administration in experimental animals, is very low but sufficient to induce a fatal disease, in agreement with the observations in naturally occurring cases of botulism.

Is only BoNT or the whole botulinum complex absorbed from the digestive tract? ANTPs are non-covalently associated to BoNT, and proteins dissociate at pH 7.5 and above, and yet L and M botulinum complexes type B were found to be undissociated in intestinal juice at pH 7 [171]. This indicates that additional factors, such as ionic strength, control the assembly of botulinum complexes and further account for the toxin stability in the intestinal content, since it is protected in the complex form. Using a reversed passive hemagglutination assay, ANTPs have been detected in the lymph of rats inoculated intraduodenally, suggesting that the botulinum complexes, in their associated or undissociated form, can pass through the

intestinal barrier. The status of BoNT and botulinum complexes in the lymph has been monitored by ultracentrifugation in gradient sucrose. L botulinum complex type B appeared in the undissociated form in the lymph, whereas M botulinum complex type B did not [172]. Botulinum complexes type A and E orally administrated have also been found in their undissociated form in the lymph [88]. This does not exclude that ANTPs and BoNT can cross the epithelial barrier in the undissociated form and reassemble in the lymph. In a natural case of type A botulism by intestinal colonization, monomeric BoNT/A was identified in the blood. The detection of ANTPs was not reported, thus making it difficult to know whether only BoNT/A was translocated into the blood circulation or the whole complex was translocated with subsequent dissociation [90]. The amount of toxin in the intestine might greatly influence the passage of BoNT alone or botulinum complexes in their associated or undissociated form into the lymph and blood. Additional experiments with more accurate and sensitive methods are required to clarify the status of BoNT, which is transported from the digestive tract to the internal medium.

3.4.2 Experimental Intestinal Colonization with C. Botulinum

Attempts to reproduce botulism by intestinal colonization have been performed in mice and rats. Conventional adult mice and rats administered intragastrically with a high number of *C. botulinum* type A spores (up to 10^9) rarely develop a toxicoinfection. In contrast, germ-free mice or rats orally inoculated with only ten *C. botulinum* spores show signs of botulism within 4–6 days. *C. botulinum* growth was evidenced in the cecum, colon, and ileum, and BoNT/A accumulates in the gut, reaching $1.3–1.8 \times 10^5$ mouse intraperitoneal lethal doses 50% (LD50) mainly in the cecum and to a lower extent in the colon and the lower ileum. Susceptible axenic mice or rats exposed to conventional animals for 3 or 6 days become resistant to an oral challenge of 10^5 spores, and they do not contain any detectable toxin in the gut. Therefore, the intestinal microflora, which is the only difference between axenic and conventionalized animals, is the main factor involved in the resistance to colonization of the digestive tract by *C. botulinum* [118, 119]. The composition of intestinal microflora responsible for the *C. botulinum* colonization resistance has not been determined. Not all intestinal bacteria, but rather a complex association of specific bacteria, are involved in the prevention of *C. botulinum* growth [183]. The role of intestinal microflora in *C. botulinum* colonization resistance is also supported by conventional mice orally treated with antibiotics (erythromycin and kanamycin), which show a much higher susceptibility to multiplication of this pathogen in the digestive tract [22].

Intestinal colonization with *C. botulinum* has also been investigated in young conventional mice and rats. When intragastrically challenged with 10^5 spores, only animals that were 7–12 days old were colonized, but did not become ill. The 9-day-old animals were the most susceptible. The 50% infecting dose in 9-day-old mice has been estimated to be 700 spores (170–3,000) per animal, and the toxin was present in the large intestine, but not in the small intestine, from 1 to 4 days and in

some cases 7 days after challenge. Toxin amounts (60–1,920 mouse intraperitoneal LD50) in the colon of suckling mice intragastrically inoculated with *C. botulinum* spores reached at least a tenfold greater level than the minimum needed to kill them by the intraperitoneal route, despite any apparent sign of botulism. In contrast, axenic adults monoassociated with *C. botulinum* showed a higher toxin titer (2,300–215,000 mouse intraperitoneal LD50) in the cecum, colon and lower small intestine and became ill. Suckling mice might be less sensitive than adults are to BoNT, but also the toxin, which is only present in the colon, is not as efficiently absorbed compared to the small intestine. This also argues that BoNT passage through the intestinal barrier is not an efficient process. The resistance of very young mice and rats less than 5–6 days, which showed neither *C. botulinum* multiplication nor toxin production in the digestive tract, cannot be explained by an absence of an intestinal microflora preventing the multiplication of *C. botulinum*. Possibly, other factors such as milk factors could be involved [118, 119, 174].

3.4.3 Experimental Botulism by Inhalation

Botulism by the inhalation route has been investigated in experimental animals in order to better understand the disease through this inoculation route. Although toxin dissemination by aerosol is not a common source of naturally acquired botulism, it represents the most likely use of BoNT as a biological weapon. Mice are susceptible to inhalational botulism [132]. The BoNT/A LD50 is estimated to be 21 ng/kg [179], which is higher than that by the intraperitoneal route: 0.3 ng/kg [115] to 1.2 ng/kg [65] for BoNT/A, 0.5–0.6 ng/kg for M complex, and 0.8–1 ng/kg for L complex of BoNT/A or BoNT/B [173]. Mice inoculated by the respiratory route develop all the characteristic symptoms of botulism [179]. Rhesus macaques are also susceptible to botulism by inhalation. The LD50 was calculated to be 17 ng/kg (550 mouse intraperitoneal LD50) for BoNT/A1, which is comparable to that estimated in human, and 280 ng/kg (21,600 mouse intraperitoneal LD50) for BoNT/B1 [11, 156]. The onset and the severity of the symptoms in mouse or monkey are toxin dose dependent [156, 179]. In addition to the common symptoms of botulism, monkeys develop abnormal signs like anorexia when challenged with low doses. Other abnormal clinical findings, including ataxia, lethargy, coughing and dysphagia, occur more frequently at middle doses of BoNT/A1 or BoNT/B1. All monkeys inoculated with BoNT/A1 by the respiratory route, even with high dose, up to 5,275 mouse intraperitoneal LD50, did not show any detectable circulating toxin, whereas toxin was evidenced in the blood of most but not all the monkeys challenged with BoNT/B1 doses from 25,000 to 82,000 mouse intraperitoneal LD50 [156]. All monkeys with detectable BoNT/B1 in the blood and some monkeys without detectable circulating toxin died [156]. This indicates that BoNT dissemination through the blood circulation is not an obligate pathway to transport the toxin to target neurons, unless blood transport of effective toxin molecules is not detectable. Albeit no specific lesions are attributable to BoNT, monkeys and mice treated by the respiratory route manifest a suppurative inflammation of the lungs, accompanied by a variable de-

gree of perivascular and generalized interstitial edema, moderate intra-alveolar/intrabronchiola hemorrhage and polynuclear infiltration. It is not clear whether BoNT can directly trigger an inflammatory response, or whether the pulmonary histological changes result from an aspiration pneumonia secondary to paralysis of muscles responsible for swallowing and thus permitting the entry of food or stomach content through the larynx [156, 179].

3.5 Passage of BoNT/Botulinum Complexes Through Cultured Epithelial Cell Monolayers

3.5.1 BoNT Passage Through Intestinal Epithelial Cell Barrier

There is evidence that BoNT undergoes a receptor-mediated transcytosis to cross the intestinal barrier. Indeed, BoNT/A transport through polarized intestinal cell monolayers is inhibited at 4°C and occurs at 37°C in a saturable manner over a 30–60-min period [2, 34, 107], which is compatible with previously reported receptor-mediated transcytosis through epithelial cells [122, 131]. This is further supported by the fact that BoNT/A is transported through epithelial cells within 2 h, without either trans electrical epithelial resistance (TEER) alteration or any organizational disturbance of tight and adherens junctions, thus excluding a paracellular passage of BoNT/A. In addition, competition experiments showing that BoNT/A transport through intestinal cell monolayers was significantly prevented by an excess of recombinant BoNT/A Hc, but not by Hc from BoNT/B or tetanus neurotoxin (TeNT), strongly argue for a specific receptor-mediated transcytosis. This is in agreement with the fact that iodinated BoNT/A H-chain as well as Hc domain are able to bind and cross epithelial cells [107]. Among the cell lines that have been tested, the mouse intestinal crypt cells m-IC_{cl2} showed the highest level of BoNT/A passage, whereas the colon carcinoma cells, Caco-2 or T84, which retain a phenotype of enterocyte [15], showed a lower passage rate. Since many pathogens preferably use M cells to enter the intestinal barrier [86, 131], Caco-2 cells cocultured with murine lymphocytes differentiated into M cells have been tested [85]. No significant difference in BoNT/A passage was observed through Caco-2 cell monolayers containing or not M-like cells [34]. This strongly suggests that BoNT can pass more efficiently through certain cell types of the intestinal mucosa, and that intestinal crypt cells may represent a preferential site of BoNT/A absorption. Indeed, using a mouse intestinal loop test, fluorescent BoNT/A Hc has been found to preferentially localize into neuroendocrine intestinal crypt cells, mainly serotoninergic cells [37].

The rate of transcytosed BoNT/A through an intestinal cell monolayer is low, about 1 % with m-IC_{cl2} and 0.1 % with Caco-2 cell monolayers after 2 h of incubation [34]. These transport rates are in agreement with the translocation efficiency through epithelial cells of bacterial or viral enteric pathogens ranging from 0.1 to

10% of the inoculum within 2 h [85, 131] and also with that of BoNT/B from rat duodenum to the lymphatic circulation (0.01–0.1%) [172] (see the earlier section). Therefore, the passage of BoNT through an epithelial barrier is a weakly efficient process in the same range as that of enteric pathogens.

The receptor-mediated transcytosis process raises the question whether BoNT receptors on intestinal cells are the same as those on neuronal cells? As for neuronal cells, the same toxin domain, Hc, is involved in the recognition of receptors on the intestinal cell surface. Thereby, BoNT/A Hc is able to bind and cross epithelial cells [107]. Moreover, BoNT/A transport through intestinal cell monolayers is significantly prevented by an excess of recombinant BoNT/A Hc, but not by Hc from BoNT/B or TeNT [34]. Gangliosides GD1b/GT1b are also part of BoNT receptor on intestinal cells. Indeed, addition of GD1b/GT1b impairs the binding of fluorescent BoNT/A Hc to intestinal cells as well as to neuronal cells as assayed by fluorescence-activated cytometry (FACS) and significantly decreases the transport of biologically active BoNT/A through intestinal cell monolayers. Furthermore, ganglioside depletion by DL-threo-1-phenyl-2-palmitoyl-amino-3-morpholino-1-propanol (PPMP), an inhibitor of glucosyl ceramide synthase, strongly decreases BoNT/A transport through intestinal cells, and loading PPMP-treated cells with GD1b/GT1B reverses the effect [34]. Synaptic vesicle glycoprotein 2C (SV2C), the protein part of BoNT/A receptor on neuronal cells, is also expressed in intestinal cells, such as CaCo-2 or m-IC_{cl2} cells. Recombinant intraluminal domain (L4) of SV2C was found to decrease BoNT/A transcytosis through CaCo-2 or m-IC_{cl2} cell monolayers suggesting that SV2C or an SV2C-related protein might be involved in the BoNT/A receptor on intestinal cells. Thus, BoNT receptors might be the same or consist of related molecules on neuronal and intestinal cells. However, BoNT/A Hc binding to intestinal cells is much lower to neuronal cells as monitored by FACS, indicating that the affinity is weaker or the number of BoNT receptors is possibly smaller on intestinal cells when compared to neuronal cells [34]. A different type of BoNT/A receptor on the colon carcinoma T84 cells has been suggested, based on the fact that a recombinant BoNT/A Hc mutated in the ganglioside binding site (W1266 L, Y1267S) was impaired in binding to neuronal cells, but retained the ability to cross the intestinal cell monolayer [46, 149]. Based on the dual nature of BoNT receptor, a ganglioside associated with a membrane protein, mutation of the ganglioside-binding site does not preclude that the receptor protein part or the global receptor organization is different on intestinal cells. A difference between BoNT receptors on neuronal and intestinal cells might be a distinct distribution in membrane subdomain or different exposition of the site(s) accessible to the toxin.

The distribution of BoNT receptors on the cell membrane is not fully understood. Considering the lipid BoNT receptor moiety, this is presumed to be localized in distinct lipid microdomains, but the protein moiety (SV2 for BoNT/A, BoNT/E, and BoNT/F, synaptotagmin for BoNT/B and BoNT/G) [41–43, 53, 106, 125, 148, 151] supposes a distribution on synaptic vesicle membranes,and these proteins have a wider membrane distribution. Lipids are heterogeneously distributed in plasma cell membranes, and the most well-known lipid organization is the lipid microdomains which are rich in cholesterol and sphingolipids [113]. Using the C-terminal domain

of *C. perfringens* perfringolysin (PFO), an extensively characterized cholesterol-binding cytolysin, [141], fused to green fluorescent protein (GFP; GFP–PFO), only a partial colocalization was observed with BoNT/A Hc on both neuronal and intestinal cells. This correlates with the absence of colocalization between BoNT/A Hc and probes of glycosylphosphatidylinositol-anchored proteins (GPI-APs), which are also localized in lipid rafts, such as *C. septicum* alpha toxin receptor or the decay-accelerating factor (DAF), as well as with the distribution of BoNT/A receptor on soluble fractions of membrane treated with Triton X100 rather than in detergent-resistant microdomains. In addition, depletion of plasma membrane cholesterol by a high concentration of methyl-β-cyclodextrin (MβCD; 15 mM) only partially prevented BoNT/A Hc binding to neuronal or intestinal cells. Incubation of cells first with GFP–PFO for 5 min, however, prevented further binding of fluorescent BoNT/A Hc [35]. Thus, the BoNT/A receptor seems to have a distinct membrane distribution, since it does not localize directly on cholesterol-enriched microdomains but in a subset of microdomains from detergent soluble fractions closely connected to the cholesterol-enriched microdomains.

The nature and membrane localization of receptors are critical to drive a specific intracellular ligand trafficking [99]. Indeed, cholera toxin binds to ganglioside GM_1 localized on lipid rafts and is transported into the endoplasmic reticulum, whereas *Escherichia coli* (*E. coli*) LTIIb, which is structurally and functionally related to cholera toxin, binds to GD_{1a}, a ganglioside not associated with lipid rafts, and it is not routed to the endoplasmic reticulum [57]. Lipid rafts do not seem to be required for BoNT/A entry into neuronal cells. Despite BoNT binding being greatly decreased to cells deficient in ganglioside synthesis [93, 150, 188], disruption of lipid rafts with MβCD or filipin does not prevent, but on the contrary enhances BoNT/A activity on N2a cells [134]. In addition, knockout mice lacking b-series gangliosides show a similar sensitivity to BoNT/A to wild-type mice, indicating that b-series gangliosides may not be essential in the initial steps of BoNT/A intoxication [89]. This suggests that BoNT receptors containing gangliosides GD_{1b}/ GT_{1b} are not restricted to lipid rafts, but have a wider distribution, possibly on distinct microdomain structures within the cell membrane. Interestingly, such a heterogeneous localization on membrane is supported by the finding that the same glycosphingolipid is distributed on distinct and contiguous membrane microdomains according to its concentration, low or high [160]. The accurate localization of BoNT receptor on neuronal and intestinal cell membranes, however, remains to be determined.

3.5.2 BoNT Passage Through Pulmonary Epithelial Cell Barrier

A transcytosis-mediated passage of BoNT/A has also been evidenced through a human pulmonary adenocarcinoma (Calu-3) cell monolayer. BoNT/A transport was significantly higher through Calu-3 cell monolayers than through Madin–Darby canine kidney (MDCK) cell monolayers suggesting a specific BoNT passage according to the epithelial barrier. A transcytosis mechanism instead of a paracellular BoNT passage across pulmonary cell monolayers was based on: (1) no modification

of the TEER of the cell monolayer, (2) no alteration of tight junctions as monitored by the absence of passage of small molecules, such as inulin, which are not endocytosed and can only diffuse upon disruption of intercellular junctions, (3) prevention of BoNT passage at low temperature compared to 37 °C indicating that an active process rather than a passive diffusion is involved and (4) inhibition of BoNT passage with specific antibodies. Transcytosed BoNT/A molecules were found to be in their active undissociated form, L- and H-chains linked by a disulfide bridge, like the native toxin [132].

3.5.3 Role of ANTPs in BoNT Passage Through an Epithelial Barrier

HA components bind to carbohydrate structures (see earlier section) and thus mediate agglutination of red blood cells. A binding function has been assigned to HAs, which seems to facilitate the binding of botulinum complexes to intestinal mucosa or directly to intestinal epithelial cells. In a guinea pig *in vivo* model, only the L form of botulinum complex type C (16S), which contains HAs, bound to epithelial cells of the upper small intestine prior to being absorbed from the intestine and then released into the serum, whereas botulinum complex type C lacking HAs (12S) or BoNT/C alone were poorly absorbed into the serum [55]. Among HA components from type C, type A or type B, HA33/35 and HA50 recognize distinct carbohydrate structures on erythrocytes and intestinal cells. HA33/35 binds to galactose moieties and HA50 binds to sialic acid moieties and are considered to be involved in botulinum complex binding and absorption through the intestinal mucosa [7, 56, 58]. HA33 and HA50/70 also facilitate the binding and transport of botulinum complexes type C or D through rat intestinal epithelial cell monolayers in a sialic acid-dependent manner. HA50/70 might bind to sialic acid on mucin and/or sialic acid-dependent receptors on the cell surface, and it is speculated that HA33 might also interact with sialic acid on the cell membrane [79, 80, 127]. Hence, sialic acid derivative molecules seem to be important motifs for the binding of botulinum complexes on intestinal cells and/or mucin. Botulinum complex type A binds to Intestine-407 cells, which are derived from human intestinal epithelium, via *N*-acetyl-lactosamine [91]. In human colon carcinoma cells (HT-29), type C 16S botulinum complex binds to surface glycoproteins like mucin [124]. However, HA components are not absolutely required, since BoNT/C or D alone displays binding and transport through intestinal cells, albeit to a lower extent [79, 80, 127].

A novel function has been attributed to HA components consisting in the disruption of intercellular junctions between intestinal epithelial cells. HA from type B and type A has been shown to alter tight and adherens junctions of CaCo-2, T84 or MDCK cell monolayers and to enhance BoNT transport. HAs act specifically on the basolateral side of cultured intestinal cells and induce a disorganization of molecules of the tight junctions (occludin, ZO1) as well as those of adherens junctions (epithelial (E)-cadherin, catenin). HA-dependent alteration of the intercellular junctions results in a paracellular passage of BoNT, 12S botulinum complex (lacking HAs), as well

as other macromolecules, without inducing cytotoxicity. In contrast, HAs from type C are ineffective in disrupting the human intestinal cell barrier, although they are able to alter cell monolayer integrity from canine (MDCK cells) or rat origin via a cytotoxic mechanism [60, 83, 110]. It has been evidenced that HAs type A and type B, but not type C, directly bind to the extracellular domain of human (E)-cadherin, but not neural (N)-cadherin nor vascular endothelial (VE)-cadherin. The combination of HAs (HA33, HA17 and HA50/70) is required for the optimum binding to E-cadherin, whereas the association of HA33 and HA50/70 is poorly effective and an individual HA does not interact with E-cadherin. Type B HAs specifically bind to human, bovine and mouse E-cadherin, but not to that of rat or chicken [169]. Tight junction disruption is apparently mediated by HA complex binding to E-cadherin. Indeed, MDCK cells expressing rat E-cadherin, which acts as dominant negative, maintain their intercellular junction integrity, although HA complex applied basolaterally binds to endogeneous E-cadherin as in control cells [169].

The HA mechanism of action on tight junction alteration via binding to E-cadherin is not yet known. A key question is, how can HAs have access to E-cadherin from the apical side? It is suggested that HAs bind to the apical surface and are subsequently endocytosed in early endosomes and then transcytosed through the basolateral side [169, 170]. This supposes that HAs recognize specific receptor(s) on the apical side and it is not clear whether the carbohydrates recognized by HAs are involved. It has been hypothesized that HA mediates the transcytosis of whole botulinum complexes through intestinal epithelial cells, which are delivered on the basolateral side allowing the access of HA to E-cadherin [61]. E-cadherin is the major player of the adherens junction, but its role in controlling tight junctions is controversial [25, 29, 70, 71, 136]. HAs bind to E-cadherin in a Ca^{++}-dependent manner without exerting a protease activity towards this substrate but rather changing its cellular localization. E-cadherin in HA-treated cells is redistributed from the cell–cell contacts to clusters on the cell surface and it is partially endocytosed [169]. Other bacterial toxins or virulence factors target E-cadherin. For example, *Bacteroides fragilis* enterotoxin proteolytically cleaves the extracellular domain of E-cadherin, which subsequently induces a degradation of the intracellular domain (probably by adenosine triphosphate (ATP)-dependent cellular proteases), reorganization of actin filaments, and increase in cell barrier permeability [157, 158]. Interestingly, the N-terminal part of HA70 shows sequence similarity with *C. perfringens* enterotoxin, which binds to claudins, forms pores and alters the intestinal barrier integrity [54, 114]. HA70 does not, however, retain *C. perfringens* enterotoxin activity on intestinal cells.

What is the significance of HAs in BoNT intestinal or pulmonary absorption *in vivo*? HA effects are late, since in cultured cells, an increase in barrier permeability is observed only after several hours (12–48 h) [60, 110], whereas BoNT transcytosis occurs in 30–60 min [34]. Another question, as discussed above, concerns how HAs have access to the basolateral side of intestinal epithelial cells? Do HAs, alone or associated with BoNT, undergo a transcytosis through the epithelial barrier *in vivo*? HAs are not indispensable, as BoNT alone can cross intestinal or pulmonary epithelial barriers in *in vitro* or *in vivo* models [34, 107, 132]. A more recent study shows

that BoNT/A and BoNT/E have an equivalent absorption rate and toxin potency when administrated by the inhalation route in mice as pure neurotoxin or complex form [4]. It has been reported that HAs enhance the passage of BoNT through intestinal cell monolayers by about 10-fold [110] and thus presumably increase BoNT absorption in food-borne botulism. Disruption of the intestinal epithelial barrier usually results in digestive symptoms like, increased secretion, diarrhea and mucosal inflammation, which are not observed in botulism. Hence, HAs might have a limited activity on the intestinal barrier, just enough to mediate a BoNT paracellular passage. HAs might be involved in botulism with classical type A or B strains, but in *C. botulinum* strains type E, F, and some type A BoNTs form complexes lacking HA components. Botulinum complexes type A, E, or F encompassing OrfX and P47 proteins are responsible for food-borne botulism, which is as severe as the classical type A and B botulism. The role of OrfX and P47 in BoNT absorption is not known.

3.6 BoNT/Botulinum Complexes Entry Into Intestinal Epithelial Cells

It is assumed that BoNT is endocytosed at the apical side of intestinal cells and subsequent transcytosis delivers the whole active toxin through the basolateral surface into the extracellular medium. TeNT, which is structurally and functionally related to BoNTs, undergoes an equivalent cellular transport from the local site of infection and toxin production to target neurons in the central nervous system. TeNT enters motor neuron endings via clathrin-coated pits, and it is sorted into organelles successively controlled by Rab5 and then Rab7 guanosine triphosphatase (GTPase), which move in a retrograde manner along the axon. TeNT shares a retrograde transport mediated by neutral pH compartments with neurotrophic factors, such as nerve growth factor and brain-derived neurotrophic factor. Transcytosis through motor neurons releases undissociated TeNT in the spinal cord or brain, where the toxin enters inhibitory interneurons via endosomes which are acidified. Low pH is required to trigger a conformational change of toxin molecules facilitating their membrane insertion and the translocation of the L-chain into the cytosol [17, 18, 23, 120]. In a similar way, transcytosis of BoNT/A through intestinal cell monolayers delivers active toxin at the basolateral side. This transport is not prevented by Bafilomycin A1, an inhibitor of vesicular ATPase, or monensin, an alkalinizing agent. Both drugs prevent acidification of endocytosis vesicles indicating that BoNT/A transits through a neutral pH compartment in intestinal cells [34, 108]. In contrast, BoNT/A entry into target neuronal cells requires endosomal acidification to facilitate translocation of L-chain into the cytosol, and so enabling its intracellular enzymatic activity. Indeed, Bafilomycin A1 inhibits synaptosome-associated protein 25 kDa (SNAP25) proteolytic cleavage in PC12 cells treated with BoNT/A by preventing L-chain release into the cytosol [32, 84, 165]. In contrast, Brefeldin A, which disrupts the Golgi apparatus, does not impair BoNT/A transcytosis, indicating that the toxin trafficks via an intracellular pathway not involving this cellular compartment [34, 108].

A differential intracellular trafficking pathway of BoNT/A in intestinal cells versus neuronal cells has been identified based on the distribution and morphology of endocytic vesicles carrying the toxin and on activity modulation of molecules involved in the main cell entry pathways. In neuronal cells, BoNT/A Hc entered endocytic vesicles which progressively migrated to the perinuclear area, whereas endocytic vesicles containing BoNT/A Hc were more scattered in the cytoplasm of intestinal cells, supporting a different trafficking in the two cell types. BoNT/A enters neuronal cells via a clathrin-dependent pathway, mostly mediated by synaptic vesicle recycling, but not exclusively [84, 182]. BoNT/A Hc markedly colocalizes with transferrin, a marker of clathrin-dependent endocytosis [35]. In addition, potassium depletion, which prevents assembly of clathrin-coated vesicles, significantly inhibits BoNT/A Hc uptake into neuronal cells [35, 134]. In contrast, BoNT/A Hc does not significantly colocalize with transferrin in intestinal cells and potassium depletion does not prevent its entry into these cells. The BoNT/A entry pathway into intestinal cells is also caveolin independent, based on a lack of significant colocalization between BoNT/A Hc and caveolin [35]. The different entry route of BoNT/A into neuronal and intestinal cells is further supported by electron microscopic analysis. BoNT/A Hc is distributed in coated vesicles in neuronal cells, whereas in intestinal cells, it is in smooth vesicles, which likely correlates with caveolae endosomes [36]. Therefore, clathrin-dependent pathway seems to be a preferential entry route of BoNT/A into neuronal cells and only an accessory entry pathway into intestinal cells.

A significant part of BoNT/A Hc was found in early endosomes, as evidenced by colocalization with the early endosome antigen EEA1 in both neuronal and m-IC_{cl2} cells [35]. Early endosomes labeled with EEA1 are a common compartment reached by clathrin-dependent and several clathrin-independent routes [100]. This indicates that BoNT/A is transported in a common early compartment in neuronal and intestinal cells. Then, the toxin is sorted into distinct pathways according to the cell type, since BoNT/A passes through an acidic compartment which facilitates the translocation of the L-chain into the cytosol of target neurons, whereas it is transcytosed in a neutral compartment in intestinal cells (as TeNT is in retrograde transport along motor neurons) [97].

Rho-GTPases, which are involved in the control of the actin cytoskeleton, differentially regulate several endocytic pathways. Thus, Cdc42 controls the endocytosis of GPI-APs, fluid phase markers and *Helicobacter pylori* VacA toxin into GPI-anchored protein enriched early endosomal compartments (GEECs), whereas RhoA is involved in the internalization of interleukin-2 receptor [112]. A dominant-negative mutant of Cdc42 efficiently inhibited BoNT/A Hc entry into intestinal cells, and to a lower extent into neuronal cells, whereas a dominant-negative mutant of Rac1 or RhoA did not affect the entry pattern of BoNT/A Hc into either cell type [35]. Thus, BoNT/A enters neuronal and intestinal cells by two distinct regulated endocytosis mechanisms. BoNT/A Hc probably follows a similar Cdc42-dependent endocytic route in intestinal cells to GPI-APs or VacA. However, the Cdc42-dependent pathway in intestinal cells delivers BoNT/A Hc into a different compartment to GEECs. Indeed, GEECs have been found to be devoid of transferrin and EEA1 markers [64, 152], and BoNT/A Hc partially colocalizes with transferrin and EEA1

[35]. In addition, cholesterol depletion with a low concentration of MβCD (2 mM), which has been found to prevent GPI-AP uptake into GEECs [27], did not impair BoNT/A Hc entry. Thus, a distinct endocytic pathway from GEEC and controlled by Cdc42 seems to be involved in BoNT/A Hc entry into intestinal cells.

An important step in endocytosis is the fission process which permits the individualization of endocytic vesicle from invaginated membrane containing a cargo and its subsequent migration. The GTPase dynamin is a key molecule implicated in the pinching mechanism of endocytic vesicles [147, 178]. Dynamin was first described to be part of the clathrin-dependent pathway, but it was subsequently found to be involved in several clathrin-independent endocytosis pathways, such as those mediated by caveolae or RhoA [112]. We observed that BoNT/A Hc endocytosis in both neuronal and intestinal cells was dynamin dependent, indicating a similar mechanism of endocytic vesicle formation in both cell types [35]. Intersectin, a major binding partner of dynamin, was first involved in clathrin-dependent endocytosis [164], but it also regulates caveolae-mediated transcytosis in endothelial cells. Indeed, intersectin recruits dynamin through its SH3 domains to the caveolae neck [139]. Overexpression of the intersectin SH3A domain efficiently inhibits BoNT/A Hc entry into neuronal cells as well as in intestinal cells, indicating the same mechanism of entry in both cell types [35]. Several isoforms of intersectin have been described; however, intersectin isoform 1 is neuron specific and is involved in synaptic vesicle formation, whereas isoform 2 is widely distributed in the different tissues [48]. In endothelial cells, intersectin 2 associates with dynamin and synaptosome-associated protein 23 kDa (SNAP23) in the fission of caveolae and transcytosis mechanism [139]. Interestingly, the long isoform of intersectin 1 or 2 contains a DH (Dbl homology) domain which functions as a guanyl nucleotide exchange factor (GEF) for Cdc42 and an SH3 domain interacting with N-WASP [48]. This suggests a possible intersectin-mediated Cdc42 activation in the entry process of BoNT/A Hc into intestinal cells. A different regulation of entry mechanism might be involved in neuronal cells, in which BoNT/A Hc entry is dynamin and intersectin dependent, but not or only partially Cdc42 dependent. This does not rule out that several BoNT/A Hc entry pathways might coexist.

Does botulinum complex enter intestinal cells? It seems unlikely that so large protein complexes are efficiently absorbed into cells. Only a few reports have addressed this question. It is presumed that botulinum complex type C, consisting of BoNT/C, NTNH, and HA components, binds to HT29 intestinal cell surface via O-linked sugar chains of mucin-like glycoproteins but not ganglioside, and enters cells via both clathrin and caveolae pathways [124, 181]. Only a small fraction of botulinum complex type C receptor localizes in lipid rafts and most is in soluble membrane fractions, whereas MβCD strongly inhibits internalization of the complex [181]. Thus, it is not clear whether the functional receptor is only the fraction localized in lipid rafts. BoNT/C apparently dissociates from ANTPs in endosomes and it is transported to the Golgi apparatus. BoNT/C and ANTPs are not apparently routed to lysosomes or endoplasmic reticulum [181]. It is supposed that ANTPs are secreted via recycling endosomes, but it remains to be determined how toxin and nontoxic proteins are exported, as well as how and where they reassociate to form a whole active toxin complex, or whether only BoNT/C is transcytosed.

3.7 BoNT Dissemination

BoNT dissemination to the target neurons after entry through an epithelial barrier is poorly understood. Only a few reports have addressed this question. The main difficulty resides in the minute amounts of toxin which have to be monitored. It is presumed that the major route of BoNT dissemination is the blood circulation. This supposes that the toxin has to pass twice through an endothelial barrier: to enter the blood circulation and then to be delivered to the neurons. Assuming that the efficiency of BoNT transport through an endothelial barrier is in the same range as that through an epithelial barrier (less than 1 %), only a very small fraction of ingested or inhaled toxin might attain the target neuron endings. When injected into the lumen of an *ex vivo* mouse intestinal loop, BoNT/A reduces the frequency of spontaneous contractions and inhibits the smooth muscle contractions evoked by electric field stimulation. As monitored by fluorescent BoNT/A Hc in this experimental model, BoNT/A Hc crosses the intestinal barrier and targets cholinergic neurons of both submucosal and myenteric plexuses. This supports that BoNT/A is able to pass through the intestinal epithelium to diffuse through the extracellular space and to interact with intestinal neurons independently of the blood circulation, thus inducing local intestinal effects characterized by constipation [37].

Does BoNT recognize the same receptors on endothelial cells as in epithelial cells and undergo an equivalent transcytotic passage? Large botulinum complex type D, and to a lesser extent BoNT/D, has been found to bind to bovine aortic endothelial cells via sialic acid [187] similarly to that reported for epithelial cells [59, 126, 127]. HA33 from botulinum complex type D seems to be the main molecule mediating the binding of botulinum complex to sialic acid [187]. The mode of passage of the other BoNT types in the endothelial compartment has not been fully investigated. However, ANTPs play no role in BoNT diffusion in the organism, since BoNT dissociates from ANTPs at neutral pH or higher [186]. Indeed, in physiological conditions, BoNT is not linked to ANTPs in the lymph and blood [155, 171]. Moreover, investigations on local injections of botulinum complex for a therapeutic purpose have confirmed that BoNT/A dissociates virtually immediately from ANTPs under physiological conditions [45, 135]. Moreover, although BoNT remains relatively localized at the site of injection by binding to the local nerve endings, side effects of BoNT therapy have been observed due to toxin diffusion [26]. Most often, the undesired effects are related to local toxin diffusion to the adjacent muscles, but in rare cases, adverse effects occur in distant muscles or result in central effects or generalized intoxication [1, 63, 66, 67, 87, 146, 185]. Systemic spreading and autonomic side effects, including mouth dryness, blurred vision, swallowing difficulties and constipation, are more often observed with BoNT/B than BoNT/A [44]. The difference between both toxins in inducing systemic autonomic disturbances is not known. Several factors can influence BoNT diffusion, such as volume of injection, toxin formulation, amount and concentration of toxin. The mode of BoNT diffusion to distant neuromuscular junctions is still under debate. Two hypotheses have been proposed: systemic distribution by the blood circulation and diffusion by a retrograde axonal transport [63]. Experimental findings support the second hypothesis.

Iodinated BoNT injected intramuscularly in cat is retrogradely transported to the ipsilateral spinal anterior horn cells [184]. A more demonstrative evidence of BoNT/A retrograde transport has been provided by Antonnuci et al. [5]. Intrahippocampal BoNT/A injection led to cleavage of SNAP25 in the ipsilateral hippocampus 1 day after administration, and in the contralateral side 3 days later. Moreover, BoNT/A injection into the optic tectum induced SNAP25 cleavage as well as reduction of cholinergic-driven wave activity in the retina that cannot be explained by a systemic spread of the toxin [5, 143, 145]. In addition, it has been evidenced that BoNT/A, BoNT/E and their recombinant Hc domains can undergo a fast axonal retrograde transport in primary spinal cord motor neurons [144]. As mentioned above, fluorescent BoNT/A Hc administered into a mouse intestinal loop preferentially targets cholinergic neurons in the submucosa and musculosa, but also to a lesser extent, it targets other neuronal cells, including glutamatergic and serotoninergic neurons. The effects of BoNT/A in these non-cholinergic neurons are not known. One possibility is that BoNT/A might use the non-cholinergic neurons to be retrogradely transported to other target neurons [37].

Blood circulation offers the possibility to rapidly spread the toxin in the organism if the toxin is free and not modified. The majority of BoNT (85–95%) remains free in the serum or plasma and does not accumulate in blood cells. Only a small proportion (24%) is bound to albumin. Moreover, BoNT is not metabolized in blood and its biological activity is unchanged [3, 142]. Therefore, free and stable BoNT in the serum permits a systemic distribution of the toxin. The general circulation can be considered as a "holding compartment" from where small but sufficient amounts of toxin are delivered to target neuron endings [3]. Indeed, in food-borne botulism, most of the patients show the presence of toxin in the serum, and the toxemia can persist until 25 days after symptom onset [163]. However, in some cases of food-borne botulism (20–30%) and in the majority of infant botulism, BoNT is not detectable in the serum. Whatever the origin of botulism, food-borne botulism, *C. botulinum* intestinal colonization or wound botulism, the sequence of symptom onset is the same with attack of cranial nerves and then spinal motor neurons, which is representative of a descending flaccid paralysis. This does not support dissemination by the blood circulation, which would distribute the toxin to all neuron endings leading to a generalized botulism. Thus, the exact pathway of BoNT distribution remains to be determined.

3.8 Concluding Remarks

BoNTs are highly sophisticated toxins which only exert an enzymatic activity towards specific neuronal proteins involved in neuroexocytosis. This raises the intriguing question how such a toxin from an environmental bacterium has emerged to recognize specific targets of the most differentiated cells of higher organisms. Is it the result of evolutionary convergence or are there evolutionary pressures driving a differentiation pathway leading from a bacterial enzyme to a neuronal

cell-specific toxin? Consequently, have BoNTs also acquired a sophisticated mode of entry and dissemination into the organism to have access to the target neurons? Many bacterial toxins have the ability to specifically enter target cells and to deliver an intracellularly active domain into the cytosol, most commonly by using a physiological entry pathway. It has been evidenced that BoNTs enter epithelial cell by a receptor-mediated transcytosis mechanism, which delivers the undissociated active toxin to the basolateral side. Then, the toxin is transported by the blood/lymph circulation, possibly by a retrograde axonal trafficking to the target motor neuron endings, where it uses a distinct entry pathway via acidified endosomes enabling the translocation of the L-chain into the cytosol. Do BoNTs recognize distinct receptors to drive a transcytosis through epithelial cells and a productive endocytosis in motor neurons? BoNTs, at least BoNT/A, seemingly recognize the same set of receptors, including gangliosides and a synaptic vesicle membrane protein or related protein, in both cell types. This does not preclude, however, that another molecule in the receptor platform or a distinct receptor distribution in membranes might drive the specific entry pathways. Does this entry through an epithelial barrier and routing to motor neurons hijack a physiological process? To date, no molecule is known to be absorbed from the digestive or respiratory tract and then specifically directed to neuronal cells. ANTPs have a role in the protection of BoNT against low pH and intestinal proteases. Their role in the passage of BoNT through an epithelial barrier is still unclear. Botulinum complexes containing HAs have been shown to alter the integrity of the epithelial barrier via E-cadherin and to facilitate a paracellular passage of BoNT, but their accessory role in the absorption of BoNT in natural conditions remains to be clarified, as does the eventual role of botulinum complexes lacking HA proteins. Albeit most of detailed molecular and structural mechanisms of BoNT activity at the neuronal cell level have been unraveled, toxin dissemination and trafficking through the organism to the target cells still remain poorly understood.

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Chapter 4
The Botulinum Neurotoxin Complex and the Role of Ancillary Proteins

Bal Ram Singh, Tzuu-Wang Chang, Roshan Kukreja and Shuowei Cai

Abstract All seven known serotypes of botulinum neurotoxin (BoNT) are produced in the form of a complex with a group of neurotoxin-associated proteins (NAPs). The BoNT complex is encoded by a gene cluster regulated by its own transcription factor, and the proteins coded by polycistronic messenger ribonucleic acid (mRNA) self-assemble into complexes of 300–900 kDa. Types A, B, C, D, and G complexes contain hemagglutinin (HA), whereas types E and F complexes do not contain HA. Sequence homology among respective BoNTs and NAPs range from 55.3 to 98.5 %, and all the proteins in the BoNT complex belong to a stable class of protein with high longevity inside mammalian cells. A new 250-kDa protein (P-250) with high immunogenicity has been identified in the BoNT/A complex which is not part of the neurotoxin gene cluster. The 33-kDa hemagglutinin (HA-33) is the most abundant NAP. The HA-33 is protease resistant and is highly immunogenic. HA-33 appears to play an important role in the translocation of the neurotoxin across the gut wall, enhancing the endopeptidase activity of BoNT and protection of BoNT against proteases. The role of other NAPs is not as clear, and their role in the biology of the bacteria is not understood at all. BoNT complexes are used as therapeutic products, although a therapeutic product without NAPs appears to retain the properties of the complex-based products. NAPs in therapeutic products may have other subtle long-term effects which need to be investigated.

Keywords Botulinum · Botox · Clostridium · Dysport · Complex · Neurotoxin · NAPs · Protein stability · Serotypes · Therapeutic · Toxin · Xeomin · Botulinum neurotoxin · Neurotoxin-associated proteins · Hemagglutinin · Progenitor neurotoxin · Gene cluster · Operon · Polycistronic · Molecular stoichiometry · Endopeptidase

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4.1 Botulinum Neurotoxin Complex

Botulinum neurotoxin (BoNT) is produced in three progenitor toxin forms: M, L, and LL forms. The M form consists of neurotoxin (150 kDa) and a nontoxic protein component (120 kDa) which is called neurotoxin-binding protein (NBP) [101] or nontoxic nonhemagglutinin component (NTNH) [22] with 12S molecular size (the molecular size of complex forms is expressed as sedimentation equilibrium values). The L form has a molecular weight of about 500 kDa and a molecular size of 16S. The LL form is about 900 kDa and 19S. The L and LL complexes consist of several hemagglutinin (HA) components besides the BoNT and NBP, and exhibit HA activity [31, 102]. In 1998, the Singh research group named the proteins other than neurotoxin present in the BoNT complex as neurotoxin-associated proteins or NAPs [30]. NAPs have at times been referred to as associated nontoxic proteins (ANTPs) to emphasize their nontoxic property [81].

NBP is distinct from other NAPs because it clearly interacts with BoNT. In fact, the M form of BoNT consists of the 150-kDa BoNT and approximately 120-kDa NBP.

There are variations in sizes and composition of complexes of different BoNT serotypes (A through G). The BoNT/A complex can exist in three forms: M, L, or LL; BoNT/B,/C, and /D complexes exist in two forms: L and M; BoNT/E and /F complexes are known to exist only in M form; and the BoNT/G complex exists only in L form [4, 76]. Some of type A and B neurotoxins which cause infant botulism exist in M form [17].

The size of the complex formed in BoNT/A and BoNT/B depends on the medium used for bacterial growth. The addition of iron or manganese to the growth medium results in a higher concentration of M and L forms of neurotoxin complexes [90]. In addition, some foods such as vegetables have high botulinogenic properties. *Clostridium botulinum* types A and B are found to produce stable L and LL forms of neurotoxin complexes in vegetables, whereas they produce the less stable M complex in tuna and pork (89).

The nontoxic components of progenitor neurotoxin, NAPs, appear to play a critical role in the toxico-infection by neurotoxin. They are presumed to protect neurotoxins against the digestive proteases and stomach acidity when the progenitor neurotoxins pass through the gastrointestinal tract (GI) [1, 90, 102]. Such protection is critical in food poisoning activity of the neurotoxin. For example, if NAPs are removed, the oral toxicity of the purified type A neurotoxin goes down by up to 358 fold [77, 89]. Tetanus neurotoxin (TeNT) which is produced by anaerobic *C. tetani* is not a food poison and is in fact inactive by the oral route because of the absence of NAPs [100]. The oral toxicity increases with incremental association of the neurotoxins with the protective proteins. In BoNT/A and BoNT/B, the larger forms of progenitor neurotoxins (19S and 16S) are more toxic by the oral route and more resistant to acid and pepsin than the smaller form (12S) of progenitor neurotoxins [90, 30, 93, 96].

The progenitor neurotoxins are believed to dissociate into the neurotoxins and NAPs in the small intestine or in an alkaline condition and the components of NAPs might also play a role in the adhesion of progenitor neurotoxins to the intestinal tissue before the dissociation of the progenitor neurotoxins [33–35, 89]. Certain carbohydrate groups which help the NAPs to anchor to the intestinal wall have been identified [50, 74].

The neurotoxins and nontoxic components can re-associate spontaneously when the pH is lowered [41]. An ordered interaction between neurotoxins and their NAPs was suggested by the observation that the L and LL forms of type A neurotoxin form crystals [90].

4.2 Structure and Composition of BoNT Complexes

The crystalline form of type A BoNT (what is now known as BoNT/A complex) was first isolated in the 1940s [62], and was considered to represent the pure form of the toxin owing to the scientific belief then, and to certain degree now, that crystallization of material occurs in its pure form allowing perfect packing of molecules. The understanding of the molecular intactness and purity of the BoNT/A complex continued until the mid-1960s when the complex was subjected to ion-exchange chromatography and sodium dodecyl sulfate (SDS)–gel electrophoresis [6, 18, 19]. Even after the 150-kDa BoNT was separated from its NAPs using chromatography, there seemed to be skepticism regarding the intactness of the toxin, and it was contended that perhaps the 150-kDa BoNT represented a fragmented piece of the intact crystalline molecule (B. R. DasGupta, personal communication [5], [19]).

Attempts have been made more recently to examine the structure of BoNT complexes for their composition, size, shape, interactions among constituent subunits, and structural stability.

4.3 Composition

Components of the BoNT complex vary with neurotoxin serotypes and the strains of *C. botulinum* producing such toxins. Because BoNT belongs to a gene cluster consisting of BoNT and its associated proteins, the components of the complex in each of the serotypes of BoNT are expected to have products of genes in the respective cluster. Two major neurotoxin gene cluster types have been identified and named type I “ha plus/orf-X minus” and type II “ha minus/orf-X plus” [16, 23, 66] (Fig. 4.1). Sebaihia et al. [91] suggested a nomenclature of the gene loci for BoNT cluster, and designated them *cnt* referring to “clostridial neurotoxin” as the prefix for all genes located inside that cluster. For example, the type A neurotoxin gene (BoNT/A previously) will be designated as *cntA*/A, type B neu-

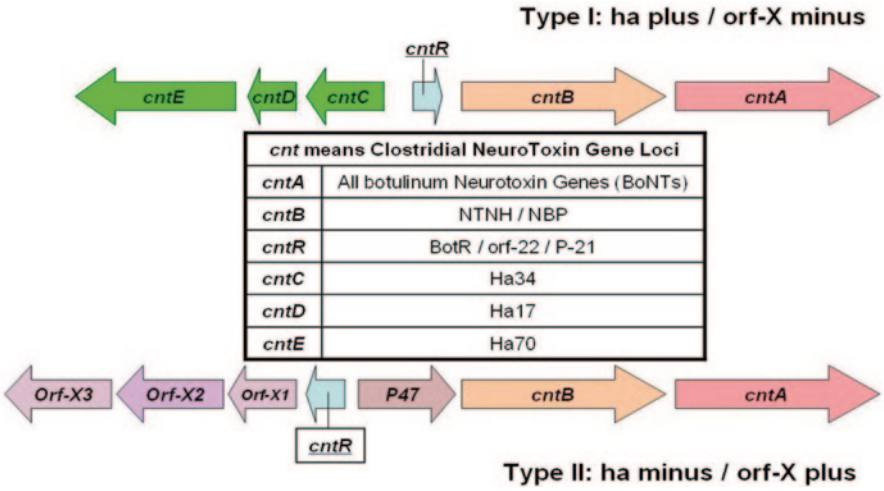

Fig. 4.1 The *cnt* nomenclature system of botulinum neurotoxin gene cluster and its corresponding translation table for their earlier names

rotoxin gene (BoNT/B) as *cntA*/B, type C neurotoxin gene (BoNT/C) as *cntA*/C, etc. The genes for encoding NAPs are designated as *cntB*, *cntC*, *cntD,* and *cntE* as shown in Fig. 4.1. For type II gene clusters (types E, F, and A2), the BoNT and NBP genes are referred to in the same way as type I gene clusters, but HA genes are referred to as OrfX1, OrfX2, and OrfX3 as shown in Fig. 4.1. Although fully understandable to name them as Orf at the beginning, it is now known that these genes in fact code for proteins which are expressed and present in the cell culture. Therefore, it is appropriate to name them accordingly. Even cntA, cntB, etc. are confusing, because the serotypes of BoNT are named A, B, C, etc. A more appropriate nomenclature would be to have each gene named after the protein it expresses. Such naming of the genes had been in practice early on when these gene clusters were discovered. While the nature of the type I gene cluster product (e.g., type BoNT complex) is clearly understood, with its component proteins identified, sizes estimated, and their composition in the complex established, no such clear information is currently available on type II gene cluster. According to Singh and Zhang [99], type E *C. botulinum* produces a complex similar to type A complex, having large (L) size, consisting of six proteins, including BoNT/E, NBP, and proteins corresponding to the three Orfs (Fig. 4.1). Although the chemical nature of the BoNT/A complex is drastically different from that of the BoNT/E complex, the two complexes have several common characteristics: stability, protection of BoNT from proteases, and enhancement of the endopeptidase activity [57, 99]. Freshly prepared BoNT/A and BoNT/E complexes from respective strains of *C. botulinum* each exhibited six Coomassie blue-stained protein bands when analyzed on a 4–20% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gel under nonreducing conditions (Fig. 4.2a). For the BoNT/A complex, the protein bands correspond to 145 (BoNT/A), 120 (NBP), 53 (NAP-53), 33 (HA-33), 20 (NAP-20),

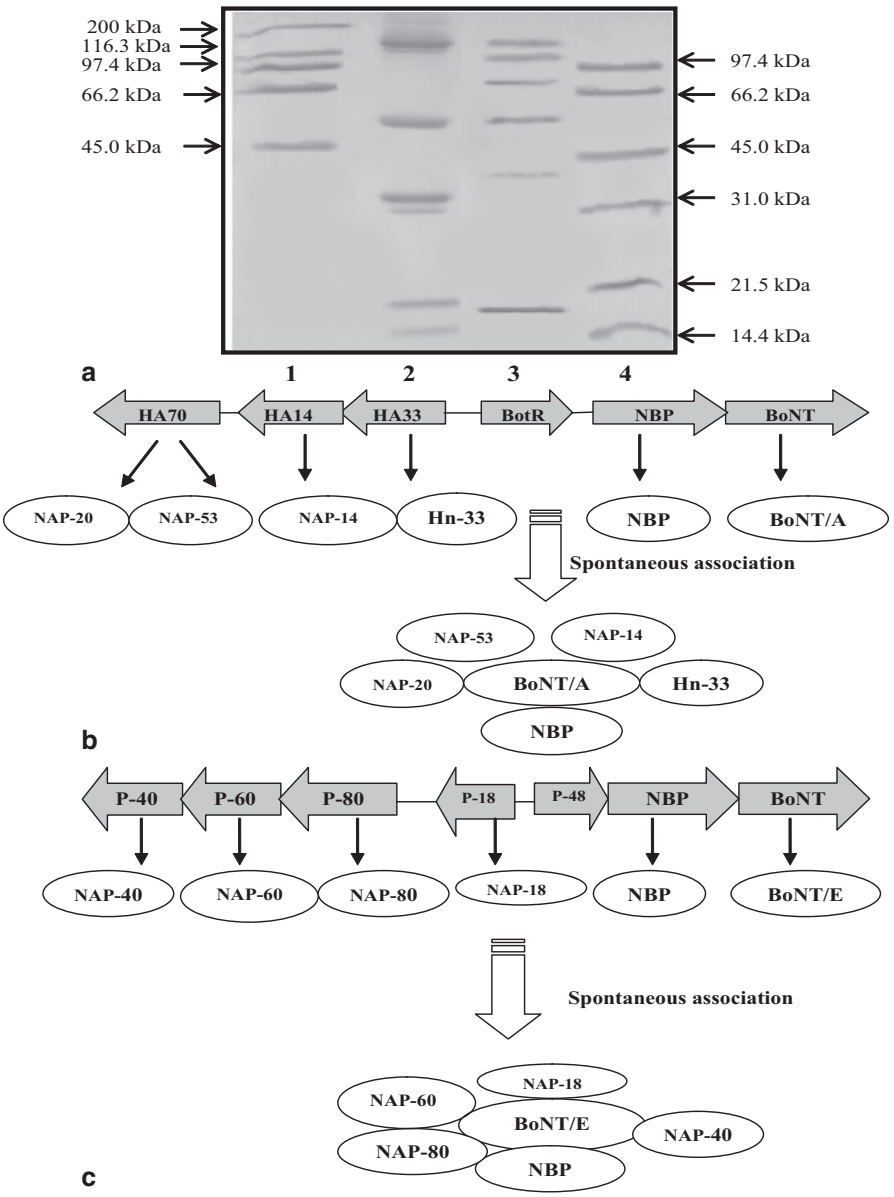

Fig. 4.2 **a** SDS-PAGE analysis of BoNT/AC and BoNT/EC under nonreducing conditions visualized by Coomassie blue staining. Lane 1: high molecular mass protein marker, lane 2: BoNT/AC, lane 3: BoNT/EC, lane 4: low molecular mass protein marker. **b** Schematic diagram showing the genomic organization of *C. botulinum* type A [36] and their expressed proteins in forming BoNT/AC. **c** Schematic diagram showing the genomic organization of *C. botulinum* type E [66] and their expressed proteins in forming BoNT/EC

Table 4.1 Comparison of structural stability and functional activity of BoNT/A in its complex and purified forms

	Neurotoxin complex	Pure neurotoxin	Neurotoxin-associated proteins
Type A	Structural stability: Tm ~75°C Endopeptidase activity at 37°C: Nonreduced ~66% (21) Reduced ~92% (21)	Structural stability: Tm ~54°C Endopeptidase activity at 37°C: Nonreduced ~4% (21) Reduced ~69% (21)	Structural stability: Tm ~71°C ND
Type E	Structural stability: Tm ~63°C Endopeptidase activity at 37 °C: Nonreduced ~42% Endopeptidase activity at 45°C: Nonreduced ~96%	Structural stability: Tm ~52 °C Endopeptidase activity at 37°C: Nonreduced single chain ~4% (18) Reduced single chain ~15% (18) Endopeptidase activity at 45°C: Nonreduced single chain ~8% Reduced single chain ~88%	ND ND ND

Tm midpoint temperature, *ND* not determined

and 14 kDa (NAP-14) [21]. For the BoNT/E complex, the protein band corresponds to 138 (BoNT/E), 118 (NBP), 80 (NAP-80), 60 (NAP-60), 40 (NAP-40), and 18 kDa (NAP-18) [66, 99, 101]. The assignments of bands observed on SDS–PAGE gels are based on previous biochemical and genetic analysis of BoNT/A and BoNT/E complexes [2, 66, 82, 99, 101]. A schematic model of BoNT/A and BoNT/E genes and their expressed proteins is shown in Fig. 4.2b and c, respectively.

Despite being similar in nature, the complex produced by type I and type II gene clusters behaved uniquely, both in physical and chemical characteristics. Kukreja and Singh [57] have shown (Table 4.1) specific differences in these characteristics: (1) BoNT/A and BoNT/E in their complex forms are enzymatically active under nonreducing conditions. The BoNT/A complex under nonreducing conditions attains optimum activity at the physiological temperature of 37°C, whereas the BoNT/E complex under nonreducing conditions achieves maximum endopeptidase activity at 45°C; (2) BoNT/E complex undergoes conformational alterations in its polypeptide folding at 45°C and this may result in its favorable binding with the substrate leading to maximum cleavage of the latter; (3) possibly due to direct interactions with NAPs, BoNT/A and E in their respective complex forms may conform to a structurally active state which is similar to that of the reduced BoNT/A and BoNT/E, respectively, leading to their enhanced endopeptidase activity; and (4) BoNT/A in its complex form is structurally more stable, while the BoNT/E complex is functionally more stable against temperature.

A recent study [69] with another member of type II gene cluster, *C. botulinum* A2 (Kyoto F) has revealed a slightly different story. In type A2, while all the OrfX genes, except for OrfX1, were shown to express their respective proteins in bacterial culture, these proteins did not seem to form an L complex. Instead,

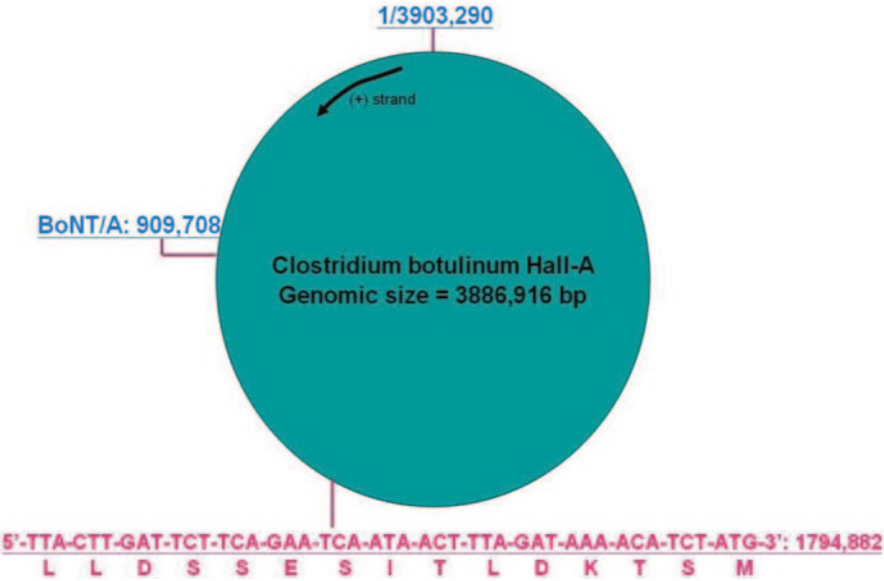

Fig. 4.3 Genomic map of *Clostridium botulinum* strain Hall, showing the location of origin, BoNT/A cluster gene, and the gene of P-250 protein (amino acid sequence in *violet*)

it showed the usual M complex consisting of BoNT and the NBP. Notably, the culture also expressed the P47 protein, a unique protein whose gene is located upstream of the NBP gene (Fig. 4.1). The gene for P47 (reported as P48 in type E *C. botulinum*; [66]) is entirely absent in type I cluster gene. P48 has not been shown to be associated with the mature complex isolated from type E *C. botulinum* [99].

4.4 Discovery of a Highly Immunogenic New Protein

Although not a part of the BoNT gene cluster, the presence of a 250-kDa protein in type A *C. botulinum* Hall strain was noted by Kukreja et al. [58] as part of the BoNT/A complex preparation. Its N-terminal sequence, LLDSSESITLDKTSM, was unique and matched with a single open reading frame (ORF) in the *C. botulinum* type Hall strain (American Type Culture Collection (ATCC) 3502) genome (National Center for Biotechnology Information (NCBI) accession number: YP_001254150). The putative ORF site encompassing the peptide identified by the peptide sequencing is located at the genome map position 1,794,882 of ATCC 3502 Hall-A, in contrast to the BoNT gene being at position 909,708. Therefore, it is clear that P-250 does not belong to the BoNT gene cluster and is located 885,114 bp downstream (Fig. 4.3). Interestingly, the gene corresponding to the peptide sequence of P-250 has the start codon methionine 190 amino residues upstream to the N-terminal sequence we obtained, suggesting that the polypeptide gets digested either during bacterial

growth or during the purification process of BoNT/A. The polypeptide segment corresponding to the downstream of the peptide sequence identified including the N-terminal 15 amino acid sequenced adds up to a total of 169 amino acid residues. The molecular size of this segment therefore corresponds to only 18.74 kDa, and so the P-250 band is likely to be an SDS-resistant oligomer of this 18.74-kDa segment, consisting of about 16 monomers. This conclusion needs to be confirmed with further experiments. P-250 showed more than 97% sequence homology with bacterial immunoglobulin (Ig)-like proteins, intimins, found on the surface of bacterial cells or phage particles. It also has sequence homology ranging between 64 and 97% with *C. botulinum* type B, F, and A3, *C. beijerinckii,* and *Bacillus* proteins which have been classified as cell adhesive proteins. A sequence homology analysis encompassing the whole protein that begins with the ORF starting amino acid showed significantly lower homology. The segment showing the maximum homology matches with the most conserved bacterial Ig-like domain (Big_2 domain). Big_2 domains are conserved domains of cell adhesion proteins found in many bacterial cells [53]. Since these proteins are present on the surface of the bacterial cell, it is possible that P-250 protein identified in the BoNT/A complex is present on the surface of the *C. botulinum* cells, and possibly gets attached to the BoNT/A complex during the latter's release from the bacterial cells. Further evidence is needed to determine that P-250 protein is indeed associated with the BoNT/A complex and is not just a minor contaminant that is copurified with the complex. As a form of indirect supporting evidence, a protein of similar size can be seen on SDS–PAGE gels of not only other BoNT/A complex preparations [48] but also types C and D BoNT complexes [49]. The relevance of the immunogenicity of P-250 protein needs to be further examined, both for detection of BoNT and also for immunogenic response in therapeutic preparations used against several neuromuscular disorders.

As discussed above, there are two major sources of information on the composition of BoNT complexes: (1) isolation of such complexes from bacterial cultures and characterization of the proteins involved biochemically and (2) identification of gene clusters of different strains of *C. botulinum* known to harbor BoNT genes. Ideally, both genetic and biochemical information would be available for defining components along with their relative abundance. Still, complications in explanations appear as shown by the example of the presence of P-250 (not a product of the BoNT gene cluster) in the BoNT/A complex or the absence of P-48 (necessarily part of type II gene cluster) in the BoNT/E complex. Furthermore, the molecular composition in terms of relative abundance of the proteins in the BoNT complex can only be ascertained by isolating and purifying the complex from *C. botulinum* cultures. It is, therefore, critical at this point to address the issue of the composition from both genetic and biochemical perspectives. It is also important to consider interactions among BoNT and NAPs of the complex, physically, biochemically, and biologically.

The genomic organizations of BoNTs and their associated proteins were suggested by several researchers [22, 37, 44, 66] and are summarized in Fig. 4.4. No characteristic signal peptide sequences are observed in any of the progenitor neurotoxin genes. The genes of progenitor neurotoxins are closely grouped as operons on the chromosome, phage, or plasmid. NBP gene is located only 26 bp

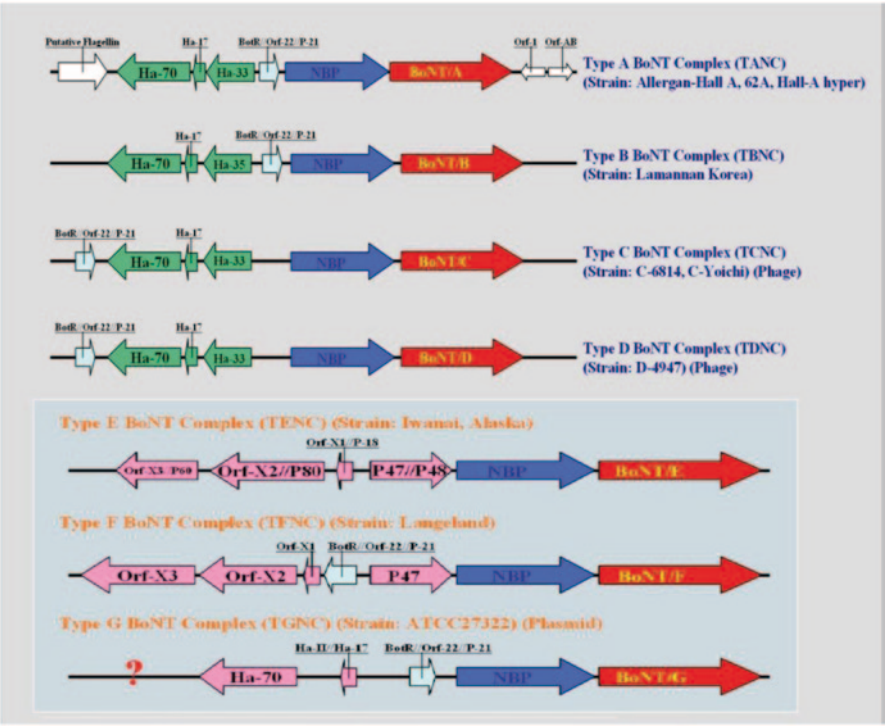

Fig. 4.4 The BoNT gene and its associated protein together form a polycistronic transcription unit. *Red* color represents the locus of the BoNT gene and *blue* color represents the locus of NBP which cotranscribed in the same direction. The *green* color represents the hemagglutinin property and the loci of NAPs. The *pink* color means their hemagglutinin attribution has not been confirmed yet. The *light blue* color indicates the locus of transcription regulator for the BoNT gene cluster. There are two types of BoNT gene cluster. Type I (HA+/Orf-) lacks Orf-X family (*pink* color, Orf-) protein but contains hemagglutinin (*green* color, HA+). Type II is the complementary (which means HA-/Orf+) form of type I

(type A, strain 667Ab or National Collection of Type Cultures (NCTC) 2916 or Centers for Disease Control (CDC) 3281) [85] or 44 bp (type A, strain ATCC 3502), 15 bp (type C, strain C-6814 and C-Yoichi) [87, 88], 15 bp (type D, strain D-4947) [55], 11 bp (type F, Langeland) [24], 12 bp (type F, strain F202) [22], 14 bp (type F, *C. baratii*, strain ATCC 43256) [24], or 85 bp (type G, strain ATCC 27322) [4] upstream of the BoNT gene. There are two promoter regions for neurotoxin genes. One overlaps the C-terminal end of NBP genes and the other is shared with the NBP genes, controlling cotranscription of the two genes. The neurotoxin gene expression is critically important in botulism pathogenesis, diagnosis, pathogen identification, and possibly in evoking host response. The neurotoxin genes are transcribed either alone or along with NBP genes via a polycistronic messenger ribonucleic acid (mRNA) and thus these two genes form an operon (Fig. 4.1), thereby suggesting that the molecular ratio of BoNT and NBP will always be 1 or more.

HA gene clusters form another operon transcribed in the opposite directions from the BoNT and NBP genes. HA gene clusters are located 262 bp (in type C, strain C-6814 and C-Yoichi) [87, 88], 261 bp (type D, strain D-4947) [55], 925 bp (in type A, strain Hall A-hyper) [20], 698 bp (type A, ATCC 3502), 811 bp (type A(B), NCTC 2916) [85] upstream of their respective NBP genes. It has been shown that HA genes are expressed in a polycistronic mRNA [45, 83]. Each HA gene contains its own transcription terminator (consensus sequence of tetra-loop UUCG) which is predicted to form a stem-and-loop structure in the mRNA (Fig. 4.4) [44, 110, 111].

In *C. botulinum* strains with type II gene cluster, P47/P48 is predicted to be polycistronically transcribed along with NBP and BoNT genes, although it is possible that bicistronic NBP–BoNT and monocistronic BoNT can also be transcribed [66, 69, 83]. All OrfX genes express proteins in type E *C. botulinum* cultures [57, 66, 99]. However, P47/P48 gene product, which has been recently shown to be present in *C. botulinum* type A2 culture [69], has not been shown in *C. botulinum* type E cultures [57, 66, 99]. It has not been established that OrfX genes can be expressed polycistornically, bicistronically, and/or monocistronically, although this information will be useful to rationalize variation in the stoichiometry of the complex. Based on the reports about the involvement of BotR regulatory protein in Ha cluster gene expression [83], there is a possibility that these genes can be expressed individually even though BotR regulation may involve mostly polycistronic transcription. Involvement of other transcription factors cannot be ruled out. Notably, the BotR gene has not yet been located in type E *C. botulinum*.

4.5 Stoichiometry

The stoichiometry of the isolated BoNT complex is a function of the relative expression of BoNT and component NAPs in the bacterial cell culture, and of course the biochemical methods involved in isolating the complex from the cell culture. Significant variations can occur even when the same bacterial culture is utilized to isolate the complex with different methods. Composition also changes with culturing conditions, including media, temperature, period of culture, etc. (Y. Zhou and B. R, Singh, unpublished results). There is also a possibility of degradation of expressed proteins in the cell culture.

Molecular stoichiometry has been determined for only a few BoNT serotype complexes. The most extensively examined so far has been the type D BoNT complex. In addition to its subunit analysis, HA activity and protection of BoNT against proteolysis, the complex has been examined by transmission electronic microscopy, which has provided structural features at a resolution enough to provide general arrangements of the subunits in a 14-mer structure [43]. As shown in Fig. 4.5, each BoNT/D complex assembly consists of one BoNT, one NBP (NTNH), three HA-70, three HA-17, and six HA-33 molecules. It is notable that HA-33

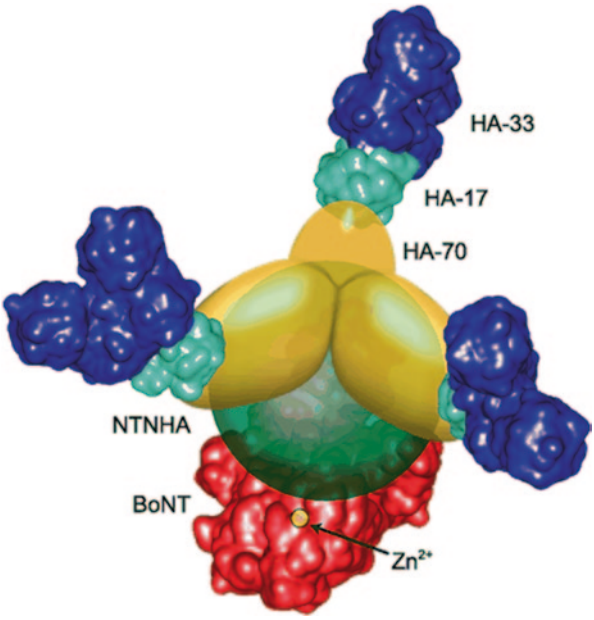

Fig. 4.5 Hypothetical 14-mer model showing the arrangement of individual components in the botulinum neurotoxin (BoNT)/D complex isolated from *Clostridium botulinum* type D strain D-4947. The BoNT is highlighted in *red*, the nontoxic nonhemagglutinin (NTNH) in *green*, three hemagglutinin-70 (HA-70) in *yellow*, six HA-33 in *blue*, and three HA-17 in *cyan*. The catalytic zinc ion in BoNT is indicated by the *orange circle* and the *arrow*. (Taken from [43])

exists as a dimer, and each of the three HA-33 dimers is bound to HA-17. Such a structure of HA-33/HA-17 has been confirmed by X-ray crystallography as well as by biochemical analysis and has been shown to be critical for the hemagglutination activity of the BoNT/D complex [43]. The BoNT/D complex structure appears to be built on a basic structure consisting of BoNT/D and its NBP as is found in M complex structure. Three molecules of HA-70 are proposed to interact directly with the single NBP on one end and each with one HA-33/HA-17 complex on the other to provide a "three-armed" structure (Fig. 4.5).

Extensive studies by Ohyama's group on the BoNT/D complex have established that the likely pathway for the assembly of the L complex includes the formation of the M complex between BoNT/D and NBP, followed by binding of the M complex with three molecules of HA-70, yielding a complex that can be observed in solution. Both M complex and M–HA-70 complex show no hemagglutination activity. For hemagglutination activity, the HA-33/HA-17 complex needs to bind to the HA-70 component of the M–HA-70 complex, and full hemagglutination is observed when one HA-33/HA-17 unit is bound to each of the three HA-70 in M–HA-70 complex (Table 4.2).

The BoNT/D complex structure was possible in part due to a unique strain of type D *C. botulinum* (D-4947) which produces BoNT, NBP, and HA-70, all in intact forms, without proteolytic nicking. Proteolytic nicking usually yields a dichain BoNT, a truncated NBP, and two fragments of HA-70 (approximately 53 and 22 kDa). In fact, this phenomenon is common to all serotypes of *C. botulinum* strains with type I gene clusters, including type G *C. botulinum*. Notably, types A, B, C, and D are known to exist in L and M complex forms, whereas BoNT/G is

Table 4.2 Molar ratio of the components of BoNT complexes

BoNT complex			
	Type A Hall [48]		Type D-4947 [43]
Components	LL	L	L
BoNT	1	1	1
NBP	1	1	1
HA-53/HA-70[a]	3	3	3
HA-33	8	4	6
HA-22/HA-70[a]	3	3	3
HA-17	3	3	3

Densitometric analysis provides significant variations in band intensity estimates. For type A, best molar estimates are from the conclusions drawn by the authors of the original article
BoNT botulinum neurotoxin, *NBP* neurotoxin-binding protein, *HA* hemagglutinin
[a] HA-53 and HA-22 are fragments of HA-70

reported to exist only in L complex form [75, 76]. Based on these published reports, it would seem that nicking of at least the NBP may play a role in the formation of the L and M complexes [55, 76]. Nicking of the HA-70 invariably occurs along with the NBP. Based on reconstitution studies of BoNT/D proteins from *C. botulinum* strain D-4947, HA-70 interacts with the M complex, as long as the NBP is intact. Once the NBP is nicked, neither H-70 nor HA-33/HA-17 binds to the M complex [55]. Binding of HA-70 to M complex protects both NBP and HA-70 from trypsin digestion, indicating either the cleavage sites are involved in binding of HA-70 to NBP or conformational changes are introduced upon their binding leading to the protection of cleavage sites.

The above description of the BoNT complex composition and the forms (L and M) of complexes may be generally true, but there are observations which point to variations from the composition and role of various proteins in the complex formation. First of all, while type G *C. botulinum* strain 2740 produces only L form of complex [75], *C. botulinum* strain G-89 produces both L and M complexes (Carl Malizio, Metabiologics, Inc., personal communication). Also, the BoNT/G complex from strain G-89 shows fragmentation of NBP and HA-70 into their usual fragments as observed for other serotype BoNT complexes, suggesting that the presence of both L and M complexes may still be consistent with the presence of both intact and nicked NBPs. Furthermore, type A1 *C. botulinum* Hall strain produces a 900-kDa BoNT complex, which has been considered as a dimer of the type A L complex [25, 67, 76]. This LL complex is presumed to be formed through an oligomeric structure of HA-33 [48, 76]. Although proteins equivalent to HA-33 are present in serotypes B, C, and D *C. botulinum*, none of these produce LL complex [76]. At the same time, type G *C. botulinum* does not seem to contain a gene for HA-33, but it still forms the L complex [4, 75]. Thus, the formation of the L complex apparently occurs in more than one way, and further studies are required with not only *C. botulinum* of different serotypes but also different strains of the same serotype of *C. botulinum*.

HA-33 is perhaps the most extensively examined NAP for its structure, composition, interaction, and function [30, 32, 34, 36, 48–50, 70, 87, 94–96, 106, 115]. HA-33 has been shown to directly protect BoNT/A from proteolysis [93], suggesting that it can directly interact with the toxin. Direct interaction of HA-33 with BoNT/A was demonstrated experimentally with size exclusion chromatography (SEC) showing a complex between BoNT/A and HA-33 [93]. Furthermore, HA-33 has also been shown to enhance the endopeptidase activity of BoNT/A and BoNT/E [95], which would be consistent with direct interaction between BoNT and HA-33.

Molecular stoichiometry of BoNT complexes of two serotypes (A and D) has been estimated from dye-stained band intensities on SDS–PAGE gels and biochemical titration studies. In type A *C. botulinum*, both L and LL BoNT complexes exist. In L complex, the molar ratio of BoNT/A, NBP, HA-52/53, HA-33, HA-22/23, and HA-14–17 was 1:1:3:4:3:3, while for LL complex the ratio was the same except for HA-33 which doubled in terms of its molar ratio [48]. The L complex data generally agreed with a later analysis by Sharma et al. [97] even though band separation and staining is affected by acrylamide gel conditions. The results have led to the conclusion that while LL complex is a dimer of L complex, the link between the two monomeric L complexes is an oligomeric HA-33 consisting of four molecules [96]. Since the HA-33 has been shown to be a dimer, the oligomeric link will consist of two dimeric HA-33.

Based on types A and D BoNT complex data, it seems that their relative stoichiometry is similar except for HA-33, which is six in the BoNT/D complex whereas it is four (L complex) or eight (LL complex) in the BoNT/A complex. The differences in the stoichiometry between the BoNT/A and BoNT/D complex notwithstanding, one question remains to be answered is the mechanism involved in the expression and assembly of HA proteins such that there is differing amount of HA-33 expressed compared to HA-70 and HA-17. This is notable in view of the polycistronic expression of all HAs, regulated by botR (Fig. 4.6).

4.6 Stability and Protection of BoNT in Complexes

NAPs are well known to protect BoNT against GI proteases and acidic conditions in the stomach [89]. This has been reflected in enhanced oral toxicity of M and L complexes. Temperature and pH effects have been examined on the BoNT/A complex, and its constituent components, the neurotoxin and NAPs, using a variety of techniques probing secondary and tertiary structures [7]. In a series of experiments with the BoNT/A complex, neurotoxin and NAPs, structural characteristics have been analyzed over a wide range of pH (3–8) and temperature (12–84 °C) using circular dichroism (CD) spectroscopy, intrinsic (Tryptophan, Trp) and extrinsic (1-anilinonaphthalene-8-sulfonic acid, ANS) fluorescence, and static light scattering. This analysis allowed construction of empirical phase diagrams showing at least five different conformational states in the BoNT/A complex and purified

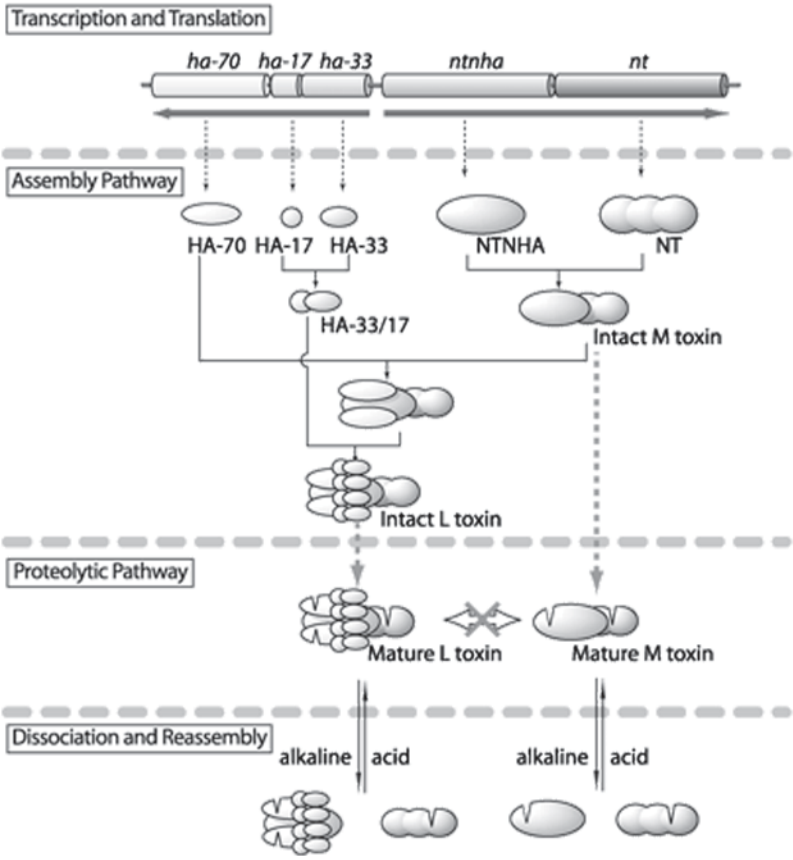

Fig. 4.6 Proposed model for the botulinum progenitor toxin assembly pathway. Genetic organization of botulinum type C and D progenitor toxins and their expressed products are represented based on nucleotide sequences and N-terminal amino acid sequences. The *letters* refer to the designation of the constituent proteins of the progenitor toxin in this study. Stoichiometry of each component was deduced by analysis of gel filtration and densitometry of the stained bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The assembly pathway from each gene product and proteolytic pathway is indicated by *solid* and *dotted arrows*, respectively. The *upper panel* represents the assembly pathway of the components to progenitor toxins, which were observed in d-4947, and the *middle panel* represents putative proteolytic pathway of the nicked progenitor toxins usually observed in other type C and D strains. The lack of mutual conversion between L and M toxins is represented by the *X symbol*. The *lower panel* represents dissociation and reassembly of the progenitor toxins depending upon pH. (Taken from [55])

neurotoxin, although these states were quite different between the two preparations. In general, neurotoxin showed significantly lower stability than the BoNT/A complex, which decreased for both with increase in pH and temperature. NAPs on the other hand had stability higher than the neurotoxin but lower than the BoNT/A complex. However, phase diagram of NAPs showed no clear conformational phase boundaries, a behavior that might be expected from a mixture of proteins. Such a

behavior suggests that in the absence of neurotoxin the NAPs are only loosely bound with each other. Based on midpoint temperature (Tm) estimates from temperature-induced structural changes monitored by CD and fluorescence spectroscopy, it was shown that secondary structures of the neurotoxin are more resistant to temperature-induced denaturation compared to tertiary structure, thus yielding molten globule state, reported for the first time from our laboratory [9, 56]. BoNT/A complex data in contrast showed tertiary structure monitored by intrinsic fluorescence to be more resistant than the secondary structure to the temperature denaturation. Overall, the BoNT/A complex compared to the neurotoxin displayed increased thermal stability over the pH range of 3–8. The increased thermal stability apparently arose from the internal interactions between the neurotoxin and NAPs [7].

As noted previously, there are two classes of NAPs derived from type I and type II neurotoxin gene clusters of *C. botulinum*. The BoNT/A complex and BoNT/E complex are the representative products of type I and type II gene clusters, respectively. NAPs of the BoNT/A complex are quite different from those of the BoNT/E complex. An examination of their cumulative role in intact complex of BoNT/A and BoNT/E provides a unique opportunity to understand their structural and functional role in neurotoxin action. Furthermore, BoNT/E targets the same intracellular substrate, synaptosome-associated protein 25 kDa (SNAP-25), as BoNT/A; thus, intense structural and biochemical analysis of BoNT/E has provided an opportunity to compare it with BoNT/A, especially for its relevance to binding and recognition of SNAP-25.

We have investigated the effect of temperature on the structural and functional stability of BoNT/A and /E complexes. The results indicated that the BoNT/A complex is structurally more stable than the BoNT/E complex against temperature, whereas the BoNT/E complex is functionally (in terms of its endopeptidase activity) better protected against temperature in comparison to the BoNT/A complex. Functional stability of the botulinum complexes against temperature plays a critical role in the survival of the agent in cooked food and in foodborne botulism.

Examination of the endopeptidase activity of the BoNT/A complex revealed that it acquires optimum activity at the physiological temperature of 37°C under conditions where the disulfide bond between the light and heavy chains was not reduced, which is a behavior similar to that of the reduced form of BoNT/A neurotoxin and the light chain of BoNT/A [8, 56]. This enhancement in the endopeptidase activity of BoNT/A in the complex has been attributed to its direct interaction with NAPs leading BoNT/A to adopt a structurally active state similar to that of reduced BoNT/A [9]. At 37°C, BoNT/A under reducing conditions exists in a molten globule conformation which is its enzymatically active structure [56]. Recently, we have also shown that the endopeptidase moiety of BoNT/A (the light chain) acquires a novel PRIME conformation at 37°C and exhibits maximum endopeptidase activity against its intracellular substrate, SNAP-25, at this temperature (36°C). Our observation of the optimal endopeptidase activity of the BoNT/A complex at 37°C reinforces the hypothesis of a conformational alteration in BoNT/A upon its interaction with NAPs in the complex, also concluded by Brandau et al. [7]. This altered conformation of BoNT/A in the complex,

similar to the molten globule conformation of reduced BoNT/A or the PRIME conformation of the light chain of BoNT/A at physiological temperature, may facilitate its favorable interaction with SNAP-25 leading to the optimal cleavage of the latter at this temperature, even under conditions in which the disulfide bond between the light and heavy chains of BoNT/A remains intact.

This implication of a substantial functional role of NAPs requires further experimental evidence to provide a confirmed mechanism of the enhanced enzyme activity. At the minimum, these results suggest a substantial role of NAPs in the critical endopeptidase activity of BoNT, which may even have relevance to the biological activity of BoNT in vivo. While the toxico-infection process of botulism assumes separation of the NAPs from BoNT before reaching the nerve cell, injection of the BoNT/A complex for therapeutic or cosmetic use does include both the components. Furthermore, currently it is not known what role the toxin plays inside the bacterial cell or in the native ecological conditions of *C. botulinum.* An activated form of the complex endopeptidase may have a critical role under those conditions.

Interestingly, the BoNT/E complex, under nonreducing conditions was found to exhibit optimum enzyme activity at 45°C. We have observed that the reduced form of single-chain BoNT/E is maximally active at 45°C [59]. Because the purified single-chain BoNT/E under nonreducing conditions is enzymatically inactive [95], it can be concluded that the enhanced endopeptidase activity of BoNT/E in the complex (wherein it exists in the nonreduced single-chain form) is likely to result from its interaction with the NAPs. This interaction must introduce specific changes in BoNT/E to adopt a structurally active state similar to that of the reduced form of single-chain BoNT/E, a phenomenon similar to the one observed in the BoNT/A complex (*vide supra*).

The BoNT/A complex comprises six NAPs including HA-33. HA-33 makes up the largest fraction of the NAPs in the BoNT/A complex [30] and strongly protects the toxin against proteases [93]. The X-ray crystallographic structure of BoNT/A indicates that the active site is buried 20–24 Å deep in the protein matrix and is partially shielded by a belt from the N-terminal domain of the heavy chain involved in disulfide bond formation and that the reduction of the disulfide bond exposes the active site [61]. Recently, it has been proposed that the interaction of HA-33 with BoNT/A in the complex exposes the enzyme active site without the need of opening the belt, leading to its enhanced enzyme activity and that HA-33 mimics the effect of all NAPs in enhancing the endopeptidase activity of BoNT/A [95]. HA-33 from the BoNT/A complex also enhanced the endopeptidase activity of BoNT/E, suggesting a common structural motif of BoNT serotypes for HA-33 [95]. However, our report, wherein the BoNT/E complex that is devoid of HA-33 also displays enzyme activity under nonreducing conditions (~42% at 37°C and 96% at 45°C), suggests that the interaction of BoNT/E with the NAPs present in the BoNT/E complex is sufficient to alter the structure of the neurotoxin where it conforms to a structurally active state similar to that of the reduced form of BoNT/E [59]. However, the efficiency of cleavage of SNAP-25 by the BoNT/E complex has been found to be less than that of the BoNT/A complex at 37°C (the BoNT/A complex displays 32% higher endopeptidase activity than that of the BoNT/E complex at 37°C). Higher toxicity of

BoNT/A in comparison to BoNT/E may in part be reflected by the fact that BoNT/A in its complex form is more enzymatically active at the physiological temperature of 37°C as compared to BoNT/E in the complex form.

The effect of temperature on the polypeptide folding of the BoNT/A complex and BoNT/E complex was examined by monitoring CD signals at 222 and 280 nm as a function of temperature. The unfolding curves of both the complexes obtained from CD recordings in the far- and near-ultraviolet (UV) regions coincide and indicate that both the secondary and tertiary structures for both the complexes unfolded simultaneously and the Tm for the unfolding was 75 and 63°C for the BoNT/A complex and the BoNT/E complex, respectively [57]. Tm values for purified BoNT/A and BoNT/A free of NAPs were estimated as 53 and 54°C, respectively [9, 59]. The temperature denaturation profile of the BoNT/E complex reveals that the unfolding transitions in this protein occur over a significantly broader temperature range than those of the BoNT/A complex (or purified BoNT/A, BoNT/E), suggesting a less cooperative unfolding of the BoNT/E complex which would be consistent with a nonrigid structure. The lower Tm value observed for the BoNT/E complex indicates that it is conformationally less stable than the BoNT/A complex.

The Tm values of around 62 and 45°C for the BoNT/A complex and BoNT/E complex, respectively, based on Tyr exposure, suggest lower exposure of tyrosine residues upon heating of the BoNT/A complex. These results suggest that significant changes in the polypeptide folding of the BoNT/E complex occur upon heating and that at a temperature of around 45°C, it exists in a conformational state that is different from the native state (25°C) or from the conformational state at the physiological temperature of 37°C. Interestingly, it is at this temperature that the BoNT/E complex exhibits optimum enzyme activity [59]. This change in the conformation of the BoNT/E complex at 45°C may be responsible for its optimum enzyme activity at this temperature. It is possible that at this temperature the interactions of BoNT/E with the NAPs in the complex are favorable to bring the enzyme active site close to the substrate cleavage site which in turn leads to maximum cleavage of SNAP-25 at this temperature as compared to that at 37°C. Paradoxically, purified BoNT/E (single chain but only after reduction of the disulfide bond) also shows maximum endopeptidase activity at 45–50°C [59]. Similar observations are made for BoNT/A endopeptidase activity, which is optimally observed (only after reduction of the disulfide bond or separation of the light chain) at 37°C [9, 56], similar to the BoNT/A complex. These observations suggest that while NAPs likely make the BoNT endopeptidase active site more accessible, temperature optimum for activity remains unaffected by their interactions.

The two complexes (BoNT/A and BoNT/E) have significant differences in their protein components, composition and conformation reflected by secondary structure analysis, near-UV CD spectra and temperature denaturation pattern of secondary and tertiary structures. However, NAPs seem to play a similar structural and functional role in protecting the toxin and enhancing its endopeptidase activity, suggesting the presence of specific interactions between the two components (BoNT and NAPs) of BoNT complexes.

While BoNT complex stoichiometry of other serotypes has not been reported, it would not be surprising if the stoichiometry for each of the serotypes is slightly different. BoNT/C complex stoichiometry is assumed to be similar to that of type D [55], although direct experimental estimates are not available. Furthermore, there are differences among different strains of even the same serotype of *C. botulinum* (e.g., D-4947 strain and D-CB16 of type D) [72].

4.7 Biological Implications of Specific Interactions of NAPs with BoNT

It is clearly established that NAPs protect BoNT conformationally against temperature and pH, and structurally against proteases whether the BoNT is produced as part of the type I gene cluster or type II. Two possible mechanisms could be involved in the protection of BoNT by NAPs: (1) NAPs may interact specifically and cover the neurotoxin in such a way that it remains inaccessible to proteolytic attack and other adverse conditions of the GI tract. (2) Specific interactions of NAPs modify the polypeptide folding of the neurotoxin so that the structure becomes more resistant to proteases. In order to understand these mechanisms vis-a-vis HA-33, we have first investigated its interaction with the neurotoxin as that would be a prerequisite to either of the two possible mechanisms. When purified HA-33 was incubated with the neurotoxin and passed through a Sephadex G-200 column, two peaks were observed in the elution. The first peak contained the neurotoxin and HA-33 with calculated apparent molecular mass of 259 kDa, while the second peak contained only HA-33. The appearance of two bands in the first peak indicated a direct interaction of HA-33 with the neurotoxin. We used a 1:1.5 molar excess of HA-33 for the experiment with an understanding that the relatively large molecule of the neurotoxin could require more than one HA-33 to interact with its surface; 94% of HA-33 remained bound to the neurotoxin. Recovery of only 6.0% of HA-33 in the second peak indicated that almost all the molecules of HA-33 directly interacted with the neurotoxin.

The binding of HA-33 with neurotoxin suggested that HA-33 could protect the neurotoxin through direct interactions in the native complex. However, binding in itself did not prove that HA-33 really protected the neurotoxin against proteases. And, if it did protect, then it was not known if the protection involved HA-33 binding-induced alteration in the polypeptide folding of the neurotoxin. These issues were addressed in a study examining proteolysis of type A BoNT in the absence and presence of HA-33.

Notably, none of the proteases used were able to proteolyze HA-33 itself. The experimental conditions used corresponded to optimal proteolysis by each of the proteases, three (pepsin, trypsin, and α-chymotrypsin) of which are known to be active in the GI tract. Treatment of the pure neurotoxin with the same enzymes clearly proteolyzed, suggesting that the enzymes were active under our experimental conditions. Proteolysis following the incubation of the neurotoxin with HA 33 at

1:1 ratio (w/w), which corresponded to 1:4.5 molar ratio, resulted in significant retention of the intact neurotoxin in all but the case of subtilisin. Such an observation could indicate a strong interaction between HA-33 and the neurotoxin, which forms too tight a complex to allow access to the proteases. Moreover, since the amount of uncleaved neurotoxin was different for different proteases, the binding appears to be very specific, thus allowing a given enzyme access to their respective cleavage sites on the neurotoxin.

Among the unique features of BoNT endopeptidase activity, it has been reported that reduction of the disulfide bond between the light and heavy chain is required for the endopeptidase activity [65, 105]. However, in the presence of NAPs there is no requirement of disulfide reduction for the endopeptidase activity [9, 57]. Interestingly, the enhanced endopeptidase activity of BoNT/A in the presence of HA-33 is virtually the same as the whole BoNT/A complex, both quantitatively and qualitatively. In qualitative terms, the HA-33-enhanced endopeptidase activity of BoNT/A did not require reduction of the disulfide bond, just like the BoNT/A complex [9]. It may be noted here that the disulfide bond of BoNT/A remains intact in the native complex form as well as in a complex with HA-33 [9, 93]. Quantitatively, HA-33 enhanced the endopeptidase activity of nonreduced BoNT/A by 21-fold for a 15-min reaction period [94], whereas the BoNT/A complex is reported to exhibit endopeptidase activity 17-fold higher than nonreduced pure BoNT/A for a 10-min reaction period [9]. Thus, HA-33 seems to be able to imitate the presence of all the NAPs in the BoNT/A complex with respect to the enhancement of the endopeptidase activity.

Although the mechanism by which HA-33 may enhance the BoNT/A endopeptidase activity is not discernible from current data, a possible explanation derived from the known X-ray crystallographic structure of BoNT/A is as follows: The active site of BoNT/A is known to be buried in a crevice of about 24 Å deep, which is occluded by a 56-amino-acid residue (residues 490–545) belt [60, 61]. It has been suggested that disulfide reduction opens up the belt and exposes the active site to the surface of the protein for its binding with the substrate peptide groups [60]. We believe that the interaction of HA-33 exposes the enzyme active site without any need to open up the belt. However, reduction of the disulfide bond and thus opening of the belt can further expose the active site to the substrate, as indicated by about 17% additional increase in the endopeptidase activity of BoNT/A [95]. Exposure of the active site upon interaction with HA-33 becomes more plausible in view of a recent report [8] suggesting that the enzymatically active structure of BoNT/A is in the form of a molten globule. Molten-globule folded structure is significantly more flexible than native folded structure [14, 73].

Interestingly, although HA-33 was isolated from the BoNT/A complex [30], its effect appears common to all the BoNT serotypes. This was demonstrated by a similar enhancement of the endopeptidase activity of BoNT/E and BoNT/A by HA-33 [95]. HA-33 effect on BoNT/E is especially noteworthy, as the latter does not have a comparable NAP in its complex form [99]. Therefore, a similar effect of HA-33 on the two serotypes of BoNT suggests a common mechanism involved in the accessibility of the active site of BoNTs to their respective substrates.

Moreover, there must be common structural motifs of the surface of the BoNT/A and BoNT/E molecules. While the three-dimensional structure of BoNT/E is not yet solved, common structural motifs are well known for BoNT/A and BoNT/B [42, 98, 104]. Future work with crystal structure of HA-33 and the BoNT complex will be required to confirm the common structural motif on BoNT surface for interaction with HA-33.

The molecular basis of HA-33-enhanced BoNT endopeptidase activity can be discerned from an additional set of published data, related to the HA-33 protection of BoNT/A from proteases. HA-33 can completely protect BoNT/A from pepsin, trypsin, α-chymotrypsin and subtilisin, suggesting that HA-33 either surrounds the protease cleavage sites on BoNT/A or introduces refolding in BoNT/A so that the cleavage sites become inaccessible. The dissociation constant of 0.4 μM derived from isothermal calorimetry experiments of BoNT/A and HA-33 indicates a strong binding between the two proteins capable of introducing polypeptide refolding in BoNT/A. This is especially likely given a single binding site ($n = 1.0$) obtained from the isothermal titration curve. While it is possible that only one binding site is involved between BoNT/A and HA-33, more than one binding site could also lead to $n = 1$, as long as different binding sites have similar binding affinities. More than one binding site are also likely considering two dimers of HA-33 bind to BoNT/A [30, 48, 96].

Each of the seven serotypes of BoNT has a group of associated NAPs, whose biological roles are not clearly understood. NAPs are known to protect the toxin from adversarial environmental conditions such as temperature and the acidity and proteases of gastric juice [35, 49, 89]. It is notable that HA-33 represents the largest fraction of BoNT/A NAPs [30] and accounts for most of the HA activity of the BoNT/A complex [8]. HA-33 by itself can protect BoNT/A from proteases [93]. Thus, the influence of HA-33 on the endopeptidase activity of BoNT/A and BoNT/E is consistent with the HA-33 effect on other biological and physical features of BoNT/A and its complex with the NAPs. HA-33 seems to imitate the role of all NAPs in the BoNT/A complex.

In a further effort to examine the possible role of NAPs (HA-33) in the biological activity of BoNT, we compared SNAP-25 cleavage in synaptosomes, representing nerve cell conditions, by BoNT/A and BoNT/E in the presence and absence of HA-33 [95]. SNAP-25 cleavage by both BoNT/A and BoNT/E is less in synaptosomes compared to in vitro conditions, but HA-33 enhanced the endopeptidase activity of both BoNT/A and BoNT/E even in synaptosomes, albeit to a lesser degree (three- to four-fold). These results suggest that the neurotoxin complex with HA-33 enters the synaptosomes. The lower cleavage of SNAP-25 in comparison to in vitro conditions is likely to result from the inaccessibility of SNAP-25 in synaptosomes. We have also made an observation [115] that HA-33 binds to synaptosomes through synaptotagmin, which will further support the possibility of HA-33 entry into synaptosomes. These results are particularly significant to the possibility of the use of the BoNT complex with HA-33 as a therapeutic agent. The entrance of HA-33 and BoNT as a complex will have enhanced and stable endopeptidase activity inside the neuronal cell. Stable endopeptidase activity

Table 4.3 Comparison of clostridial neurotoxins showing percent identity (*lower left*) and percent similarity (*upper right*)

	TeNT	BoNT /A	BoNT /pB	BoNT /npB	BoNT /C1	BoNT /D	BoNT /E	BuNT /E	BoNT /F	BaNT /F	BoNT /G
TeNT		55.3	61.8	62.1	56.2	55.3	57.8	58.0	59.0	57.9	60.8
BoNT/A	35.4		60.0	60.2	55.2	55.2	61.0	60.5	61.8	63.2	58.8
BoNT/pB	42.4	44.1		96.0	59.1	57.3	61.6	61.3	61.7	62.5	74.9
BoNT/npB	42.7	40.7	**92.8**		58.4	56.6	61.9	61.3	61.8	62.8	74.2
BoNT/C1	35.1	33.9	36.0	35.0		69.1	55.9	56.0	56.3	56.4	57.4
BoNT/D	**34.1**	34.3	36.6	36.4	53.8		57.1	57.1	57.1	56.8	57.6
BoNT/E	37.9	41.3	40.0	40.2	34.3	35.6		**98.2**	77.3	77.2	60.0
BuNT/E	37.9	41.5	40.0	40.1	34.8	35.4	**96.9**		77.1	76.8	60.0
BoNT/F	38.5	41.4	41.0	41.7	34.6	36.4	62.2	62.1		80.7	58.6
BaNT/F	40.5	44.1	42.8	43.3	35.1	35.5	63.9	63.6	70.5		59.5
BoNT/G	41.6	41.2	58.4	57.7	35.0	37.1	39.5	40.6	39.3	40.7	

BuNT/E: neurotoxin produced by *C. butyricum* type E **BoNT/npB: non-proteolytic type B neurotoxin**
BaNT/F: neurotoxin produced by *C. baratii* type F

inside the neuron is a critical phenomenon for the long-lasting effects of botulinum either as a toxin or as a therapeutic agent [28, 52]. Even though there have been ample reports to suggest that the toxin (or the light chain) itself is encoded with information for its longevity inside the neuronal cells, HA-33 may still be able to enhance intracellular activity and stability.

4.8 Protein Sequences

Protein sequences of various protein components of different serotypes of the BoNT complex vary in their homology. A general survey of their sequence homology is provided in the following section to give a general idea of the variability that exists in these related proteins. Within the BoNT complex, biological function of NAPs is not clearly established, at least for physiological conditions, and little is known about the molecular basis of NAPs' protection of the toxin from adverse environmental conditions, such as low pH and proteases in the GI tract, and high temperature in the environment and in cooked food. Primary sequences can be examined to estimate their potential stability (*vide infra*).

4.8.1 BoNTs

The homology among all types of BoNT genes ranges from 55.2 to 98.2%, as listed in Table 4.3. As a reference for a related but different toxin, we have also listed sequence comparison with TeNT. The homology of TeNT gene to other BoNT genes ranges from 55.3 to 62.1%, which is lower than that among BoNT genes. The homology of serotype E between *C. botulinum* and *C. butyricum* is the highest (98.2%), whereas the lowest homology is between serotype D and serotype A (see Table 4.3). Overall, the homology is generally higher within the same serotype (such as /B, /E and /F) than that among different serotypes whether it is compared

Table 4.4 Percent amino acid identities (*lower left-hand triangle*) and similarities (*upper right-hand triangle*) of NBP

NBP/A		90.5	83.0	83.0	79.0	81.5	86.0
NBP/B	81.5		81.5	81.5	76.0	78.0	87.0
NBP/C	65.4	66.5		95.0	73.0	75.5	82.0
NBP/D	65.4	66.5	93.0		72.0	73.5	80.0
NBP/E	65.8	59.0	54.0	53.0		77.0	69.0
NBP/F	74.0	61.5	56.5	55.5	65.0		73.0
NBP/G	72.0	75.0	65.0	64.0	54.0	58.0	

NBP neurotoxin-binding protein

from different species (*C. botulinum*, *C. butyricum*, and *C. baratii*) or different proteolytic strains (proteolytic type B neurotoxin (pB) versus nonproteolytic type B neurotoxin (npB)). The highest homology among different serotypes is observed between /B versus /G (74.2–74.9 %) and /E versus /F (76.8–77.3 %). These results strongly suggest the existence of common ancestors of the "/B–/G" group and the "/E–/F" group during molecular evolution.

4.8.2 Protein Sequence Homology Among Different NBPs

The homology among all types of NBP genes ranges from 69 to 95 % as listed in Table 4.4. The highest homology is between /C and /D (95 %), whereas the lowest homology is between /E and /G (69 %). These results strongly suggest that NBP genes from types /C and /D have a homologous relationship.

There is a short (14 residues) repeat in the amino acid sequence of the NBPs which could appear as L-L/N-S-L-I/M/V-S/T-T/S-A/T-I-P-F-P/L-Y/F-G from residue 97 to 111 and from 136 to 150. The second repeat may play an important role in distinguishing the M complex from other types of progenitor neurotoxins as indicated from the following observations. First, the amino acid residues from 99 to 149, which include the second repeat from 136 to 150, were deleted in NBPs from M complex forming serotypes (NBP/E, NBP/F, and NBP/E from *C. butyricum*). Second, NBPs from M and L complex forming serotypes (NBP/A, NBP/C, and NBP/D progenitor neurotoxins) were cleaved in the second repeat region to yield approximately 13 and 106 kDa components [37].

NBPs have sequence homology to both BoNT (all types) and TeNT in the light-chain area. The conserved domain is named peptidase_M27 area (see Fig. 4.7). This protein sequence possesses similarity to a known Zn-dependent peptidase (peptidase M27) and even has a truncated Zn-binding motif in its M27 region. NBP lacks the consensus sequence "abXHEbbHbc"; therefore, it loses the capability of binding with Zn atom [84]. Based on this observation, we suspect NBP to be in fact a pseudo-toxin, originated from the early genomic duplication events (also known as paralogous events). Interestingly, a crystal structure of NBP was recently presented at the Toxins 2011 meeting by Dr. R. Jin, which seems to be very similar to the BoNT itself in its general organization (personal communication, [39]).

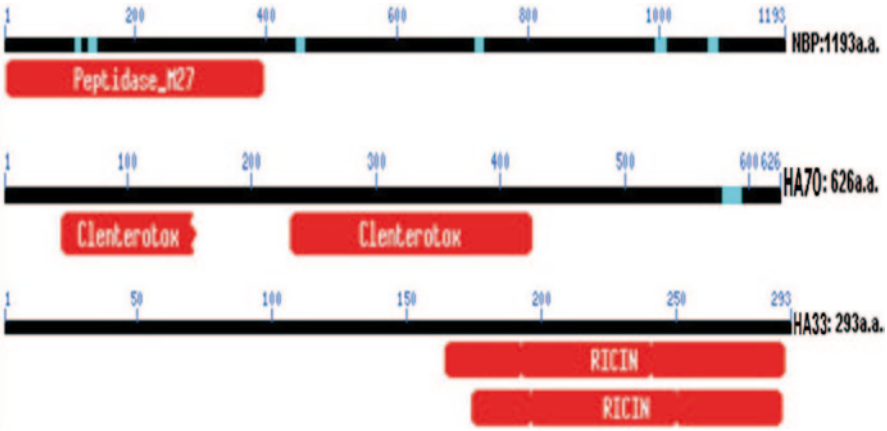

Fig. 4.7 Sequence homology of segments of neurotoxin-binding protein (NBP), hemagglutinin-70 (HA-70) and HA-33, with known functional proteins (see text for details)

Table 4.5 Percent amino acid identities (*lower left-hand triangle*) and similarities (*upper right-hand triangle*) of HA 33

	HA33/A	HA33/npB	HA33/pB	HA33/C	HA33/D
HA33/A		93.0	88.5	58.0	58.0
HA33/npB	89.0		89.0	58.0	58.0
HA33/pB	83.5	82.0		58.0	57.0
HA33/C	38.5	41.0	39.0		78.0
HA33/D	38.5	40.0	35.0	65.0	

HA hemagglutinin, *npB* nonproteolytic type B neurotoxin, *pB* proteolytic type B neurotoxin

4.8.3 Protein Sequence Homology Among Different NAPs

Homology among all types of HA-33 genes ranges from 57 to 93 % and is listed in Table 4.5. Interestingly, serotypes /A and /B form one group, whereas serotypes /C and /D form another. However, the homology between /C and /D is smaller than that between /A and /B (88.5–93 %), irrespective of whether serotype B is from a proteolytic or nonproteolytic strain. These results may suggest that the serotypes /C and /D may have split earlier than serotypes /A and /B in molecular evolution.

Homology of HA-70 genes among all serotypes ranges from 76 to 98.5 % (Table 4.6). The highest homology is between the pair of /A and /B (98.5 %) on one hand and the pair of /C and /D (95.5 %) on the other. The lowest homology is observed between the pair of /C and /G (76 %). These results suggest that the HA-70 genes are more conserved than all the other NAP family genes.

Two conserved domains are present in HA-70 (one completely conserved domain and one 3'-end-truncated conserved domain) of a domain known as Clenterotox (InterPro Database ID as "IPR003897"), which has the common name "clostridium enterotoxin." This domain appears to exist in two proteins—"clostridium perfringens enterotoxin" (CPE) and "clostridium enterotoxin." The Clenterotox

Table 4.6 Percent amino acid identities (*lower left-hand triangle*) and similarities (*upper right-hand triangle*) of HA-70

	HA70/A	HA70/B	HA70/C	HA70/D	HA70/G
HA70/A		98.5	82.5	82.5	83.0
HA70/B	96.0		82.5	85.0	84.0
HA70/C	68.5	71.5		95.5	76.0
HA70/D	68.5	71.0	93.5		77.0
HA70/G	72.0	73.0	65.0	65.0	

conserved domain of HA-70 is related to an enterotoxin which is secreted by *C. perfringens* (sequence similarity 44%). The enterotoxin has been shown to be the major virulence factor and the main reason for food poisoning and other GI illnesses caused by *C. perfringens* [86]. This conserved domain is believed to be involved in the disintegration of tight junctions between gut endothelial cells. This mechanism is mediated by host claudin-3 and claudin-4 receptors, situated at the tight junctions [51, 64]. Based on these sequence analyses, we believe that the function of HA-70 might be related to the translocation of BoNT across the intestinal wall.

HA-33 has a putative conserved domain in its C-terminus with ricin B-related lectin (*Polyporus squamosus*) [112]. HA-33 also contains another conserved domain (shorter but overlapping with ricin domain) which could only match with a eukaryotic protein called pierisin. Pierisin is an apoptosis-inducing protein, first reported from cabbage butterfly [114], which is possibly involved in the adenosine diphosphate (ADP) ribosylation of pierisin itself [71]. HA-33 also has a whole protein sequence similarity with a mosquitocidal toxin secreted by *Bacillus sphaericus* [12]. The fact that HA-33 has protein sequence similarity with prokaryotic as well as eukaryotic organisms possibly indicates that HA-33 plays significant biological roles in both coevolution and pathogenicity between eukaryotic and prokaryotic organisms.

4.9 Prediction of the Half-Life of BoNTs and NAPs: The N-End Rule

The N-end rule was proposed by Bachmair and his colleagues [3]. The rule is useful to predict the half-life of a protein with known sequence. It has been demonstrated that the sequence identity of the N-terminal residue in a protein actually plays an important role in determining the protein stability in vivo [3]. It seems that the first several N-terminal residues initiate the process of ubiquitin-mediated proteolytic degradation [15, 109]. Therefore, it is possible to predict the half-life of a protein under in vivo condition by comparing their N-terminal sequences with standard protein sequence of known half-life [15, 109].

The prediction of half-life of BoNTs and NAPs using the N-end rule algorithm was carried out with a computer program provided by ExPASy Proteomics Server (http://us.expasy.org/tools/protparam.html) named ProtParam tool. The results

Table 4.7 List of potential PTM sites on different components of BoNT/A complex, as predicted through the ExPASy website

	BoNT/A	NBP	HA-70	HA-33	HA-17
N-Myristoylation site	13	12	5	2	
Casein kinase II phosphorylation site	21	27	4	5	
Tyrosine kinase phosphorylation site	6	2	1	1	1
Protein kinase C phosphorylation site	11	15	8	7	1
N-glycosylation site	19	22	13	6	1
Amidation site	1				
Tyrosine sulfation site	3	5	3	1	1
Zinc-binding region	1				
cAMP and cGMP-dependent protein kinase phosphorylation site		1			
Cell attachment sequence			1		
Leucine zipper pattern			1		
Lectin domain of ricin B				2	

BoNT botulinum neurotoxin, *NBP* neurotoxin-binding protein, *HA* hemagglutinin, *cAMP* cyclic adenosine monophosphate, *cGMP* cyclic guanosine monophosphate

show the same pattern for BoNTs and NAPs, all belonging to high-longevity protein [13]. All proteins of the BoNT/A complex have half-life longer than 30 hours in mammalian reticulocytes (cell culture, in vitro). But this conclusion is somewhat contradictory to the conclusions from the "instability index" [13, 40]. According to the instability index (calculated based on an algorithm for the presence of unique dipeptides, [92]) calculation results, at least one of the NAPs (HA-70) is not predicted to be stable because its instability index is larger than 40, which is considered beyond the threshold for a stable protein [40]. While specific experiments need to be carried out to examine actual stability of these proteins intracellularly, the prediction from instability index is somewhat consistent with the observation during the purification of the BoNT complex. HA-70 is known to degrade into two fragments of HA-53 or NAP-53 and HA-20 or NAP-20 [48, 57].

4.10 Estimation of Post-Translational Modification Sites

The prediction of putative post-translational modification (PTM) sites for an ORF of known sequence can be used to derive its biological function and metabolic pathway. Currently, the PTM prediction has become a very useful tool in proteomics and functional genomics. Putative PTM sites for BoNTs and NAPs were searched using a set of bioinformatics tools available at ExPASy web site (http://us.expasy.org/tools/#ptm). Table 4.7 summarizes 12 kinds of predicted PTM sites for BoNT/A and the members of its NAPs. It shows the "N-glycosylation" site as the most dominant PTM site throughout the whole BoNT complex (total of 61 sites). The "casein kinase II phosphorylation" site is the second dominant PTM site (57 sites), and there is only one "cell attachment" site that appears in HA-70 to suggest its

potential biological role in the BoNT complex. BoNT/A has a total of 75 predicted PTM sites, while NBP has 84 sites. These two proteins have the most predicted PTM sites in neurotoxin complex, and thus most possibly play important functions in the biochemical behavior of the neurotoxin. There are already reports that phosphorylation and ubiquitination may play a role in the intracellular biological activity and stability of the toxin [27, 47].

4.11 Composition of Therapeutic BoNT Products

Current major BoNT therapeutic products include the BoNT/A complex (marketed as Botox® and Dysport®), BoNT/B complex (marketed as Myobloc® and Neurobloc®), and purified BoNT/A without NAPs (marketed as Xeomin®). While the in vivo longevity of the BoNT/A products does not seem to be dependent on the presence of NAPs, there has been spirited debate on the role NAPs may play in the stability of the BoNT formulation and diffusion of the injected BoNT for therapeutic purposes [11, 103]. In general, BoNT in the complex form is resistant to environmental stress, such as pH, temperature and proteases. However, commercial products contain additional formulations, which may affect the stability of the product. In the BoNT/A complex preparations, adding either sodium chloride (Botox®) or lactose (Dysport®, Ipsen, Ltd.) protects the steric conformation of BoNT [78]. Human serum albumin is also added to prevent loss from surface adsorption. The toxin is then dried either with freezing (Dysport®) or without freezing (Botox®, Allergan, Inc.) [78]. These, as well as the pure BoNT/A product, Xeomin®, are lyophilized products which are reconstituted with saline solution maintained near physiological pH.

The botulinum toxin type B product (Myobloc®, Neurobloc®) is provided in liquid form at pH 5.6, as opposed to a lyophilized powder that requires reconstitution in saline. It nevertheless is also based on the complex of BoNT/B neurotoxin and NAPs. BoNT/B has shown stability for months when stored appropriately at 2–8 °C, whereas BoNT/A must be stored at −5 °C as a powder and must be used within hours once reconstituted according to the manufacturer's recommendation [54]. At pH 5.5, the BoNT/B complex appears as a 700-kDa single peak on size exclusion chromatography (SEC), but when exposed to pH 7.8 overnight, a small portion of the neurotoxin appears to dissociate from the complex [10]. A similar observation has been made for the BoNT/A complex dissolved in 50 mM Tris–HCl, pH 7.6, which showed the complex appear as a 569-kDa single peak on a Sephadex G-200 SEC analysis [9].

Although the BoNT/B product is generally considered safe, case reports by Dressler and Benecke [21] indicate that side effects include visual disturbances, dry eyes and dry mouth which suggest systemic diffusion of physiologically active quantities of toxin from the sites of injection. This interpretation is limited, however, by the small number of cases [63]. One thing that has clearly been observed is that there is more antibody response to BoNT/B-based drugs because of the requirement of dramatically higher dose (about 100-fold higher) compared to other serotypes of BoNT-based drugs [26].

One of the major issues in the literature is whether the size or the nature of the BoNT complex in the drug formulation will have impact on the diffusion characteristics. Using radiolabeled BoNT/A complex (900 kDa) and purified BoNT/A (150 kDa), it has been clearly established that there is no significant difference in the diffusion of these reagents at physiological doses [29, 107]. In fact, the diffusion was not significant for either of the samples. So, diffusion-related problems with BoNT/B drugs are likely due to the higher dose used.

The size and composition of the BoNT/A complex in therapeutic preparations have been examined, and it has been shown that the active ingredient used in Botox® preparations consists of a 900-kDa BoNT/A complex as has been presented by Allergan and other researchers [7, 67, 68, 80]. The size of the complex as an active ingredient of Dysport® has not been published, although components of the BoNT/A complex used for the formulation has been presented. A recent study [25] has compared sizes of the BoNT complex present in the commercial products of Botox® and Dysport® at different pH and salt conditions. This study has also compared results from a nonformulated preparation of the BoNT/A complex at different concentrations under various pH and salt conditions. Eisele et al. [25] have shown that the nonformulated BoNT/A complex mostly dissociates into 150 kDa BoNT/A and its associated proteins at neutral pH within 1 minute. At a concentration of 100 μg/ml, the BoNT/A complex dissolved in 50 mM citrate buffer, pH 5.5, exists as a 900-kDa particle. Lowering of the concentration to 0.1 μg/ml in the same buffer reduces the size to 500 kDa. Concentrations between 0.1 and 100 μg/ml show a varying mixture of 900- and 500-kDa BoNT/A complexes. In phosphate-buffered saline (PBS), pH 7.4, the BoNT/A complex dissociated into 150-kDa BoNT/A and its associated protein even at 40 ng/ml. Analysis of commercial products, Botox® and Dysport®, in PBS, pH 7.4, showed no intact complex of even 500 kDa; the whole complex was dissociated into 150-kDa BoNT/A and its associated proteins (NAPs). Interestingly, NAPs appeared to exist as a complex of their own with a molecular size of 400 kDa even in PBS, pH 7.4. Even at pH 5.5 (50 mM citrate), Botox® had less than 17% 500-kDa complex, whereas Dysport® showed the presence of no intact complex. In contrast, nonformulated BoNT/A complex preparation showed all the protein to exist as a 500-kDa complex at 40 ng/ml. While the relative amount of 150-kDa BoNT/A appeared to be similar in Botox® and Dysport® as well as nonformulated BoNT/A complex, Dysport® appeared to have lost much of the NAPs complex [25].

It has been pointed out that composition and perhaps stability of the BoNT/A complex depend on the culture and purification conditions [80]. Long-term stabilizing effects of NAPs have been questioned from the stability data of pure 150-kDa BoNT/A preparations used in Xeomin® formulations (NT 201, [38]) under temperature conditions of up to 60 °C in the presence of human serum albumin and sucrose excipients.

The presence of NAPs in therapeutic products based on the BoNT/A complex (Botox® and Dysport®) and BoNT/B complex (Myobloc®, Neurobloc®) may or may not be needed for stability and biological activity, but is currently present as part of the formulation. The question is whether their presence has any unintended consequences, both positive and negative. This is important to note since BoNT

complexes are currently in use as therapeutic drugs, and even if BoNT and NAPs separate either before injection or after injection, nerves and surrounding tissues are exposed to both components. Recent reports on the exposure of neuronal and other cells suggest that there is a massive genomic and cytokine response to the complex, and some of these responses appear to be exclusive to the BoNT and NAPs [108, 113]. Since the complex has remained a safe drug for a couple of decades now, it is possible that the cellular responses to NAPs and BoNT may provide a balance in the cellular physiology. This would be an interesting area of research.

4.12 Concluding Remarks

The origin, gene expression, assembly, secretion, purification, physicochemical characteristics and biological activities of the BoNT complex has been examined to a certain degree for more than 60 years. The information on the seven serotypes of the BoNT complex has been intriguing with variability in their genetic organization, sequences, composition, physical characteristics and biological activity. Their native biological role is hardly understood and physicochemical properties are just beginning to be examined. The complex is an evolved system that appears to be a nanoparticle in an anaerobic bacteria, and certainly plays a role in the survival of the toxin in the adversarial environment of high temperature and proteases of digestive tract. It is quite possible to utilize these characteristics of this structure in biotechnology and biomedicine. When full information on its physicochemical characteristics becomes available, the scope of its utility in modern medicine may be fully realized.

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Chapter 5
Neurotoxin Structure

Subramanyam Swaminathan

Abstract The crystal structures of a number of neurotoxins are now available and reveal that all botulinum neurotoxins (BoNTs) have similar structures in general. However, there are differences. These variations and their relation to functional differences will be reviewed. BoNTs A, B, and E have similar structural domains responsible for specific functions in toxicity but have different domain organization. This leads to the difference in speed of onset of toxic effect and its efficacy. Individual domains of botulinum toxins also exhibit differences and these can be correlated to their functional differences. Structural information is also being used in developing countermeasures for botulism. The strategies and their results are discussed.

Keywords Botulinum neurotoxin · Structure–function relationship · Crystal structures · Domain organization · Substrate–enzyme complex · Exosites

5.1 Introduction

The seven antigenically distinct serotypes (A–G) of *Clostridium botulinum* neurotoxins (BoNTs) are the most poisonous proteins known to humans with the lethal dosage in the range of 1 ng/kg weight for humans [1, 2]. They cause flaccid paralysis leading to death by blocking neurotransmitter release. The neurotoxins are produced by various strains of *Clostridium botulinum* and several other species of *Clostridia* [3] (see Chap. 10 of this book). The ease with which they can be produced makes them the most dreaded potential biowarfare agents and, as such, declared as category A biological agents by the Center for Infectious Diseases, USA. Interestingly, these poisons also serve as therapeutic agents for treating many ailments and are approved by the Food and Drug Administration (FDA) and other regulating agencies [4–11]. While all serotypes are poisonous, BoNT/A, B, E, and F affect humans, BoNT/C affects avian, and BoNT/D is toxic to animals [12].

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BoNTs are produced as an inactive single polypeptide chain (150 kDa) and are cleaved by endogenous proteases into dichains before release. All BoNTs are released as dichains except BoNT/E and BoNT/D which are cleaved by host proteases. The dichain molecule consists of a 100-kDa heavy chain (HC) and a 50-kDa light chain (LC) held together by a disulfide bond [2]. All serotypes of BoNTs consist of three functional domains of approximately equal molecular mass. The N-terminal half of the HC (50 kDa) is the translocation domain, the C-terminal half (50 kDa), the receptor-binding domain, and the LC is the catalytic domain. BoNTs first bind to the cholinergic nerve terminals via the binding domain and are internalized into neurons by a temperature- and energy-dependent process. The internalized BoNTs undergo conformational change when the endosomal pH becomes acidic by the H + pumping adenosine triphosphatase (ATPase), triggering the insertion of the translocation domain into the endosomal membrane to form channels for the catalytic domain to escape into the cytosol where it cleaves one of the three proteins of the soluble N-ethylmaleimide sensitive factor attachment protein receptor protein (SNARE) complex [13]. The SNARE complex is required for the docking and fusion of synaptic vesicles containing neurotransmitters to target membranes and the formation of the complex is prevented when one of the proteins is cleaved resulting in inhibition of neurotransmitter release leading to botulism.

BoNTs are unique in that their substrates are large polypeptides and each serotype specifically cleaves one SNARE protein at a specific peptide bond. The catalytic domain responsible for cleavage is a zinc endopeptidase with a characteristic HExxH + E zinc-binding motif. BoNTs A, E, and C cleave synaptosomal-associated 25-kDa protein (SNAP25) at specific peptide bonds while BoNTs B, D, G, and F cleave the vesicle-associated membrane protein (VAMP) also at specific bonds. BoNT C is the only BoNT which cleaves two SNARE proteins, SNAP25 and syntaxin [2]. Intriguingly, BoNTs are so specific that BoNT A and C precisely cleave adjacent peptide bonds of SNAP25 and so do BoNT D and F in VAMP. The large substrate making multiple specific contacts with the enzyme is responsible for the specificity of cleavage of the peptide bonds. Also, though the sequence pair of the scissile bond may appear in multiple places, BoNTs target a unique pair for cleavage.

Over the past three or four decades, a large body of biochemical, biophysical and structural information has been accumulated and has helped in understanding the structure–function relationship. This chapter will describe the structural details that have emerged from macromolecular crystallography and will correlate them with the function of this most important toxin. Crystal structures of holotoxins of A, B, and E have been determined and the function of individual domains analyzed [14–16]. To further understand their function structures of individual domains with and without their interacting partners have been determined. The common and distinct features of these structures and how they relate to their differences in functions and most importantly to the development of therapeutics to treat botulism are discussed in detail.

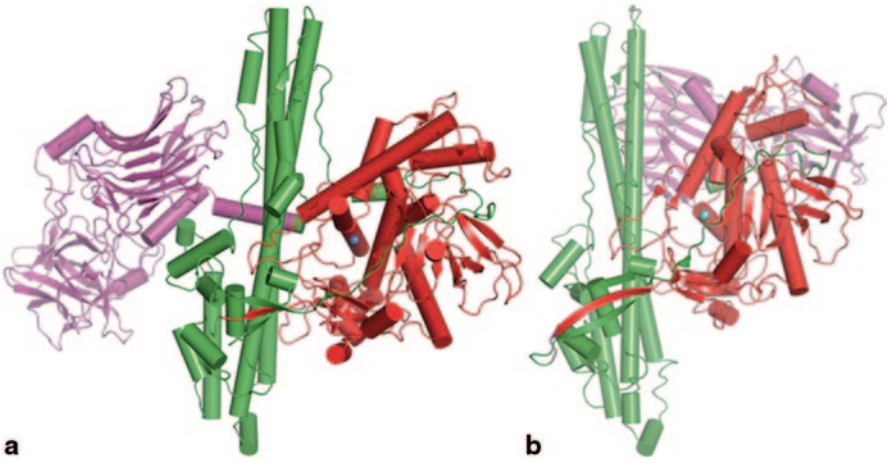

Fig. 5.1 a *Ribbons* representation of holotoxin botulinum neurotoxin (BoNT) B. The catalytic, translocation, and receptor-binding domains are shown in *red*, *green*, and *magenta*, respectively. Zinc is shown as a *sphere*. **b** *Ribbons* representation of BoNT E with individual domains shown in the same color. The alpha helices are shown as *cylinders*. While in B the domains are arranged in a *linear* fashion, they are more *globular* in E with catalytic and binding domains on the same side as the translocation domain

5.2 Crystal Structures of Holotoxins: Similar and yet Different

5.2.1 Serotypes A and B

Crystal structures of BoNT A and B have been determined and provide a detailed three-dimensional arrangement of the functional domains [15, 16]. Both of them exhibit three distinct structural domains corresponding to the three major functions of the toxicity steps [13]. BoNT A and B have 39% sequence identity and 56% similarity and possess similar fold and domain organization [17]. The structures agree with a root-mean-square deviation (rmsd) of ~2.9 Å when the binding domain is excluded from least squares fitting. This is because the binding domain in A is inclined at a larger angle with the axis of the translocation domain than in B. The three domains are arranged in a linear fashion with the translocation domain in the middle flanked by the binding and catalytic domains. There is no interaction between the binding and the catalytic domains and interactions between the translocation and binding domains are minimal (Fig. 5.1).

The N-terminal half of the HC (H_N), the translocation domain, comprises two long α-helices (105 Å) forming a coiled coil. These two together with other short helices form helical bundles at the two extremes of the long helices and resemble the structure of colicin A. The remainder of this domain is mostly in random coil

conformation except for short stretches of β-strands. Remarkably, a long region of the N terminal part of H_N (residues 492–545 in BoNT A and 481–532 in BoNT B) forms a loop and wraps around the catalytic domain like a belt and is aptly called the "belt region." Though this region is in the primary sequence of the translocation domain, in three dimensions it is closely associated with the catalytic domain. The function of this belt region is emerging slowly via various biochemical and biophysical studies. The putative transmembrane region has been identified and takes a random coil conformation in both the crystal structures. This is speculated to take a helical conformation when the pH of the endosome becomes acidic allowing the hydrophobic residues to be exposed for insertion into the vesicle membrane.

The C-terminal half of the HC (H_C), the receptor-binding domain, consists of two subdomains, the N terminal H_{CN} and the C terminal H_{CC} domains. H_{CN} consists of 14 β-strands forming two 7-stranded β-sheets. The two β-sheets form a jelly roll or lectin-binding fold [18]. The H_{CN} domain is connected to the H_{CC} domain by a short α-helix. H_{CC} contains a β-trefoil fold formed by three β-sheets [19]. Otherwise, H_{CC} is mostly made up of random coil and long loops. The receptor-binding pockets reside in cavities formed by the β-trefoil motif as described later. The purpose or function the H_{CN} domain is still not understood well.

The catalytic domain has an α/β fold with the active site zinc occupying a deep cavity in the middle of the domain. The catalytic zinc is coordinated by two histidines and one glutamate of the HExxH + E motif. A water molecule provides the fourth coordination and acts as a nucleophile for the catalytic action. The deep cavity has a high negative electrostatic potential and is partly occluded by the belt region of the translocation domain.

5.2.2 Serotype E

The crystal structure of BoNT E also shows the three domain structure [14]. BoNT E shares ~38% sequence identity with A or B. The individual domains agree very well with the corresponding domains of A or B. The rmsd between the corresponding catalytic, translocation, and binding domains of BoNT E and B are 1.4 Å, 1.4 Å, and 1.9 Å, respectively. Since the holotoxin used for crystallization is an un-nicked single-chain molecule, the interchain disulfide loop is well defined in the crystal structure. The catalytic zinc is coordinated as in A or B. However, there is a major difference between the crystal structures of A or B and E. The domain organization is completely different. While the three domains are arranged in a linear fashion in A or B, they form a globular molecule with both binding and catalytic domains on the same side of the translocation domain in BoNT E. Accordingly, the binding and catalytic domains have interactions which are absent in A or B. Each pair of domains has shared interfaces. Part of the belt region is between the binding and catalytic domains making three-way interactions (Fig. 5.1).

The interactions between the domains are listed in Table 5.1. There are three interactions in BoNT E which are absent in A or B and interestingly these are salt-

Table 5.1 Interactions at the interface of various domains in BoNTs (botulinum neurotoxins) A, B, and E Interactions in A and B are putative when the B domain takes the same position as in BoNT E

BE Atom 1	BE Atom 2	BA	BB
T:K672 NZ	B:S849 OG	T:S700-B:N875	T:T687-B:N862
T:K672 NZ	B:D1051 OD1	T:S700-B: D1076	T:T687-B:N1063
T:K826 N	B:F843 O	T:Q852-B:Y869	T:D839-B:Y856
T:K826 NZ	B:D869 OD1	T:Q852-B:A895	T:D839-B:G882
T:K826 NZ	B:D869 OD2	T:Q852-B:A931	T:D839-B:G882
T:D675 OD2	B:Y905 OH	T:N703-B:A931	T:N690-B:N916
T:L473 O	B:N1068 ND2	T:L504-B:N1093	T:L493-B:Y1080
T:N474 OD1	B:N1070 ND2	T:T505-B:G1095	T:D494-B:E1082
C:N261 ND2	B:Y839 O	C:K272-B:T865	C:S278-B:M852
C:N261 OD1	B:R846 NH1	C:K272-B:N872	C:S278-B:N872
C:I263 O	B:R846 NH2	C:1274-B:872	C:L280-B:N872

bridge interactions. The catalytic and translocation domains have a buried surface area of 8,574 Å, the catalytic and binding domains have a buried surface area of 830 Å, and the translocation and binding domains have an interface area of 2,986 Å. While the belt region is exposed to solvent in A and B, about 750 Å is buried in E since it is sandwiched between the catalytic and binding domains.

This unique domain organization is not an artifact of crystallization. Both A and E were crystallized at pH 7 and have a different domain organization. A and B crystallized at pH 7 and 6, respectively, have the same domain organization. But A and B are nicked dichain molecules while E is a single-chain molecule. The effect of this also can be ruled out since a reconstructed low-resolution electron microscope image of nicked dichain molecule of E shows somewhat similar arrangement as in the crystal structure of E. However, in the electron microscope the translocation domain could not be placed well [20]. In a later section, a possible implication of this difference in domain organization is discussed.

5.3 Structures of Individual Domains and their Functional Relationship

5.3.1 Binding Domain

Crystal structures of binding domains of all serotypes are now available, except for BoNT E [12, 18, 21–30]. However, the binding domain structure of BoNT E is available in the crystal structure of holotoxin E. All of them have the same fold except for the length of some loops. In addition, crystal structures of the binding domain in complex with receptor molecules are available. The function and purpose of the H_{CN} domain are not yet clear. However, it has recently been shown

that BoNT A H_{CN} binds to microdomains of the plasma membrane, especially to phosphatidylinositol phosphates [31]. It is also suggested that this helps in orienting the translocation domain for insertion into membrane. However, structural work is needed to prove this hypothesis.

The H_{CC} domain has been studied extensively. Early biochemical and biophysical studies have shown that the C-terminal region of H_{CC} is involved in ganglioside binding. Sialic acid-containing gangliosides like GT1b, GD1b, etc. have been identified as receptor molecules for the binding domain of BoNTs [32–34]. These are in abundance on the presynaptic membrane surfaces and help in accumulating BoNTs at the surface before being internalized. Neuraminidase-treated cultured cells have reduced affinity for BoNT A. Also, bovine chromaffin cells lacking in polysialogangliosides became sensitive to BoNT A when pretreated with gangliosides [35–37]. Fluorescence quenching experiments on ganglioside binding implicated tryptophans at the C terminus to ganglioside binding [38]. Photoaffinity labeling occurred predominantly at His1292 of tetanus neurotoxin (TeNT) [39]. Further, the region was narrowed down to 34 residues containing the conserved sequence motif H…SxWY…G as essential for ganglioside binding [40]. In the cocrystal structure of BoNT B with sialyllactose molecule [16], this region was identified as the GT1b-binding site since sialyllactose partly mimics GT1b. His1240, Ser1259, Trp1261, and Tyr1262 of the conserved motif H…SxWY…G in BoNTs form this pocket with residues Glu1188 and Glu1189 also taking part. The sialic acid sits between His1240 and Trp1261, and the stacking interaction between the sialic acid and Trp1261 is critical. The sialyllactose molecule has extensive interaction with the protein molecule. Sequence comparison with BoNT A and TeNT confirms that similar residues can form the same pocket for ganglioside binding. This pocket is called site 1 in this chapter (also referred to as lactose-binding site) [22, 32]. In a recent study on BoNT A-binding domain in complex with GT1b analog, this has been further confirmed with some minor variation [29]. The BoNT E H_{CC} domain also has a similar pocket except that the first H in the conserved motif is replaced by K. BoNT F and BoNT G have a similar GT1b-binding pocket except the H in the H..SxWY…G motif is replaced by G in BoNT G. This site is present in A, B, E, F, and G. However, this motif is absent in C and D though a tryptophan residue occurs in a region close to this motif [12, 27].

While BoNTs A, B, E, F, and G have a single ganglioside-binding site, BoNT C and D possess two ganglioside-binding sites [32, 41]. The dual binding site for ganglioside binding has been shown for TeNT both structurally and biochemically [25, 42]. One site corresponds to site 1 of BoNTs and the second site is a nonoverlapping adjacent site to site 1. The second site is called site 2 or sialic acid-binding site in TeNT. A crystal structure of TeNT H_C with a GT1b-analog (GT1b with beta-linked sugar) showed that GT1b-β cross links two molecules [23]. However, this could be an artifact of crystal packing or the nature of the sugar linkage in GT1b-β. Biochemical evidence supports two ganglioside-binding sites for TeNT and only one for BoNTs A and B and no cross-linking is seen in solution. Recent studies also support two ganglioside-binding sites for C and D which lack the H..SxWY…G motif. One site is close to site 1 but the other site has not been identified structurally. The

primary sequence of C and D contains a Trp in a loop close to the conserved motif in other BoNTs. This Trp affects ganglioside binding and accordingly this loop is called ganglioside-binding loop [12, 32]. This unique loop in BoNTs C and D might allow them to function differently.

5.3.2 Double Receptor Model

Since the affinity and toxicity of BoNTs could not be explained solely based on ganglioside binding, it was concluded that there is a second receptor involved in the uptake of BoNTs by neuronal cells. Reduction in TeNT binding was observed when rat brain membranes were treated with proteases suggesting proteins may also be involved in uptake [43, 44]. Other biochemical evidences also suggested a second receptor molecule, specifically a membrane protein or a glycosylated protein. These led to the idea of a double receptor model [34]. The low-affinity, high-density gangliosides allow BoNTs to concentrate on the surface of the cell and they move laterally to bind to a high-affinity, low-density second protein receptor. GT1b is a common receptor for almost all BoNTs. However, each BoNT has specific protein receptor(s). The dual receptors increase the binding constant and explain the nanogram level LD_{50}. Specific receptors have been identified for BoNT A, B, E, and G. BoNT A uses three isoforms of synaptic vesicle 2 proteins (SV2A, SV2B, and SV2C) [45]. Synaptotagmins (Syt I and Syt II) act as receptors for BoNT B and also for BoNT G, though with a lower affinity [46–48]. Recently, glycosylated SV2A and B have also been identified as receptors for BoNT E [49]. SV2 may also be the receptor for BoNT F and probably the keratan sulfate moiety of SV2 binds to the second receptor site [28]. BoNT C and D do not need a second protein receptor and use dual ganglioside binding [12, 27]. But the details are still to emerge. Though biochemical evidences were gaining ground, structural support started coming only recently. Crystal structures of TeNT HC with a tripeptide (Tyr–Glu–Trp) and BoNT B in complex with Syt II peptide support the double receptor model [21, 25, 26].

Recent exquisite structure determination of BoNT B with the luminal peptide of Syt II gave structural evidence for the double receptor model [21, 26]. Stevens and coworkers reported the structure of the holotoxin B cocrystallized with Syt II peptide [21] while Brunger and coworkers determined the crystal structure of the H_C domain fused to Syt II peptide by a large linker peptide [26]. Both structures showed an identical binding site for Syt II peptide which is adjacent to site 1 identified to be the ganglioside-binding site. The peptide, which is unstructured in the native protein, is induced to form a helix when it binds to BoNT B. It occupies a hydrophobic pocket. Phe47, Leu50, Phe54, Phe55, and Ile58 of Syt II peptide are buried into the binding groove and make hydrophobic and stacking interactions with BoNT B residues. Charged residues Glu57 and Lys51 interact with residues having complementary charges in the protein. There are other hydrogen bonds and electrostatic interactions for the peptide to bind strongly. Syt II in both occupies a hydrophobic cavity in the β-trefoil fold. Interestingly, this site is the same as

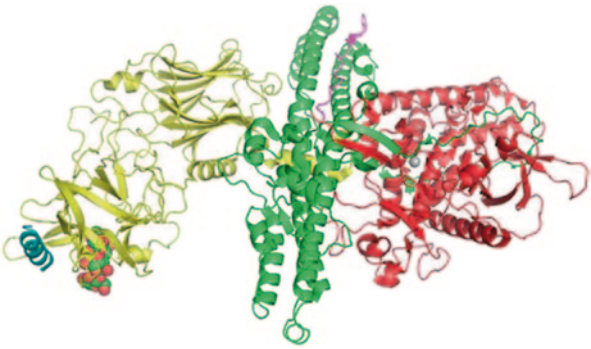

Fig. 5.2 A composite figure of botulinum neurotoxin (BoNT) B with GT1b and Syt II. GT1b was transferred from BoNT A structure. They occupy adjacent cavities in the receptor-binding domain. GT1b is shown in *sphere* model while Syt II peptide is shown in *ribbons* representation in cyan

identified in TeNT as site 2 where GD3 sugar or a tripeptide binds [25]. In TeNT, it may be that the ganglioside binding to site 2 is displaced by a high-affinity protein receptor [42].

5.3.3 Modeling of GT1b on the Structure of BoNT B–Syt II Complex

As of now, there is no crystal structure available for a ternary complex of GT1b–BoNT B- Syt II. Since BoNT A and BoNT B have identical pockets for GT1b binding, it is reasonable to transfer GT1b from the BoNT A–GT1b complex onto BoNT B after least square fit of the two protein structures (Fig. 5.2). This gives some interesting perspective of how these receptor molecules sit adjacent to each other and even make some contacts. This also opens up possibilities for therapeutic intervention using both sites simultaneously with a possible linker.

Syt I and Syt II are protein receptors for BoNT G which has a similar binding pocket and they are expected to bind in a similar fashion [47]. It is expected that in BoNT A, E, and F, their protein receptor will bind in site 2. However, no structural information is available yet. It will also be helpful to determine structures of BoNT A, E, F, and G with their corresponding protein receptors to identify the similarities and difference in binding. Additionally, the ternary complex of ganglioside, toxin, and receptor protein is desirable.

5.3.4 Translocation Domain

The translocation domain plays an important role in the toxicity pathway of BoNT. The internalized neurotoxin is inside the vesicle and has to escape into the cytosol where its targets reside. The pH of the endosome undergoes a change to acidic environment and helps in a conformational change which in turn allows the translocation domain to insert into the membrane. It is believed that the translocation

domain forms a channel to allow the catalytic domain to escape. A few low-resolution electron micrographs support a tetrameric pore formation but no structural details are available. The transmembrane region has been predicted in BoNTs. In the crystal structures, this region, 653–673 in BoNT/B and 650–672 in BoNT/A, does not take helical conformation and is at one tip of the translocation domain apposing one of the long helices. It is speculated that this region will take a helical conformation when the pH changes to acidic. However, structure determination of BoNT/B at various pH (as low as 4) did not show any change in this region [50] though it could be because of crystal packing. In any case, the size of the channel is predicted to be 15 Å wide which is not enough for catalytic domain to pass through without unfolding. It is possible that the catalytic domain partially unfolds in the vesicle due to pH change and then escapes into cytosol where it folds back at neutral pH. While structural information on individual domains is available in plenty for the binding and catalytic domains, it is lacking for translocation domain. Especially, the role of the belt region needs to be understood more. It is known that when the catalytic domain separates from the translocation domain, the hydrophobic region occupied by the belt is exposed and is used by the substrate that binds to it. Accordingly, the belt region protects the active site from premature cleavage of substrate. It is interesting that when the catalytic domain separates, the conformation of the belt region should change since otherwise it will be too flappy and disordered. This may be one reason why it is difficult to crystallize the translocation domain by itself. Attempts are underway by many groups to crystallize the translocation domain with or without the belt region. At present, there is no crystal structure available. The state of oligomerization is also not yet known for the pore formation.

In contrast to the lack of structural work, a large body of information is available via biochemical and biophysical studies. Early studies by Montal's group have suggested that the HC and catalytic domain help each other as chaperons for changing the conformation to guide the catalytic domain into the cytosol from the endosome. This study combined single current measurements with cleavage assays to show how the catalytic domain is transferred [51]. When the endosomal environment changes to acidic pH the H_N domain penetrates the membrane and translocates LC from N to C terminals during which the channel is occluded by the LC. Since the catalytic domain has to separate from the toxin, it is imperative that the disulfide bridge is reduced before it can escape into the cytosol. It is proposed that the translocation is not complete until the disulfide bond is reduced [52]. With the LC-H_N fragment, it is shown that the binding domain is not required for translocation [53]. Also, it is now believed that the catalytic domain of this fragment (LC-H_N) can be translocated into cytosol even when the pH of endosome is neutral contrary to the fact that it has to be 5.4 when the holotoxin is concerned [53–55]. The physiological relevance of this is yet to be understood since it is difficult to understand the partial unfolding of the catalytic domain without a change in pH. The role of the belt region is not yet understood well. Recent studies show that it plays a role in translocation. The lowering of pH neutralizes the acidic residues in the belt region and nullifies the repulsion between the negative charge on the membrane and the

protein [56]. However, this contradicts the translocation at neutral pH in the LC–H_N complex. These conflicting results must be resolved. These discrepancies need to be addressed in future. The belt region also acts like a pseudosubstrate and protects the aggregation of H_N. The belt region is displaced by the substrate when the catalytic domain separates and enters cytosol [57, 58].

5.4 Substrate Recognition by BoNTs

Crystal structures of catalytic domains of all serotypes, including TeNT have been determined. The catalytic domains of all serotypes of BoNTs share significant sequence homology (31 to 34% sequence identity) and they all show similar fold and possess a deep electronegative cavity containing the catalytic zinc and the active site residues. Most importantly, around the catalytic zinc the sequence identity is conserved and the interactions between the conserved residues are also maintained. The characteristic zinc-binding motif, HExxH…E, is in the middle of the primary sequence of LC. The zinc ion is coordinated by two histidines and one glutamate with the fourth coordination provided by a nucleophilic water molecule. The nucleophilic water molecule makes a strong hydrogen bond with the first Glu in the zinc-binding motif which acts as base for the catalytic action. Remarkably, the active sites of all BoNTs share similar architecture and significant sequence conservation. The conserved residues within 10 Å of the zinc make identical contacts. In BoNT A, the zinc ion coordinates with His223, His227, Glu262, and the nucleophilic water [59–61]. His223 and Arg363 are bridged via hydrogen bonds with the conserved Glu351 in the middle. Similarly, His227 and His230 are linked via hydrogen bonds to the conserved Glu261. The nucleophilic water makes a hydrogen bond with the conserved Tyr366. These interactions are conserved in all BoNTs, including the nucleophilic water to Glu224 hydrogen bond [62, 63] (Fig. 5.3). Mutational analyses on these conserved residues have confirmed that they are all involved in the catalytic activity. Most important of these are Tyr366 and Glu224, where catalytic activity is not detectable following mutation. Mutations of Arg363, Glu351, and Glu261 drastically reduce the K_{cat} compared to wild type. This is equally true for BoNT E and by extension to others also [62, 64–66]. However, there are some exceptions. In BoNT A in a high-resolution structure, two water molecules coordinate zinc as also in BoNT E holotoxin structure [14, 59]. Such variations have also been observed in thermolysin, a similar zinc endopeptidase. Since the interactions in the vicinity of the zinc ion are identical the catalytic mechanism is common to all BoNTs.

While the similarity of structures and the active site architecture of catalytic domains confirm that the catalytic mechanism is similar and points to the critical residues involved in catalytic activity, they do not inform anything regarding mode of selection of specific peptide bond for cleavage by each serotype. Obviously, the substrates for BoNTs, being large polypeptides, are expected to make multiple contacts with the enzyme when the scissile bond is positioned and properly oriented for

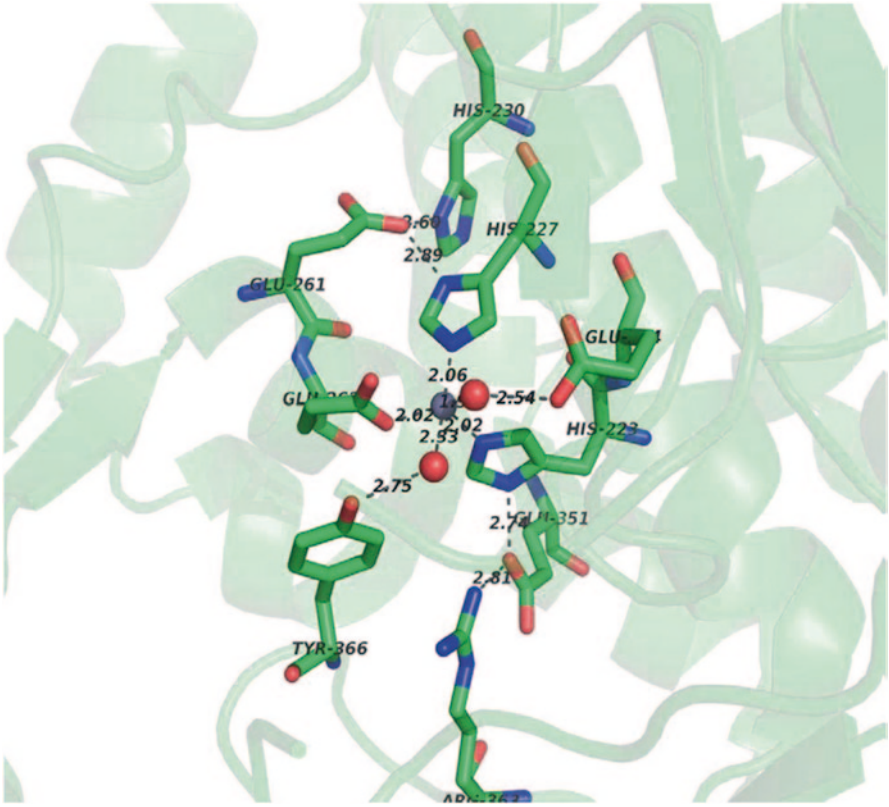

Fig. 5.3 The common interaction in all botulinum neurotoxin (BoNT) catalytic domains is shown as in BoNT A. Figure was produced using PDB id 3BON. The conserved residues are shown in stick model, zinc, and waters in *sphere* model in *grey* and *red*, respectively. *Dashed lines* represent coordination and hydrogen bonds

cleavage. These interactions will determine the specificity of the enzyme with some being critical. How could the adjacent bonds of SNAP25 be exactly placed near the catalytic zinc for cleavage by BoNT A and C (Q197-R198 for A and R198-A199 for C)? To understand the specificity and selection of the unique scissile bond, crystal structures of the enzyme–substrate complex are required. Unfortunately, first the enzyme–substrate complex is difficult to crystallize since the substrate is immediately cleaved leaving only the products possibly bound to the enzyme which may or may not mimic the Michaelis–Menten complex. Second, the substrates are large polypeptides (12 to 25 k) and it may be difficult to form the enzyme–substrate complex for cocrystallization. In the case of BoNTs, two strategies have been adopted so far. For the BoNT A–SNAP25 complex, an inactive double mutant with cleavable SNAP25 has been used while for BoNT F, it is an active enzyme with an uncleavable substrate-based inhibitor [57, 67].

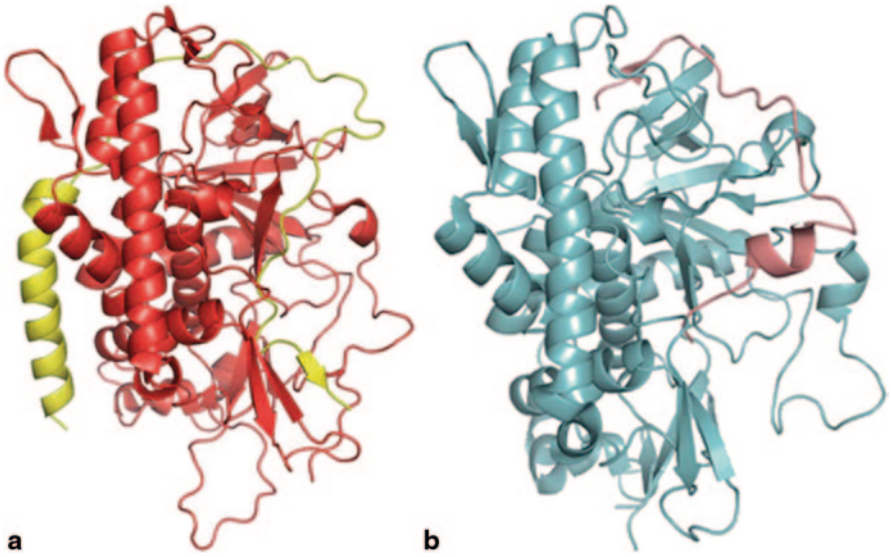

Fig. 5.4 a The botulinum neurotoxin (BoNT) A catalytic domain with synaptosome-associated protein 25 kDa (SNAP25) (PDB id 1XTG). The enzyme is in *red* and the substrate peptide in *yellow*. Note the helical and beta strand segments at the two ends. **b** The BoNT F catalytic domain with vesicle-associated membrane protein (VAMP) peptide. BoNT F is in light *blue* and the VAMP peptide in *magenta*. Both (**a**) and (**b**) are in same orientation and the substrate peptides occupy similar grooves

5.4.1 BoNT A–SNAP25 Complex

An inactive mutant (E224A, Y365F) of BoNT A was cocrystallized with a SNAP25 peptide (141–204) for structure determination [67]. The shorter SNAP25 was chosen since it is used as a substrate in many biochemical experiments and contains the scissile bond region and is cleavable. It makes extensive contacts with the enzyme at many places and the substrate wraps around the catalytic domain. Some of these contacts have been shown to be critical for binding and positioning the substrate for scissile bond cleavage. Unfortunately, since a double mutant was used some crucial interactions near the active site were lost. For example, the scissile bond region is not near the zinc and is away from it. Moreover, the region near the active site is disordered. But at regions remote from the active site, there are strong interactions between the enzyme and the substrate. These sites, called exosites, provide detailed information about the contacts which may be used to explain specificity. The substrate which is unstructured in the native state acquires some secondary structure on binding to the enzyme, especially the helix formation in the region 147–168 of SNAP25 (Fig. 5.4).

The crystal structure of an uncleavable shorter peptide with the native enzyme is helpful in understanding interactions between the enzyme and substrate near the active site mimicking the native enzyme and substrate contacts. Residues (192) DEAN**QR**ATK(200) of SNAP25 is the minimum region required for cleavage by

BoNT A (scissile bond Q197-R198 in bold) [68]. Since an uncleavable peptide that would bind, but not cleaved by BoNT A, was required, the substrate peptide (197)**QR**ATKM(202) (P1 to P5') was chosen for cocrystallization. This peptide binds at the active site as expected and provides detailed information about the substrate–enzyme interactions at the active site [60].

In the hexapeptide (QRATKM) complex, the carbonyl oxygens of P1 (Gln197) and P1' (Arg198) make strong hydrogen bonds with the side chains of Tyr366 and Arg363, respectively. The amino nitrogen of P1 displaces the nucleophilic water and coordinates with zinc. In BoNT A, P1' (Arg198) makes a salt bridge with Asp370 of the enzyme (Fig. 5.5). These interactions demonstrate the critical role played by these residues in addition to the zinc-coordinating residues and explain the mutational analysis [62, 64]. Based on this, a catalytic mechanism has been proposed (see Fig. 6 in [61]). This is supported by mutagenic studies on several BoNTs. Conserved Tyr and Arg help to position, orient, and stabilize the substrate for cleavage. Glu224 acts as a general base by absorbing a proton from the nucleophilic water. The nucleophilic water attacks the carbonyl carbon of the scissile bond, which forms a tetrahedral transition intermediate. The zinc ion and Tyr might stabilize this transition intermediate. The shuttling of protons with the help of Glu224 assists subsequent formation of a stable leaving amino group. The crystal structure of BoNT E with a tetrapeptide containing the scissile bond showed similar interactions as far as the carbonyl oxygens and conserved Tyr and Arg are concerned [69]. These interactions will be present in other BoNT–substrate complexes also. This model is consistent with the model proposed for BoNT B, E, and F [57, 62, 70] and will be applicable for all BoNTs. Structures of these two BoNT A–substrate complexes have helped in mapping the subsite for each residue of the substrate and the exosites. This information gives an overall picture of the substrate–enzyme complex and helps in understanding the substrate recognition by BoNT A better.

5.4.2 BoNT F–VAMP Complex

Crystal structures of BoNT F in complex with two VAMP peptides, VAMP 22–58/Gln58-D-cysteine and VAMP 27–58/Gln58D-cysteine, use an active enzyme with an uncleavable substrate inhibitor peptide with $K_i \sim 1$ nM [57]. These crystal structures give an overall picture of the enzyme–substrate interactions to help understand the substrate recognition strategy. Three exosites were identified that govern the substrate specificity. Due to induced fitting, the side chains take different rotamer positions to accommodate the substrate. Biochemical and mutational studies confirmed that BoNT F recognizes VAMP via these unique exosites. This structure established that Arg133, Arg171, and Glu164 are important residues and play a critical role in determining the substrate specificity. Both biochemical and structural results agree well [57, 71]. Extending the substrate beyond the C terminal of the inhibitor peptide improved hydrolysis suggesting additional interactions of the region 59–65 of VAMP [72]. However, the substrate inhibitor in the BoNT F complex structure stops at P1 and does not provide any information in this regard (Fig. 5.4).

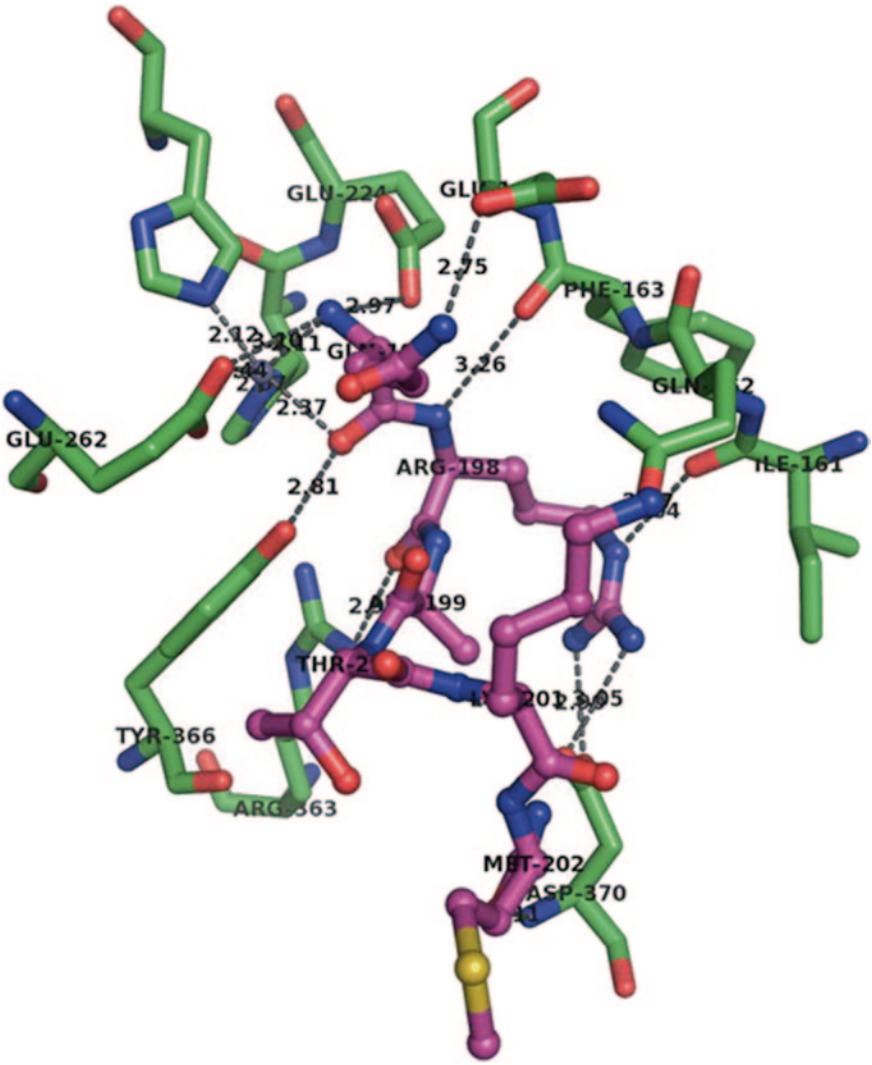

Fig. 5.5 The active site interactions. Critical interactions of QRATKM peptide with botulinum neurotoxin (BoNT) A catalytic domain. The substrate peptide is shown in *pink ball* and *stick* model while enzyme residues in *green stick* model

Although the VAMP peptide is small compared to the SNAP25 used in the BoNT A complex, it showed distinct exosite interactions. The orientation in which the substrate binds to the enzyme is the same in both and the active site interactions are conserved. However, VAMP is differently positioned compared to SNAP-25. The three major exosites in BoNT F are completely different from those in BoNT A (Fig. 5.6). Again as in BoNT A, the unstructured substrate takes a helical conformation induced by contacts with the enzyme. Exosite 1 forms a short helix and its hydrophobic side chains point towards the hydrophobic core of the enzyme.

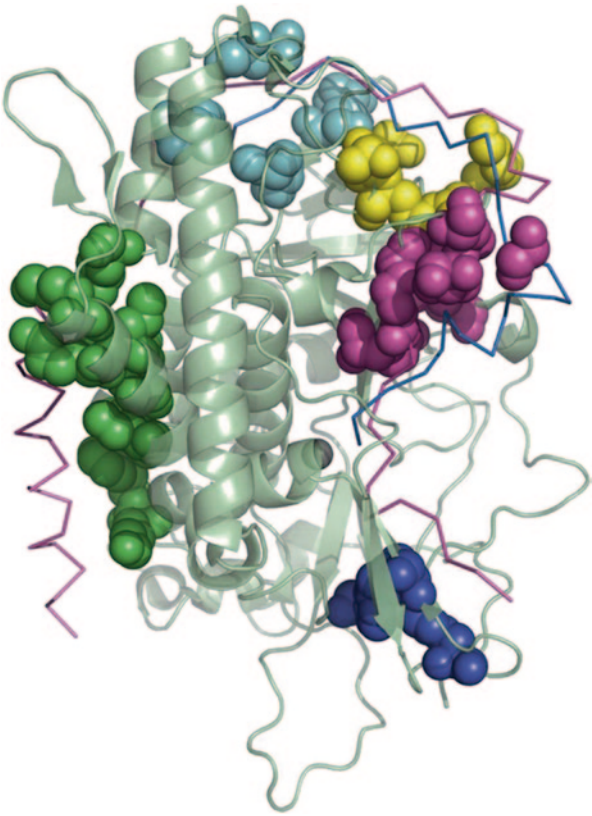

Fig. 5.6 Comparison of exosites in botulinum neurotoxin (BoNT) A and F. α and β exosites of BoNT A are shown in *green* and *blue sphere* model, respectively. Exosites 1, 2, and 3 of BoNT F are shown in *magenta*, *yellow*, and *light blue sphere* model. The exosites of BoNTs A and F do not overlap and are distinct

Surprisingly, the V1 SNARE motif is not a helix but the adjacent region (N-terminal side) is helical. In summary, the enzyme-complex structures help in understanding the interactions between the two, leading to drug design against botulism. Whether the exosites alone can be used as targets for drug design should be explored.

5.4.3 α Exosite of BoNT A-SNAP25 Complex could be Common to all BoNTs

In all three holotoxin structures known so far, the N-terminal region of the translocation domain wraps around the catalytic domain. The loop is mostly unstructured but for a short β-strand and an α-helix. Interestingly, the position and region of the helix is the same in the holotoxin structures. In BoNT A, residues 495–503 form a helix (LDLIQQYYL) and in BoNT B the helix is formed by residues 485–493 (FPINELILD), while it is residues 466–473 (NDLDQVTL) in BoNT E. These are positioned exactly in the same place in three dimensions in all three structures, on the back of the molecule (opposite side of the active site cavity). Since all BoNTs have similar fold, it is expected that this region will be α-helical in the remaining

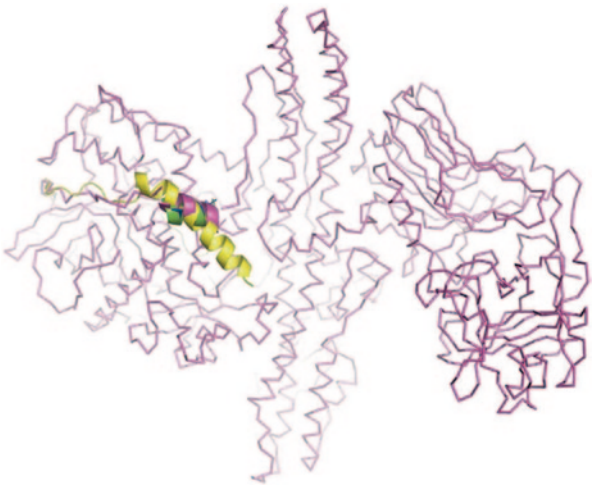

Fig. 5.7 Botulinum neurotoxin (BoNT) A *line* drawing is shown with the helical region of the belt. *Magenta, green*, and *blue* helices are from BoNTs A, B, and E. *Yellow* α helix is from synaptosome-associated protein 25 kDa (SNAP25). Note they all occupy the same region and hence all substrate could use this region as exosites

BoNTs, as also shown by sequence comparison. The α-helices are in a hydrophobic cavity formed by the catalytic domain. The substrate peptide in the BoNT A–SNAP25 complex superposes with the belt region of holotoxin BoNT A. This is also true for BoNT F. Interestingly, in the complex structure of BoNT A-LC with SNAP25, residues 147–168 of SNAP-25 forms an α-helix and occupies the same region (Fig. 5.7). This region makes extensive contacts with the catalytic domain and the site is called the α exosite. SNAP-25 which is unstructured in its native state is induced to take this conformation. In the BoNT F–VAMP complex, this region is not present in the peptide used but if present could take helical conformation and occupy a similar pocket. By extension, it can be speculated that substrates for the other BoNTs may also take a helical conformation to complement the hydrophobic cavity in the enzyme. This tempts the author to speculate that this exosite may be common to all BoNTs and may be used as a target for inhibitor design which does not involve the active site without any need for chelating zinc. A short helical segment may be used to occupy this space thereby preventing the substrate occupying the same space. This could be a pan active inhibitor for all BoNTs.

So far, structures of only three complexes are known. Structures of substrate–enzyme complexes for all serotypes would be useful in understanding the differences in specificity and how precisely BoNT A and C target adjacent peptide bonds in the same substrate. This must be a top priority for researchers working on BoNTs.

5.5 Different Domain Organization of BoNT A and E Explains the Rate of Toxicity

All BoNTs are poisonous but BoNT A is the most potent of all. However, the rate at which toxicity happens differs. Even though BoNT A is the most potent and persists longer than any other BoNTs in the neuronal cell, it is BoNT E that acts fastest. This

difference in potency and speed of action provides opportunity to create chimeric molecules that will act fast and at the same time be very potent. Dolly's group has undertaken this study to explore this possibility. The faster action of BoNT E is attributed to its ability to translocate the catalytic domain faster into cytosol. The unique domain organization of BoNT E might give some clues to explain this speedy action of BoNT E. It is presumed that the transmembrane region has to enter the membrane first to create a channel. A comparison of the structures of BoNT A and E shows that the transmembrane region and the receptor binding sites are on the opposite ends of the molecule in A and B while they are on the same end in BoNT E. It has been shown that the ganglioside binding is not affected by lowering the pH or in other words the toxin remains bound to the endosomal wall as the pH drops to an acidic environment. This means that the toxin has to reorient itself so that the transmembrane end could reach or come close to the membrane wall. This could happen in two ways. Either the molecule could rotate about the anchor point to bring the transmembrane region close to the membrane wall or the translocation domain and the catalytic domain could rotate about a hinge region without disturbing the anchor points [73]. The latter possibility seems more plausible considering there are two anchor points—ganglioside and the protein receptor. Also, in BoNT E this is the case and could explain the speed of action. It is also proposed that BoNT E is in a translocation ready conformation and this accounts for the faster action. Whereas in BoNT A or B, it has to go through a two-step receptor binding and then the rotation which will make it slower [14].

Extensive work on different combinations of BoNTs A and E has been done. The major goal is to identify a chimera which will act faster than BoNT A and at the same keep the potency of BoNT A. The studies with the chimeric molecule showed that the chimera EA containing the catalytic and translocation domains of type E and the receptor domain of A (1–844 of E and 871–1296 of A) acts as fast as BoNT E, whereas the chimera AE containing the catalytic and translocation domains of type A and the binding domain of E (1–874 of A and 845–1252 of E) is the slowest (E>EA>A>AE) [74]. This experiment clearly indicated that the speed of translocation does not depend on the binding domain since AE containing the binding domain of E had the slowest translocation. It depends only on the catalytic and translocation domain. Comparing A or B with E, the only difference is the conformation of the linker region between the binding and translocation domains. In BoNT E, this region is 830–844 of the translocation domain and makes interaction with both the other domains. Remarkably, chimera EA contains this region. It will be worthwhile to study a chimera of E with this linker peptide replaced by that corresponding to A. It will also be worth doing some mutational analysis with some individual residues in the interface or putative interface of A and B.

This chimeric study has opened up possibilities for various chimeras with different combinations. It is possible to combine the H_C, H_N, H_{CC}, H_{CN}, H_N, and LC in different permutations to obtain desired results. Since BoNTs are used in therapeutics, it may be important to have a high potency BoNT that could be used in minimum dose to limit antibody generation. This will become an important tool in pharmacology.

5.6 Rational Structure Based Drug Design for BoNTs

BoNTs are potential biowarfare agents and a public health hazard but effective drugs are yet to be developed, especially for post intoxication with botulinum toxins. Antibody therapeutics are emerging but have some limitations [75]. An equine antitoxin is also available for postexposure therapeutics. Recently a cocktail of antibodies of different serotypes were tested and found effective. The added advantage here is that it acts on multiple serotypes and therefore there is no need to identify the causative serotype first. Small molecule therapy is also being developed for both pre- and post-intoxication. Antitoxins for botulism could be developed by targeting any one of the three major steps in its toxicity pathway: binding, translocation, or catalytic activity. This can be done by designing small molecules to block any of these sites. In the following section, we discuss how structural information could be exploited to achieve this goal.

5.6.1 Binding Domain as Target

By now, the ganglioside-binding pocket of various BoNT serotypes has been characterized. In addition, for BoNT B the second protein receptor site has also been mapped. These sites and their structural characterization should help in designing small molecules to block their binding site. This was first attempted on the TeNT-binding domain and small molecules were identified to block the ganglioside-binding site. Computational methods and virtual screening were used to identify small molecules that would bind to H_C and were subsequently confirmed by electrospray ionization spectroscopy-mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) spectroscopy [76]. This study identified doxorubicin (Dox), a DNA-intercalator that was shown to compete with GT1b. The crystal structure of the TeNT-binding domain with Dox identified the interactions and provided enough information for modification to improve the binding affinity. Dox binds in a cavity formed by Glu1189, Glu1190, His1240, Trp1261, and Tyr1262 which also form the conserved sequence H…SxWY…G in BoNTs [77]. This site is the same as occupied by sialyllactose in earlier studies and also the GT1b-binding site. Dox has numerous contacts with the protein molecule and shows good binding. Since GT1b and Dox bind at the same site, it is believed that Dox could become a lead molecule for developing drugs for botulism. It would be helpful to study more derivatives of Dox to find a potential inhibitor. However, this work was not continued further.

Recently, interest in the binding domain has gained momentum since the binding sites (both gangliosides and protein receptors) have been identified [32]. These sites will be good targets for developing small molecules to prevent the toxin binding to neuronal membranes. Since these two sites are independent, adjacent and non-overlapping, two molecules connected by a linker to block both sites will be a good approach. To date, there are no published results in this direction. However, it has to be shown that they are useful for post-intoxication.

5.6.2 Translocation Domain could be a Target

The translocation domain also offers itself as a drug target since it is possible to develop small molecules to block the channel formed by this domain. The triterpenoid toosendanin, a natural product from the bark of the tree *M. toosendan*, has been used in China to protect monkeys from botulism [78]. More recently, it has been shown that toosendanin blocks the channel formed by HC and inhibits LC translocation [79]. For the first time, small molecules have been identified to block the toxicity of botulism by blocking the translocation channel. Since it blocks both the channels formed by BoNT A and E it may work as a broad-spectrum target. However, structural details of the channel formation which are needed for drug development are yet to emerge.

5.6.3 Substrate-Based Inhibitors to Block Catalytic Activity

The most important aspect of structure-based drug design is to identify the interactions between the target enzyme and the drug molecule that could mimic the substrate–enzyme interactions. Crystal structures of enzyme complexes help in achieving this goal. Drug-like small molecule libraries and virtual screening protocols could be used to identify potential lead molecules that could be accommodated at the active site to block the site for substrate binding. Even though this could be done with the native enzyme structure, structures of enzyme–substrate complexes have some advantage over the former. When a substrate binds, both the enzyme and the substrate suitably change their conformation for induced fitting. It is better to take this complex structure as a starting point by removing the substrate from the model since it makes the enzyme model in a conformation ready to accept a substrate or suitable molecule. Crystal structures of BoNTs or their domains with substrates or binding partners provide the information fundamental to this process. Most of the efforts in drug design are now focused on BoNT A, though other BoNTs are also being considered lately. Crystal structures of BoNT A with SNAP25 peptides, both large and small, have provided enough information for drug development based on structures and are being used for drug discovery. Most of the time, the strategy used is to modify the small peptides to improve their interactions with the enzyme and increase the binding affinity.

Crystal structures of enzyme–substrate peptide complexes provide enough information for structure-based drug discovery. Subsites occupied by residues spanning the active site have been identified in the tetra and hexapeptide complexes with BoNT LC [57, 60, 61, 69]. This provides the basis for designing serotype specific inhibitors which could be transformed into effective drugs for BoNTs. These structures also provide a model for pharmacophore, based on which either small molecule, peptidomimetic or non-peptidomimetic inhibitors could be developed. Several research groups are involved in developing small molecule inhibitors for BoNTs using this information at some stage for optimizing the molecule

for better efficacy [80–84]. Pang and Smith groups have used virtual screening to identify small molecules [85–88]. Janda and coworkers have combined synthetic chemistry and high-throughput screening and identified small molecules (based on hydroxamates) and optimized them using structure–activity relationship (SAR) [89, 90]. Brunger and coworkers have started from CRATKML heptapeptide spanning the scissile bond and have developed peptidomimetics [91, 92]. Swaminathan and coworkers have started from their structure of BoNT/A with hexa- and terapeptides to develop substrate peptide inhibitors [60, 61, 93]. A large number of tetrapeptides have been identified and modification of these into peptide or peptidomimetic inhibitors is in progress. This group is also working on small molecule inhibitors based on virtual screening. Currently, most of the inhibitor work is focused on BoNT/A.

The crystal structure of N-Ac-CRATKML, an inhibitor developed by Schmidt, with BoNT A protease domain is also available [94]. This structure brought some interesting facts to light. The subsite S1' of BoNT/A is large and could accommodate larger molecules than arginine. Also, this site formed by three loops adjusts to accommodate different molecules caused by induced fitting. Interestingly, when the complex structure of Ac-CRATKML, a moderate inhibitor (K_i = 2 μM), is compared with QRATKM complex, the peptide as a whole slides by one residue but still Arg occupies the P1' site [59]. This gives some indication for designing a larger side chain at P1′ without perturbing the subsite. A number of tetra, hexa and hepta substrate peptide complexes with BoNT/A LC have added more information about the interactions. These structures have shown that the P1 residue could be changed to Arg without affecting the binding efficiency and in fact it has proved to be a better inhibitor since it complements the charge in that region. It is known that changing it to cysteine improves binding [94] but oxidation of Cys may cause a problem. The structural environment of P1 and P1' also suggests that an amino acid containing an aromatic ring may be better suited as it would improve stacking interactions. Various possibilities exist and could be used for obtaining an effective inhibitor. Tetrapeptides allow one peptide to be modified at a time to study the SAR. However, modifying one residue could change the conformation and the binding mode might change. But if done in an iterative fashion, this will produce an efficient inhibitor. There are endless possibilities that can be tried with the information provided by these structures. More importantly, it will be worthwhile designing a common molecule which could act as a pan-active inhibitor.

In summary, the structural information provides the basis for structure-based drug discovery.

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Chapter 6
The Dual-Receptor Recognition of Botulinum Neurotoxins

Polysialo-Gangliosides and Synaptic Vesicle Proteins Mediate the Neuronal Entry of Botulinum Neurotoxins by Binding to its C-terminal 50-kDa H_C fragment

Andreas Rummel

Abstract The neurospecificity of the botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT) is one important reason for their extraordinarily high toxicity. This neurospecific binding is mediated by the interaction with two receptor components. All BoNTs and TeNT bind first to complex polysialo-gangliosides abundantly present on the outer leaflet of neuronal membranes. The ganglioside binding occurs in BoNT/A, B, E, F, G and TeNT via a conserved ganglioside-binding pocket within the most carboxyl-terminal 25-kDa domain H_{CC}, whereas BoNT/C and D display two different ganglioside-binding sites within this H_{CC} domain. Subsequently, upon exocytosis, the intraluminal domains of synaptic vesicle proteins are exposed and can be accessed by the surface-accumulated neurotoxins. BoNT/B, DC and G bind with their H_{CC} domain to a 15–17mer membrane-juxtaposed segment of the intraluminal domain of synaptotagmin-I and synaptotagmin-II, respectively. In contrast, the H_C fragment of BoNT/A and E interacts via an extensive surface with the intraluminal domain 4 of the synaptic vesicle glycoprotein 2 (SV2). Whereas BoNT/A interacts with all three SV2 isoforms, BoNT/E only binds SV2A and SV2B. Also, BoNT/D, F and TeNT are supposed to employ SV2 for neurospecific uptake although a direct interaction has to be demonstrated. Thereafter, the synaptic vesicle is recycled and the bound neurotoxin is endocytosed. Acidification of the vesicle lumen triggers membrane insertion of the translocation domain while the protein receptor binding is maintained, pore formation and, finally, translocation of the enzymatically active light chain.

Keywords Botulinum neurotoxin · Tetanus neurotoxin · Clostridial neurotoxins · Polysialo-gangliosides · Synaptic vesicle membrane proteins · Synaptotagmin · Synaptic vesicle glycoprotein 2 (SV2)

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6.1 Introduction

The botulinum neurotoxins (BoNTs) exert an extreme toxicity which is predominantly caused by their high target cell specificity. They bind highly specifically to nonmyelinated areas of cholinergic motor nerve terminals [1]. It was found that, like for cholera toxin, gangliosides, complex polysialic acid-containing glycolipids, play an important role in binding BoNTs. A protease-sensitive interaction of BoNT with neuronal membranes led, however, to the dual-receptor hypothesis which postulates an interaction with gangliosides and a proteinaceous receptor [2]. Subsequently, the mode of ganglioside interaction as well as the nature of the proteinaceous receptor was characterised in recent years, thereby explaining the extraordinary target cell specificity of BoNT on the molecular level.

6.2 Complex Polysialo-Gangliosides Accumulate BoNT on the Neuronal Membrane

Tetanus neurotoxin (TeNT), sharing a 35% amino acid identity with BoNT [3], together with clostridial neurotoxins (CNTs), was first identified to bind polysialogangliosides, glycosphingolipids that are found particularly in the outer leaflet of neuronal cell membranes [4, 5]. A decade later, it was demonstrated that preincubation of either BoNT/A, B, D or E with gangliosides, especially GT1b, resulted in its detoxification [6, 7]. Overlay binding assays employing ganglioside mixtures separated by thin layer chromatography demonstrated binding of BoNT/A, B, C, E and F to GT1b, GD1b and GD1a with varying affinities [8–13]. BoNT/A, B and E adhered to GT1b better than to GD1a and much less to GM1, and as the ionic strength increased less binding was observed [14]. Employing surface plasmon resonance, however, BoNT/A bound to GT1b when the ionic strength was increased from 0.06 to 0.16 M with a similar K_D ($\sim 10^{-7}$ M) for each ionic strength [15]. Use of immobilised, isolated, individual gangliosides on polystyrene surfaces of microtitre plates complemented the understanding of ganglioside preference. TeNT prefers the b-series gangliosides GT1b, GD1b and GQ1b [16–18]. Isolated GT1b also binds BoNT/A, B and with higher affinity BoNT/G [19, 20]. In addition, BoNT/G interacts equally well with GD1a, tenfold weaker with GD1b, 250-fold weaker with GM3 and hardly with GM1a [21], somewhat similar to BoNT/F which predominantly binds GD1a and GT1b but hardly GD1b or GM1 [22]. Comparing different serotypes, GD1a is bound best by BoNT/F, followed by BoNT/E and A [23], thereby supporting the GD3 synthase knockout (GD3S-KO) mice data [24]. In contrast, BoNT/C is efficiently immobilised by GD1b and to a lesser extent by GT1b and GD1a, while the closely related mosaic serotype BoNT/DC preferentially binds GM1 and much weaker GD1a but hardly GT1b and GD1b [25]. BoNT/D, like TeNT, displays a ganglioside preference for GT1b, GD1b and GD2 pinpointing the absolute requirement of the disialyl moiety (Fig. 6.1d) [26]. Furthermore, matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy

demonstrated binding of isolated GT1b to BoNT/A, B and D [19, 27]. Co-crystallisation studies exhibited that BoNT/B binds sialyllactose via its terminal sialic acid [28] and GD1a-oligosaccharide by interacting mainly with the terminal galactose of the NAcGalβ3–1Galβ moiety (Fig. 6.1b) [29]. Similar to the latter mode, BoNT/A coordinates the terminal galactose of the NAcGalβ3–1Galβ moiety in GT1b-oligosaccharide (Fig. 6.1a, highlighted in green, NAcGal-3 and Gal-4) [30].

At the cellular level, removal of sialic acid residues by neuraminidase treatment of cultured cells isolated from spinal cord [31] and adrenergic chromaffin cells [32] reduced BoNT/A potency as well as TeNT action [33]. Furthermore, binding of BoNT/C to neuroblastoma cell lines as well as rat brain synaptosomes was diminished upon neuraminidase treatment [10, 34] indicating interactions between sialic acid moieties and BoNT/A, C and TeNT. Conversely, bovine chromaffin cells lacking complex polysialo-gangliosides were rendered sensitive to TeNT and BoNT/A by pretreatment with gangliosides [35, 36]. In addition, a monoclonal antibody to GT1b antagonised the action of BoNT/A on rat superior cervical ganglions [37]. The inhibition of ganglioside biosynthesis with fumonisin in primary spinal cord neurons or with D,L-threo-l-phenyl-2-hexadecanoylamino-3-morpholino-propanol in the mouse neuroblastoma cell line Neuro-2a resulted in insensitivity towards TeNT and BoNT/A, respectively [38, 39].

Employing a genetic approach, mice lacking the genes encoding NAcGal-transferase and/or GD3-synthetase have been created (Fig. 6.2). NAcGal-transferase-deficient mice only expressing lactose ceramide (Lac-Cer), GM3 and GD3 resisted treatment with TeNT as well as BoNT/A, B, D and G in time-to-death experiments [40–42]. Furthermore, nerve stimulation-evoked endplate potentials at isolated neuromuscular junctions derived from NAcGal-transferase-deficient mice remained unaltered upon incubation with BoNT/A [43]. In addition, binding and entry of the seven BoNT serotypes was reduced in cultured hippocampal neurons of NAcGal-transferase-deficient mice but could be rescued by adding exogenous bovine brain ganglioside mix [40, 42, 44]. On the other hand, GD3-synthetase knockout mice expressing only Lac-Cer, GM3, GM2, GM1 and GD1a were resistant to TeNT, but kept their sensitivity towards BoNT/A, B and E [45] which indicates that the disialyl moiety of GT1b plays a minor role in the binding of BoNT/A, B and E. A conclusive combination of both gene knockouts resulted in GM3-only mice which displayed high resistance towards all seven BoNT serotypes at motor nerve terminals using an ex vivo phrenic nerve hemidiaphragm preparation [24, 27, 46, 47]. Hence, complex polysialo-gangliosides such as GD1a, GD1b and GT1b are essential to specifically accumulate all BoNT serotypes on the surface of neuronal cells as the first step of intoxication.

6.3 Synaptic Vesicle Proteins Are Receptors of BoNTs

As mentioned earlier, many pieces of evidence argued against gangliosides as the sole receptors of CNTs at nerve terminals, for example, a much higher affinity of BoNT *in vivo* compared to that observed in binary interaction studies *in vitro* or the

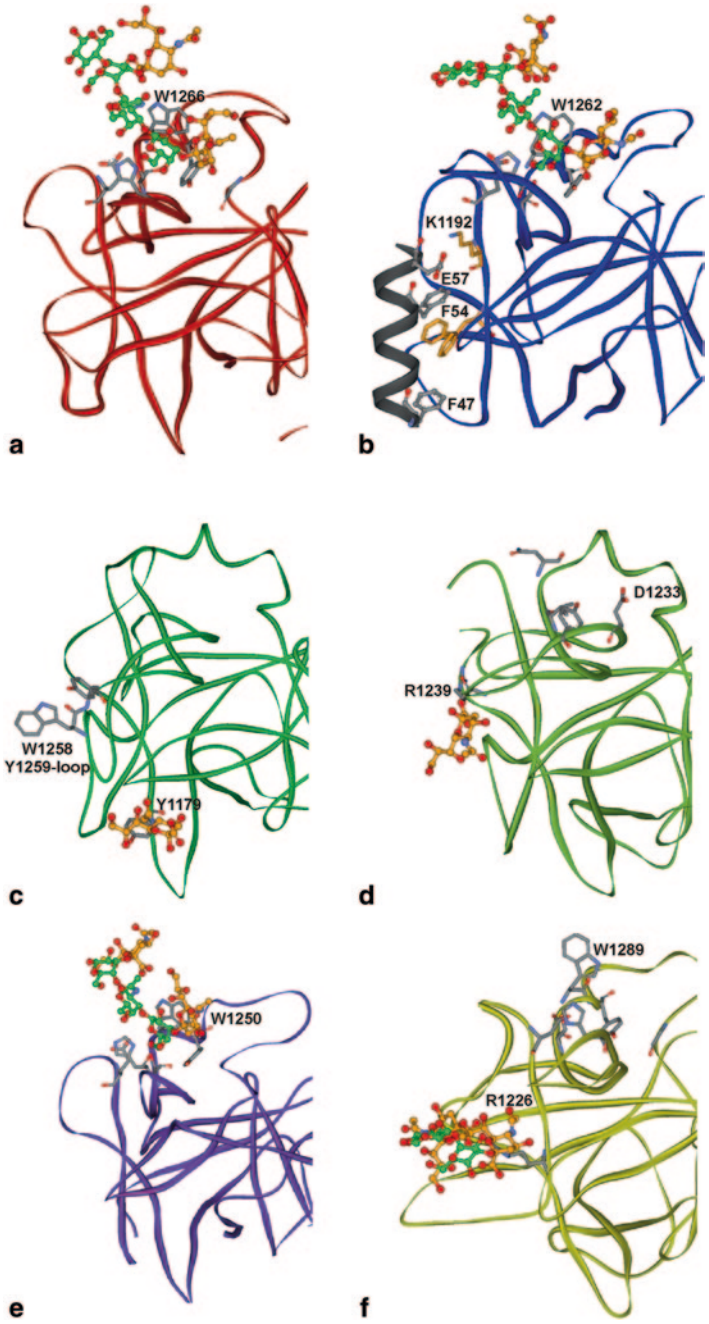

Fig. 6.1 Binding sites in the H_{CC} domain of clostridial neurotoxins. Neurotoxin residues of binding sites are displayed as stick presentations, whereas the bound carbohydrates are displayed in ball and stick presentation. **a** Synthetic GT1b binds via its terminal NAcGalβ3–1Galβ moiety (Gal carbon scaffold in *green*) to the conserved ganglioside-binding site within the botulinum neurotoxin A (BoNT/A) H_{CC} domain (2VU9.pdb). The hydrophobic side of the Gal ring packs parallel against the key residue W1266 (*grey stick representation*). The 8-2NAcNeuα could not be resolved by X-ray crystallography. **b** Binding of the transmembrane-juxtaposed 17mer peptide

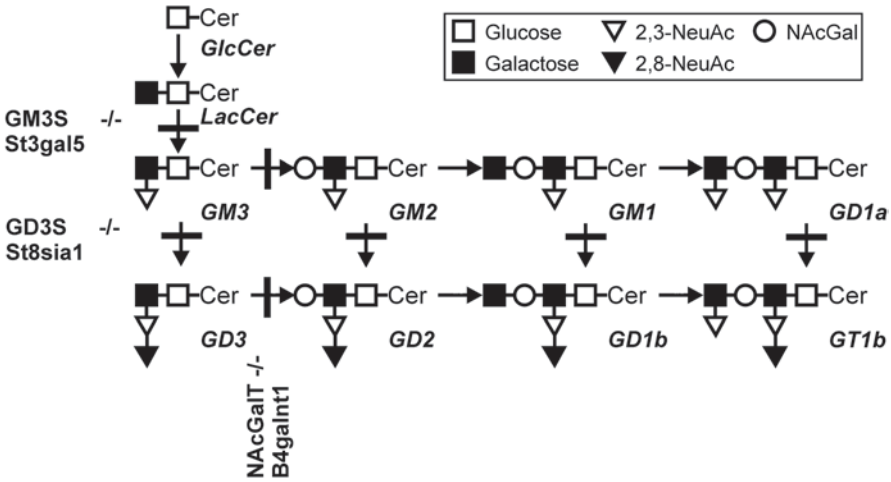

Fig. 6.2 Schematic representation of the carbohydrate building blocks and the biosynthetic pathway of complex polysialo-gangliosides. The enzymes GM3-synthetase (*GM3S*), GD3-synthetase (*GD3S*) and N-acetylgalactosamine-transferase (*NAcGalT*) are involved in ganglioside biosynthesis. Their encoding genes are deleted in the respective knockout mice leading to an altered ganglioside expression pattern

marked reduction of TeNT binding to rat brain membranes upon protease pretreatment [48, 49]. To account for these findings, a dual-receptor model involving two sequential binding steps was proposed [2] which suggests an initial low-affinity accumulation of CNTs by the abundant polysialo-gangliosides and a subsequent binding to the sparsely distributed protein receptor(s). Simultaneous interaction with ganglioside and protein receptor results in a high-affinity binding and is requisite for the subsequent specific endocytosis step of the neurotoxin.

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of the intraluminal domain of synaptotagmin-II (Syt-II) in an α-helical conformation (*black ribbon*, key amino acids in *ball and stick* presentation) to the saddle-like site in the distal tip of the H_{CC} domain of BoNT/B (residues in *orange stick* presentation). K1192 forms an important salt bridge to E57 of Syt-II. W1262 is the key residue of the conserved ganglioside-binding site (*grey stick* representation). Synthetic GD1a binds via its terminal NAcGalβ3–1Galβ moiety (Gal carbon scaffold in green) to the conserved ganglioside-binding site within the BoNT/B H_{CC} domain (4KBB.pdb). **c** BoNT/C H_{CC} lacks the conserved ganglioside-binding site but instead displays the WY-loop and a separate sialic acid-binding site. The coordination of sialic acid (carbon scaffold in *orange*) by Y1179 is of a hydrophobic nature, and an arginine such as is found in BoNT/D and tetanus neurotoxin (TeNT) is missing (3R4S.pdb). **d** BoNT/D H_{CC} possesses a ganglioside-binding site at the homologous position but with different amino acid configuration (D1233.Y1235…V1251.N1253; *grey stick* representation) (3OBT.pdb). In addition, H_{CC}D displays a sialic acid-binding site like in TeNT. R1239 (*grey stick* representation) coordinates the carboxyl group of the sialic acid (carbon scaffold in *orange*). **e** Synthetic GD1a binds via its terminal NAcGalβ3–1Galβ moiety (Gal carbon scaffold in *green*) to W1250 (grey stick representation) in the conserved ganglioside-binding site within the BoNT/F HCC domain (3RSJ.pdb). **f** Binding of disialyllactose via its NAcNeuα8–2NAcNeuα element (sialic acid carbon scaffold in *orange*) to the sialic acid binding site in the TeNT H_{CC} domain (1YYN.pdb). R1226 (*grey stick* representation) mediates the main salt bridge to the carboxyl group of the terminal NAcNeu. W1289 is the key residue of the conserved ganglioside-binding site (*grey stick* representation)

Nevertheless, the nature of protein receptors for BoNT remained obscure for a long time although several studies demonstrated an accelerated uptake of TeNT [50, 51], BoNT/A [52–54], BoNT/C [47, 55], BoNT/D [47], BoNT/E [47, 56] and BoNT/F [47] into the phrenic nerve prepared together with the adjacent diaphragm upon electrical stimulation which resulted in an earlier onset of neurotransmitter blockade. Others reported that high K^+ concentrations stimulating neurons lead to an accelerated uptake of BoNT/A, B, D, E, F and G into spinal cord and hippocampal preparations [22, 40, 42, 44, 57]. As neuronal stimulation causes increased rates of exo- and endocytosis of synaptic vesicles, the intravesicular domains of synaptic vesicle membrane proteins are frequently extracellularly exposed and thus accessible to the membrane-associated BoNT.

However, classical biochemical separation techniques of total rat brain protein, not selective searches among synaptic vesicle proteins, identified the synaptic vesicle protein synaptotagmin-I (Syt-I) as the protein receptor for BoNT/B [58]. The two homologous Syt isoforms I and II of this type I transmembrane protein [59, 60] link synaptic vesicle fusion to Ca^{2+} influx [61, 62]. Subsequent binding studies using recombinantly expressed Syt-I and Syt-II isoforms showed that BoNT/B exhibits a ten-time higher affinity to Syt-II [63]. Stable transfection of Chinese hamster ovary (CHO) cells with Syt-II identified the amino-terminal, intraluminal domain of Syt-II as the binding region for BoNT/B [64]. Deletion mutants of Syt-II lacking the two cytosolic Ca^{2+}-binding C2 domains could still act as protein receptors of BoNT/B [37]. The physiological role of this neurotoxin–Syt interaction was further demonstrated by means of loss-of-function and gain-of-function approaches employing PC12 cells as well as BoNT neutralisation assays in mice employing a Syt-II fragment containing the luminal and transmembrane domain and a ganglioside mix [65].

Shortly thereafter, Rummel et al. demonstrated that BoNT/G, whose cell-binding domain H_C is 42 % identical to the one of BoNT/B, interacts with both Syt-I and Syt-II in vitro as well (Table 6.1). It was concluded that either of the Syt isoform acts as protein receptor, as preincubation of BoNT/G with the luminal domain of Syt-I or Syt-II drastically decreased its activity at mice phrenic nerve hemidiaphragm preparations [66]. In addition, the relatedness of their binding domains is reflected in the fact that binding of BoNT/G also occurs at the membrane-juxtaposed 20-amino-acid segment of Syt-I and Syt-II [66]. However, their binding properties differ in one striking aspect. In contrast to BoNT/B, BoNT/G exhibits lower but similar affinities to both Syts in vitro [46] which is caused by a different binding mode of the Syt peptides in the H_{CC} domain of BoNT/G versus B [21]. Final confirmation for activity-dependent uptake and Syt-mediated neuronal cell entry of BoNT/B and G was provided by mouse hippocampal Syt-I knockout neurons and restoration of toxin sensitivity by Syt-I/Syt-II expression in those neurons [42]. Surprisingly, the mosaic BoNT/DC, a combination of N-terminal 2/3 derived from BoNT/D and a C-terminal cell-binding domain H_C closely related to BoNT/C, also employs Syt-I and Syt-II as protein receptors. However, its H_{CC} domain is only 28 % identical to H_{CC}B and H_{CC}G but 61 % identical to H_{CC}C which does not interact with any Syt isoform. That might be the reason for higher apparent dissociation constants of BoNT/DC for Syt-II than BoNT/B under similar assay conditions (330 versus 8.6 nM) [67].

Table 6.1 Identified neurotoxin receptors and corresponding binding sites

	AA motif in the conserved ganglioside-binding site	Key AA of the sialic acid-binding site	SV protein receptor[a]	Key AA of SV protein receptor-binding site
BoNT/A	E…H…SXWY…G		SV2C/A/B[b]	TBD
BoNT/B	E…H…SXWY…G		Syt-II/Syt-I	K1192, F1194, F1204
BoNT/C	WY-loop[c]	Y1179	TBD	TBD
BoNT/DC	WF-loop[c]		Syt-II/Syt-I	Y1180, I1264, P1182
BoNT/D	DXY…VXN	R1239	SV2B/C/A	TBD
BoNT/E	E…K…SXWY…G		SV2A/B	Y879, E1246, K1084
BoNT/F	E…H…SXWY…G		SV2A/C/B	TBD
BoNT/G	Q…G…SXWY…G		Syt-I/Syt-II	Q1200, F1202, F1212
TeNT	D…H…SXWY…G	R1226	SV2	TBD

BoNT botulinum neurotoxin, *SV2C* synaptic vesicle glycoprotein 2C, *Syt* synaptotagmin, *TeNT* tetanus neurotoxin

[a] Order expresses decreasing neurotoxin affinity

[b] N-glycosylation of LD4 is not obligatory

[c] No conserved ganglioside-binding site present

To identify protein receptors of the remaining BoNT serotypes, synaptic vesicle proteins were preselected on the basis that their intravesicular segments exceeded 20 amino acids in size and comprised both intravesicular segments of the tetraspanin proteins synaptophysin, synaptoporin, synaptogyrin-I and synaptogyrin-III as well as the large intravesicular domain of the synaptic vesicle glycoprotein 2 (SV2) isoforms A, B and C. Glutathione S-transferase (GST) pull-down experiments employing their luminal domains discovered the interaction between the large luminal domain 4 (LD4) of SV2 and BoNT/A [68, 69]. The function of this integral membrane glycoprotein with 12 putative transmembrane domains [70–73] is likely linked to synaptic vesicles priming or rendering primed vesicles fully Ca^{2+} responsive [74–76]. Recent studies showed that a lack of SV2 results in elevated Ca^{2+} levels in the presynaptic terminals and also reduces the rate of compensatory membrane retrieval after synaptic vesicle release [77]. Interestingly, it has been demonstrated that SV2 associates with Syt and may regulate the endocytosis of Syt [78, 79]. The unglycosylated, isolated LD4 of SV2C exhibited the highest affinity to BoNT/A, inhibited binding and entry of BoNT/A into hippocampal neurons and motor nerve terminals [68] and efficiently decreased BoNT/A neurotoxicity [69]. Loss-of-function and gain-of-function studies in hippocampal neurons [68], RNA interference (RNAi) experiments using wild-type PC12 and Neuro-2a cells as well as transiently SV2-expressing PC12 and Neuro-2a knockdown cell lines verified that all three SV2 isoforms can act as physiological receptors for BoNT/A [68, 80]. Since all three SV2 isoforms are expressed in α-motoneurons [68], it is still not fully clear which SV2 isoform is most relevant for the physiological uptake of BoNT/A.

In contrast to BoNT/A, BoNT/D and BoNT/E exclusively co-immunoprecipitated SV2B and SV2A, respectively, from rat brain extracts [40]. Subsequent studies, employing BoNT treatment of hippocampal neurons derived from SV2A/B knockout mice and rescue of toxin sensitivity by expression of SV2 isoforms in

SV2A/B knockout hippocampal neurons, revealed that the biological activity of BoNT/D requires the presence of SV2B and to a lesser extent SV2A and C [40] and that BoNT/E only interacts with SV2A and SV2B, but not with SV2C [44]. Furthermore, TeNT presumably employs only SV2A and SV2B at the central neurons [81] (Table 6.1). However, TeNT, BoNT/A, D and E utilise seemingly diverse SV2-binding mechanisms. Whereas BoNT/A interacts with the unglycosylated LD4 of all SV2 isoforms and N-glycosylation of N573 in SV2A only slightly enhances its uptake rate, BoNT/E requires N-glycosylation of LD4 and does not bind to any *Escherichia coli*-derived LD4-SV2 peptide. The biological activity of BoNT/E proved to be strictly dependent upon N-glycosylation of N573 in SV2A, the carboxyl-terminal of three conserved putative N-glycosylation sites among the LD4 of SV2 [44]. BoNT/D and TeNT appear to have an SV2-recognition strategy distinct from BoNT/A and BoNT/E. First, any mutation of the three putative N-glycosylation sites in SV2A like N573Q has no significant effect on the entry of BoNT/D and TeNT [40, 81]. Second, the SV2-LD4 domain expressed in low-density lipoprotein receptor-based or synaptogyrin-based chimeric proteins can function as the receptor for BoNT/A and E but failed to mediate the entry of BoNT/D. Furthermore, BoNT/D does not compete for uptake in hippocampal neurons with BoNT/A or E [40]. On the other hand, the cell-binding domain H_C of TeNT inhibits the uptake of BoNT/E and BoNT/D at the mice phrenic nerve hemidiaphragm preparation [47] as well as SNAP-25 cleavage by BoNT/E in hippocampal neurons [81]. It has to be noted, however, that a direct protein–protein interaction of SV2 and BoNT/D or TeNT remains to be shown. In conclusion, these data suggest that the CNT–SV2-binding mechanism has yet to be understood.

In addition, there is dispute about the involvement of SV2 as the protein receptor for BoNT/F, whose cell-binding domain H_C displays only 55 and 43 % amino acid sequence identity with BoNT/E and A, respectively. As BoNT/A and E H_C are also 38 % identical, it is imaginable that BoNT/F harnesses SV2 for cell entry as well. Although BoNT/F uptake at cultured hippocampal neurons has been reported to occur independently of synaptic activity [82], increased stimulation of motoneurons accelerates uptake of BoNT/F [47] indicating the involvement of synaptic vesicular structures. Furthermore, BoNT/A H_C inhibits the neurotoxicity of BoNT/F and BoNT/F H_C inhibits the neurotoxicity of BoNT/E at mice phrenic nerve hemidiaphragm preparations [47] which can be explained by competition for SV2. Moreover, two independent studies co-purified all three SV2 isoforms by BoNT/F H_C from Triton X-100-solubilised synaptic vesicle lysates [22, 47]. On the other hand, cleavage of synaptobrevin 2 by various concentrations of BoNT/F is not impaired in SV2B and SV2B/SV2A knockout hippocampal neurons [40, 81]. Residual SV2C present in hippocampal neurons and a different SV2 expression pattern in motoneurons might explain these discrepancies [40, 68]. However, an ultimate experiment employing pan-SV2 knockout motoneurons has not been conducted.

For BoNT/C, Kozaki and colleagues concluded that proteins are not a receptor component, as protease treatment or boiling of solubilised rat brain synaptosomes had no effect on binding [10]. The binding of BoNT/C H_C to phosphoinositide-containing liposomes has been described but is unlikely to replace protein receptor

interaction [83]. Along this line are observations that BoNT/C H_C failed to pull down the synaptic vesicle proteins Syt-I, Syt-II or SV2A/B/C from Triton X-100-solubilised synaptic vesicle lysates [47, 84]. In addition, cleavage of SNAP-25 and syntaxin 1A by BoNT/C is not impaired in SV2B and SV2B/SV2A knockout hippocampal neurons [40]. However, as mentioned above, the stimulation-dependent uptake of BoNT/C at mice phrenic nerve hemidiaphragm preparations points towards the involvement of synaptic vesicular structures [47, 55]. Clearly, thorough studies are required to clarify this issue. If entry of BoNT/C turns out to depend upon proteinaceous receptors, it will be interesting to find out whether different proteins than the synaptic vesicle proteins Syt or SV2 act as receptors.

6.4 The Four-Domain Structure of CNT

Each CNT is initially synthesized as an ~150-kDa single-chain protein, which is subsequently cleaved by specific bacterial or host proteases. The resulting ~50-kDa light chain (LC) and ~100-kDa heavy chain (HC) remain attached via a single disulphide bond and non-covalent interactions mediated by an HC-derived peptide loop wrapping around the LC within the substrate cleft. The LCs represent the active components which operate as zinc endoproteases with strict substrate specificities [85]. Their apo structures have all been determined (reviewed in [86] and Chapter 5 of this book). The structural differences among the LCs are mostly limited to solvent-exposed loops and potential substrate interaction sites. The LCs are ordinary proteases, i.e. nontoxic molecules, without linkage to their HCs. However, the LCs become highly poisonous agents when so linked, as a consequence of the HCs ensuring that the catalytic LCs come across their neuronal target cells and conquer the plasma membrane to reach the site of action, the cytosol. In order to fulfil these tasks, the HCs comprise two functional subunits, an ~50-kDa largely α-helical domain at the N-terminus, called H_N, and at the C-terminus an ~50-kDa fragment, H_C, in which the two ~25-kDa domains H_{CN} and H_{CC} can be defined (Fig. 6.3). Structural comparison among the H_C fragments of BoNT/A (PDB code: 2VU9 [30]), B (2NM1 [87]), C (3R4S [88]), D (3OBT [27]), E (3FFZ [89]), F (3FUQ [22]), G (2VXR [90]) and TeNT (3HMY [91]) showed that there is a varying twist between H_{CN} and H_{CC}, culminating in H_CC by about 17.2 degrees (Fig. 6.3). Nevertheless, separate pairwise structure comparisons of all eight H_{CN} and H_{CC} domains demonstrated that the structures are conserved within each domain. In spite of the conserved core structure, large structural differences are found in many surface-exposed loops. Five of such areas reside in H_{CN} and nine loops in the H_{CC} domain (Fig. 6.3).

The function of the lectin-like jelly-roll H_{CN} domain of H_C that connects H_N and H_{CC} is still not fully resolved. Whereas the H_{CN} domain of TeNT does not bind to rat primary dorsal root ganglia cells [92] and nerve growth factor (NGF)-differentiated PC12 cells [93], a low-affinity binding of H_{CN} of BoNT/A to phosphatidylinositol monophosphate incorporated in sphingomyelin-enriched microdomains of the immortalized motor neuron cell line NSC-34 was reported recently [94]. Nevertheless,

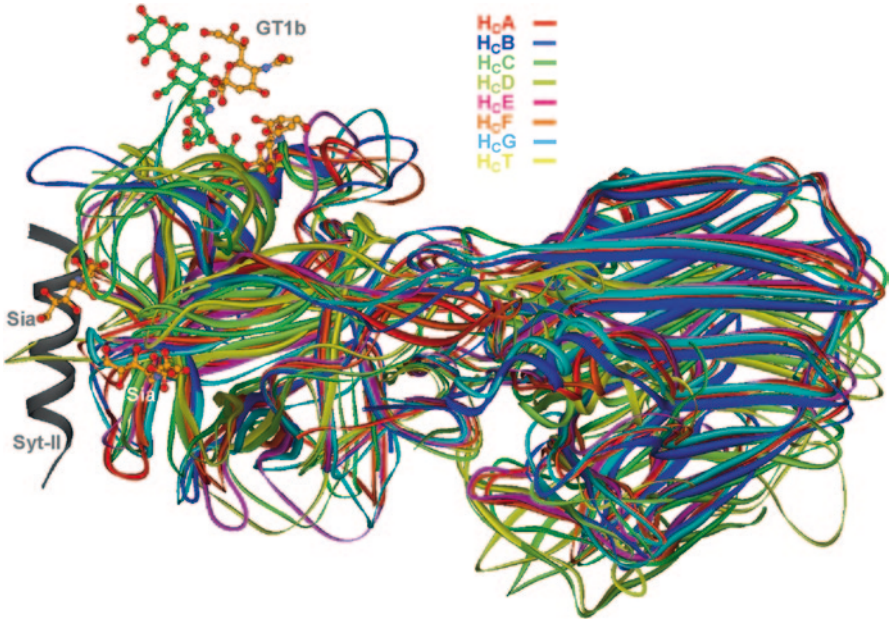

Fig. 6.3 Superimposition of the H_C fragment crystal structures of tetanus neurotoxin (*TeNT*) and all seven botulinum neurotoxin (BoNT) serotypes. Synaptotagmin-II (Syt-II) (*grey ribbon*) bound to H_CB (*dark blue ribbon*, PDB code 2NM1) was superimposed with H_CA (*red ribbon*) in complex with GT1b (*ball and stick*, 2VU9), H_CC (*dark green*) in complex with sialic acid (*ball and stick*, 3R4S), H_CD (*light green*) in complex with sialic acid (*ball and stick*, 3OBT) and H_CE (*pink*, 3FFZ), H_CF (*orange*, 3FQU), H_CG (*light blue*, 2VXR) and H_CT in complex with disialyllactose (*yellow ribbon*, 1YYN)

a direct involvement of H_{CN} of BoNT/A in the translocation step has recently been ruled out [95]. On the other hand, the β-trefoil domain H_{CC} harbours the main features required for target cell recognition and internalisation, as demonstrated for TeNT and BoNT/B, DC and G (see later).

6.5 The Mode of Molecular CNT–Ganglioside Interaction

It has been many years since it was shown that the specific binding to peripheral nerve endings at the neuromuscular junction involves just the H_C fragment [50, 96–100] and complex polysialo-gangliosides. In one of the initial approaches to identify the ganglioside-binding site, a cross-linking experiment employing ^{125}I-azido-GD1b and TeNT H_C fragment led to radiolabelling of H1293 in the proximity of a large cavity within the H_{CC} domain [101]. 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

[102]. The mutation of the TeNT residue Y1290, forming the bottom of this cavity, to phenylalanine, serine or alanine also reduced the affinity to GT1b as well as binding to synaptosomal membranes [103]. Independently, the anticancer drug doxorubicin, an anthracycline antibiotic, could be docked in silico into this pocket and was able to inhibit the binding of the TeNT H_C fragment to liposome-integrated GT1b [104]. The first structural insight was revealed by the co-crystallisation of the TeNT H_C fragment and four carbohydrate subunits of GT1b which exhibited four distinct binding sites, including the one in the proximity of H1293. Here, a lactose molecule interacts with the residues D1222, T1270, S1287, W1289, Y1290 and G1300 (Fig. 6.1f). A separate site comprising R1226 as the key residue coordinated either a molecule of NAcGal or sialic acid (N-acetylneuraminic acid, NAcNeu). Two additional sites were identified in complexes of TeNT H_C with Gal or NAcGal [105]. However, the latter two sites were considered not to function as binding pockets for polysialo-gangliosides, due to insufficient space (Gal) for a complete ganglioside to bind or a too flexible carbon backbone (NAcGal). Isaacs and co-workers refined their co-crystallisation approach by using a synthetic GT1b-β analogue lacking the ceramide portion. Indeed, the terminal disaccharide NAcGalβ3–1Galβ bound to the lactose-binding site next to H1293 (Fig. 6.1f), while the disialic acid branch of another GT1b-β molecule interacted with the sialic acid-binding site comprising R1226 [106] (Fig. 6.1f). Independent co-crystallisation of the TeNT H_C fragment with disialyllactose as well as the carbohydrate portion of GT2 confirmed the binding of the disialic acid branch to the sialic acid-binding site [91, 107]. Mutation of residues D1222, H1271 and W1289 in the lactose-binding site led to reduced binding of the TeNT H_C fragment to GT1b in surface plasmon resonance experiments and NGF-differentiated PC12 cells [108]. The importance of the lactose-binding site for binding and entry of TeNT was conclusively demonstrated by the application of corresponding, recombinant full-length TeNT mutants in the mouse phrenic nerve (MPN) hemidiaphragm assay leading to a 350-fold reduction in neurotoxicity in the case of the single amino acid mutation W1289 L [18]. Furthermore, these experiments also demonstrated that the sialic acid-binding site is essential for TeNT action, since the TeNT mutant R1226F possesses a 70-fold reduced activity in the MPN assay. Mass spectroscopy experiments indicated simultaneous binding of two molecules of GT1b to the TeNT H_C fragment, but no ganglioside-mediated cross-linking was observed in size exclusion experiments [18]. Based on these results, the ganglioside specificities of the individual lactose- and sialic acid-binding sites could be biochemically refined [16], confirming the above-mentioned crystallographic studies by Fotinou et al. [106] and Jayaraman et al [107]. Although the binding of a ganglioside to the sialic acid-binding pocket was shown, it is conceivable that there is subsequent substitution by or a direct interaction with either glycosylphosphatidylinositol (GPI)-anchored glycoproteins or sialylated LD4 of SV2 [81, 109, 110]. At present, it is still unclear whether the two ganglioside-binding sites of TeNT relate to its retrograde intra-axonal transport.

The lactose-binding site in TeNT is built by the peptide motif D…H…SXWY…G (Fig. 6.1f; Table 6.1) which is conserved among BoNT/A, B, E, F and G. This cavity displays the typical features necessary for carbohydrate interaction found also in

other protein toxins such as ricin and cholera toxin. An aromatic residue, preferably tryptophan or tyrosine, supplies the surface for the hydrophobic face of the sugar ring. Polar residues like aspartate or glutamate and serine are located opposite to interact with the sugar hydroxyl groups. These polar interactions are supported by a histidine or by a lysine like in BoNT/E.

Co-crystallisation studies with BoNT/B and sialyllactose or doxorubicin suggested that the lactose-binding site is the ganglioside-binding pocket in BoNTs [28, 111]. Detailed mutational analyses defined the contribution of various residues within the homologous lactose-binding pocket of BoNT/A and B (Fig. 6.1a, b). Again, the mutations of the key aromatic residues, W1266 and W1262 in BoNT/A and B, respectively, to leucine lead to dramatic reductions of neurotoxicity using the MPN assay [19]. In contrast to TeNT, mass spectroscopy data revealed the binding of only a single GT1b molecule to the H_C fragment of BoNT/A and B [19]. Recently, these physiological and biochemical data were confirmed by the crystal structure of a synthetic GT1b-analogue bound to the H_C fragment of BoNT/A [30] (Fig. 6.1a) and a GD1a-oligosaccharide BoNT/B H_C–Syt-II ternary complex structure (Fig. 6.1b) [29]. Interestingly, whereas the mutational data of the lactose site suggest a shared ganglioside-binding mode of BoNT/A and B differing to that of TeNT [19], the crystallographic results indicate that BoNT/A and B, like TeNT, predominantly interact with the NAcGalβ3–1Galβ moiety and only BoNT/B displays additional contacts with α2,3-linked N-acetylneuraminic acid residue (denoted NAcNeu-5) attached to Gal4 of the oligosaccharide core [29, 30, 106]. The previously reported coordination of the terminal sialic acid of sialyllactose by W1262 in BoNT/B [28] might be due to different crystallisation conditions and the use of sialyllactose instead of an entire ganglioside like GD1a. Structural data of BoNT/E, BoNT/F H_C and BoNT/G H_C displayed the presence of a conserved ganglioside-binding site like in BoNT/A and B [22, 89, 90] but only the mutation of the tryptophan residues 1268 in BoNT/G, 1224 in BoNT/E and 1250 in BoNT/F, which are all part of the conserved motif E(D)…H(K)…SXWY…G, to leucine demonstrated their key role in ganglioside interaction and biological activity [46, 47, 112] (Table 6.1). Again, the crystal structure of a GD1a-oligosaccharide–BoNT/F H_C complex confirmed the previous biochemical identification of the conserved ganglioside-binding site in BoNT/F. Here, like in BoNT/B, W1250 mainly coordinates the 1Galβ moiety, and R1111 and R1256 create additional contacts with NAcNeu-5 (Fig. 6.1e) [23]. In contrast, the conserved ganglioside-binding site is not present in the BoNT/C H_C structure [25, 88] (Fig. 6.1c), albeit the mutation of W1258 in BoNT/C, which aligns in the similar motif GXWY, clearly reduced the binding to gangliosides in synaptosomal membranes as well as biological activity [47, 113]. W1258 is a part of a long loop extending out of the core structure of $H_{CC}C$ and therefore also termed the ganglioside-binding loop (GBL). Molecular dynamics simulations indicate that this WY-loop is not a crystallographic artefact [88]. However, the mode of ganglioside coordination at this peptide remote from the $H_{CC}C$ core structure remains unclear. Also, the closely related H_C fragment of BoNT/DC, displaying 75 % amino acid sequence identity to H_CC, lacks the ganglioside-binding motif but presents an

analogous WF-loop. Consequently, H_CDC mutant W1252A completely lost binding to GM1, its preferred type of ganglioside, although the WF-loop backbone of the mutant nicely superimposes with the H_CDC wild-type structure [25]. Another study detected a partial electron density of sialyllactose at a position homologous to the conserved ganglioside-binding site, and the subsequent mutational analysis identified residues important for the binding of BoNT/DC H_C to GM1 and P19 cells (neuronally differentiated embryonal carcinoma cells) within the conserved ganglioside-binding site as well as the WF-loop [114]. Furthermore, a second ganglioside-binding site within H_{CC}C was exhibited by the co-crystallisation of sialic acid with H_CC (Fig. 6.1c). This pocket, called the Sia-1 site, locates at the tip of the H_{CC} domain in the neighbourhood of the WY-loop, but constitutes an autonomous ganglioside-binding pocket and displays a mode of sialic acid binding different to the one observed in the sialic acid-binding site of TeNT [88]. Thereafter, a molecule of sialic acid was identified at the location of the conserved ganglioside-binding site in a BoNT/C H_C crystal structure, but coordination only occurred via H bonds. Biochemical analysis revealed that this site, called GBP2, binds the NAcNeu-5 moiety of GD1a/GT1b whilst the Sia-1 site binds the NAcNeu-7 of GD1b/GT1b [115]. It remains open if BoNT/C even employs up to three molecules of gangliosides to specifically enter neurons and can afford to lack high-affinity binding to a protein receptor. Due to a high amino acid sequence divergence and the lack of crystal structures, no ganglioside-binding site could be identified in BoNT/D until recently. The co-crystallisation of sialic acid with BoNT/D H_C revealed a sialic acid-binding site around R1239 in a position similar to the sialic acid-binding site in TeNT [27] (Fig. 6.1d). Further structural data exhibited that mutation W1238A disorders the backbone, thereby also influencing R1239, whereas F1240 seems to be directly involved in ganglioside binding [26]. Mutagenesis studies demonstrated a binding mode similar to TeNT and could identify a second carbohydrate-binding site at the location of the conserved ganglioside-binding site in BoNT/A, B, E, F, G and TeNT, but with completely different configuration of amino acids, which might explain the low affinity of BoNT/D to gangliosides [27]. Structural analysis of the closely related BoNT/CD H_C exhibited a sialic acid-binding site consisting of W1242, R1243 and F1244 homologous to the one of BoNT/D [116].

In conclusion, BoNT/A, B, E, F and G harbour a single ganglioside-binding site made up of the conserved amino acid motif E(Q)…H(K)…SXWY…G at a homologous location within the H_{CC} domain (Table 6.1). The lactose-binding site of TeNT is highly similar to this conserved ganglioside-binding site, whereas the second site complexing sialic acid is closely related to the one in BoNT/D. The other carbohydrate-binding site in BoNT/D is unique with respect to its amino acid configuration, but locates similarly to the conserved ganglioside-binding site of, for example, BoNT/A. Although BoNT/C also displays a minimum of two ganglioside-binding sites like TeNT and BoNT/D plus an exposed aromatic loop, neither their amino acid configuration nor their positions are related to any other known carbohydrate-binding pocket of CNT.

6.6 Mode of the BoNT–Protein Receptor Interaction

Since BoNT/A, B, E, F and G do not possess a second carbohydrate-binding site, the question arises, whether protein receptors such as Syt-II for BoNT/B bind in a pocket that is homologous to the sialic acid-binding site within the H_{CC} domain of TeNT or BoNT/D. The different affinities of H_C fragment hybrids generated of the H_{CN} and H_{CC} domains of BoNT/B strains Okra and 111, respectively, to GT1b/Syt-II-endowed liposomes point in that direction [117].

The mode of interaction of BoNT/B with its protein receptor Syt-II has been addressed by two parallel co-crystallisation approaches. In one study, full-length BoNT/B was co-crystallised with a 20mer peptide that corresponded to the proposed interacting segment of mouse Syt-II [118]. In the second study, a recombinant fusion protein consisting of the H_C fragment of BoNT/B linked via a Strep affinity tag to the complete luminal domain of rat Syt-II was crystallised [87]. Analysis of both crystals revealed that Syt-II involving amino acids 44–60 bound to a saddle-like crevice at the distal tip of the H_{CC} domain in the direct neighbourhood of the ganglioside-binding pocket (Fig. 6.1b). Interestingly, the in-solution unstructured luminal domain of Syt-II formed an α-helix upon binding to BoNT/B. Its interaction with the toxin relies mainly on hydrophobic interactions by F47 and F54 via two adjacent pockets on the surface of BoNT/B, but it also involves important salt bridges mediated by E57 of Syt-II. Yet another parallel study starting from computer-assisted binding pocket predictions followed by mutational analyses also identified the Syt-I- and Syt-II-binding sites in the BoNT/B H_{CC} domain [46]. As determined by isothermal titration calorimetry, Syt-I has an at least two orders of magnitude lower affinity for BoNT/B compared to Syt-II [87], though it displays only two conservative replacements of residues at positions that were shown to be involved in the Syt-II/BoNT/B interaction: M47 and L50 instead of F55 and I58 in Syt-II. The individual conversion of M47 and L50 to phenylalanine and isoleucine, respectively, increased the binding affinity of Syt-I, and the corresponding double mutation converted Syt-I to a Syt-II-like high-affinity receptor [87]. This result substantiates a conserved binding mode for both Syt isoforms in BoNT/B. Recently, following the approach of Jin et al., the structure of a ternary complex consisting of GD1a-oligosaccharide and the mouse luminal domain of Syt-II fused to H_CB was solved and displayed a virtually identical positioning of the Syt-II helix in the presence of GD1a [29]. Hence, simultaneous ganglioside binding does not cause any significant structure changes in BoNT/B H_C, and both receptors interact independent of each other on the neuronal cell surface despite their close proximity (15 Å).

The structural analysis of Syt-II derived from different species revealed that F54, the key hydrophobic residue in mouse and rat Syt-II for mediating high-affinity binding to BoNT/B, DC and G, corresponds to L51 in human and chimpanzee Syt-II. Introducing the corresponding mutation F54 L into Syt-II of mouse or rat origin resulted in a complete loss of binding of BoNT/B, DC and G and cannot be rescued by the presence of gangliosides [67, 119]. Interaction of BoNT/B with Syt-I, whose corresponding residue F46 is strictly conserved in all sequenced species, can only

partially compensate this single mutation. Hereby, the disparity in the potency of BoNT/B in humans and mice as well as the 40-fold higher dosage of rimabotulinumtoxinB versus onabotulinumtoxinA is explained at the molecular level.

The binding of Syt-I and Syt-II to BoNT/G occurs at the homologous position as in BoNT/B [46]. However, only the general shape of the pocket and a few amino acids forming the surface of that binding area are conserved [90]. Multiple mutations in Syt-II showed comparable effects on binding to BoNT/G except for mutant Syt-II-E57K which did not bind to BoNT/B, but exhibited wild-type-like binding affinity in the case of BoNT/G [21]. The replacement of BoNT/G-Q1200, the counterpart of BoNT/B-K1192, which forms the major salt bridge with Syt-II-E57, by the corresponding lysine residue of BoNT/B did not improve binding to Syt-II but clearly diminished binding and neurotoxicity [46]. Detailed mutational data along with molecular dynamics simulation experiments allowed atomistic models of BoNT/G−Syt recognition. Here, the Syt-I helix that forms upon contact with BoNT/G is shortened C-terminally, whereas the Syt-II helix displays a bend after F55 as compared with BoNT/B [21]. Interestingly, the mutation of Syt-II residues depleting BoNT/B binding also decreased interaction with BoNT/DC, although the superimposition of BoNT/DC H_C and H_CB–Syt-II crystal structures revealed that the surface in H_CDC homologous to the Syt-II site in H_CB differs greatly. An alternative, partially overlapping hydrophobic area was analysed. Mutations Y1180K, I1264Q and P1182S/S1183Y reduced the binding of both Syt-I and Syt-II, whereas L1196R and L1226K selectively diminished the binding of Syt-II without affecting Syt-I binding significantly [67]. In conclusion, Syt-I and Syt-II always adopt an α-helical structure of 14–17 residues to interact with the H_{CC} domain of BoNT/B and G at homologous sites, whereas BoNT/DC binds Syt-I and Syt-II at a closely related site with a unique recognition motif.

So far, the SV2-binding site in BoNT/A has not been identified. According to current data, however, it is conceivable that BoNT/A interacts with the non-glycosylated segments of its protein ligand SV2 at a site within the H_{CC} domain similar to the Syt-II-binding site in BoNT/B. In contrast to BoNT/A, BoNT/E requires N-glycosylation of LD4 of SV2A for neuronal uptake. Only the establishment of an HEK293 cell-based SV2A expression system allowed the mapping of the SV2-binding interface of BoNT/E at the corresponding Syt-binding site of BoNT/B and at an extended surface area located on the back of the conserved ganglioside-binding site, comprising H_{CN} and H_{CC}. Mutations impairing the affinity also reduced the neurotoxicity of full-length BoNT/E at MPN hemidiaphragm preparations, and are part of an epitope of a monoclonal antibody neutralising BoNT/E activity [112]. The SV2A–BoNT/E interaction displays a novel site of binding for BoNTs that exploit SV2 as a cell surface receptor.

As outlined above, there is multiple evidence from crystallisation and mutagenesis studies that TeNT exhibits a second binding site for sialic acid. This sialic acid-binding site was later also shown to accommodate the tripeptide Tyr-Glu-Trp (YEW) [107]. It is located in the area that corresponds to the Syt-binding site of BoNT/B and G but is smaller and chemically different containing several hydrophilic residues and a central arginine (R1226). A parallel situation was described

for BoNT/D [27]. As both BoNT/D and TeNT were reported to employ the N-glycosylated SV2 [40, 81], it is imaginable that the sialic acid-binding site interacts with the terminal sialic acids of the N-glycosyl branches as well as with the peptidic backbone of SV2 to mediate the neuronal specificity.

Neither the exact binding affinities of the three SV2 isoforms to BoNT/A, D, E and TeNT nor the interacting segments and residues, respectively, of the corresponding LD4 have been determined. Ultimately, as for BoNT/B–Syt-II, co-crystal structures of SV2–BoNT H_C complexes are required to elucidate the molecular interactions within the SV2-binding pockets.

6.7 The BoNT Membrane Approach

Although the ganglioside- and protein receptor-binding sites are in close proximity (Fig. 6.1), they function independently and do not require preformation of a ganglioside/protein receptor complex [29, 46]. Moreover, mutants of BoNT/B with both the ganglioside- and Syt-binding sites (individually or in combination) deactivated, do not exhibit appreciable toxicity excluding any significant contributions of other cell surface molecules to the binding and entry of BoNT/B and G [46]. A recent study investigated the vesicle membrane approach by making it occur at the surface of neurons due to blockade of the vesicular proton adenosine triphosphatase (H^+-ATPase) by bafilomycin A1. This study showed that anchorage via two receptors is a strict prerequisite for a productive low pH-induced conformational change followed by membrane translocation, and TeNT, BoNT/B, C and D change structure and interact with the membrane in the same range of pH values as occurs in the SV lumen [120]. In contrast, Chapman et al. reported that binding only to GT1b enables BoNT/B to sense low pH, undergo a significant change in secondary structure and transform into a hydrophobic oligomeric membrane protein. Imaging of the toxin on lipid bilayers using atomic force microscopy revealed doughnut-shaped channel-like structures that resemble other protein translocation assemblies [121].

The position of the toxin on the membrane may be important for the subsequent endocytosis and translocation steps. Geometric restrictions are imposed by the simultaneous binding to the ganglioside and the protein receptor [87]. Different binding modes are conceivable. It has been suggested that the predominant negatively charged molecular surfaces of BoNT/B H_N favour a perpendicular orientation of the translocation domain and thus bend Syt into a membrane tangential direction. In this scenario, four solvent-exposed lysine residues conserved among Syt-I and Syt-II might interact with the phospholipid head groups of the membrane [87]. This view has recently been supported by modelling GT1b into the Syt-II/BoNT/B complex based on its binding to the conserved ganglioside-binding pocket of BoNT/A [30]. This model further predicts an extended loop of BoNT/B, that contains a very hydrophobic tip (G1246–F1249) and extends out between the ganglioside- and Syt-binding pockets, to be additionally involved in the interaction with the membrane. The H_CD crystal structure revealed a corresponding loop whose hydrophobic

segment F1242–Y1246 is likely to be involved in membrane association because its mutation leads to a clear loss in toxicity of BoNT/D. The fact that the BoNT/A H_C fragment reorientates 140° in the pH-resistant M-PTC and that mutation of the H_{CN}-H_C-linker helix clearly decreased the biological activity of BoNT/A indicates that such a rearrangement could also occur within the acidic environment of the vesicle lumen in the absence of nontoxic nonhemagglutinin (NTNHA) [122]. The results of future experiments have to link the known membrane anchorage of the BoNT with the mode of membrane contact and insertion of the translocation domain H_N to form LC-translocating channels.

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Chapter 7
Translocation, Entry into the Cell

Matthew Beard

Abstract Efficient targeted delivery of bioactive proteins into the cytoplasm of living cells is a major challenge for the pharmaceutical and biotechnology industries. Botulinum toxins have evolved an elegant mechanism to achieve exactly this. They specifically target pre-synaptic active neurons with sub nanomolar affinity and deliver an enzymatically active 50 kDa protease into the cytoplasm without affecting cell viability. Recent progress in understanding the molecular details of this delivery has opened new possibilities to understand and treat the disease botulism and to harness and adapt this delivery mechanism for other uses. This review describes our current understanding of the structure, function and interactions between protein domains in botulinum toxin, which act together to translocate a large soluble protein across cell membranes. As our understanding of these processes increases so too does the potential to incorporate botulinum toxin protein domains into new engineered proteins and achieve cytoplasmic delivery of other therapeutic molecules.

Keywords Botulinum neurotoxin · Protein translocation · Endocytosis · pH gradient · Redox gradient · Transmembrane potential · Chaperone proteins · Translocation inhibitors · Targeted Secretion Inhibitors (TSI) · Reducing environment

7.1 Botulinum Toxin Mechanism of Action

Botulinum neurotoxin (BoNT) proteins are extremely potent bacterial toxins, which act to weaken muscles by inhibiting neurotransmitter release at peripheral neuromuscular junctions. There are seven different serotypes of BoNT (A–G). These are produced by a genetically diverse set of bacteria that are classified together because they cause the disease botulism and not because they share strong genetic relationships at the whole organism level. BoNT-producing bacteria include the species *Clostridium botulinum* and some strains of *C. argentinense, C. baratii* and *C. butyricum* [2, 73]. In contrast to the bacteria that produce them, the various different serotypes of BoNT

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protein are all related genetically. This is possible because the coding sequences for BoNTs are located on plasmids and bacteriophages, which allow them to become acquired by horizontal gene transfer as well as by classical inheritance [73].

BoNT proteins of all serotypes are highly related. They share a common mechanism of action that consists of: (1) delivery into the circulation, (2) high-affinity binding to motor neurons, (3) endocytosis into recycling synaptic vesicles, (4) translocation into the cytoplasm and (5) inhibition of secretion. Delivery into the circulation occurs when BoNT (usually as part of a large protein complex) crosses a mucosal membrane such as the gut (following ingestion) or the lung (following inhalation) or when it becomes introduced directly either intentionally by injection (as in the cases of medical BoNT products) or unintentionally through broken skin (as in the case of wound botulism). Once in the circulation, BoNT proteins potentially have access to all peripheral cell types. They bind specifically and with high affinity to active nerve synapses, internalize by endocytosis and then translocate an enzymatically active protease subunit into the target cell cytoplasm. Together, these binding, internalization, translocation and enzymatic activities give rise to the high potency and specificity that BoNTs show towards active neuromuscular junctions [8, 55].

BoNTs are multidomain dimeric proteins. Structures are available for individual functional domains from all of the serotypes (BoNT/A–G). Full-length protein structures are available for the complete protein sequences of BoNT/A, B and E serotypes [19, 31, 50, 51] (see Chapter 5 of this book). There are clear structure–activity relationships between individual protein domains and functional steps in mechanism of action of BoNT proteins. BoNT protein dimers are composed of light- and heavy-chain subunits covalently linked by a single disulphide bond. They are synthesized as single-chain proteins and then converted post-translationally into the di-chain form by proteolysis; this process is often called activation [96]. The light-chain subunit comprises a single folded domain and functions as a highly specific protease, which acts to block neurotransmitter release by cleaving and inactivating a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein, which is a core element of the secretory apparatus [95]. The heavy chain comprises two major functional units. The N-terminal region (H_N) encodes the translocation activity that transports the light chain across cell membranes. The C-terminal region (H_C) contains two high-affinity receptor-binding sites, both of which are located towards the extreme C-terminal end of H_C (called H_{CC}). These function together to provide neuronal-specific cell targeting (Fig. 7.1).

This chapter focuses on the activity of the H_N translocation domain and on the processes by which BoNT proteins take advantage of neuronal physiology to harness energy and deliver a large (50 kDa) enzymatically active protein across cell membranes. The process relies on well-coordinated structural changes. The H_N translocation domain forms a protein-conducting channel only after endocytosis into a recycling synaptic vesicle compartment. The conditions in this compartment allow the light chain to unfold and to be guided into the channel. Then interactions between the light chain and the channel itself act to drive protein translocation across the membrane and into the target cell cytoplasm, where the prevailing neutral pH and reducing environment allow the light chain to refold and dissociate from the channel so that it can act on its target SNARE proteins. For a long time, the events

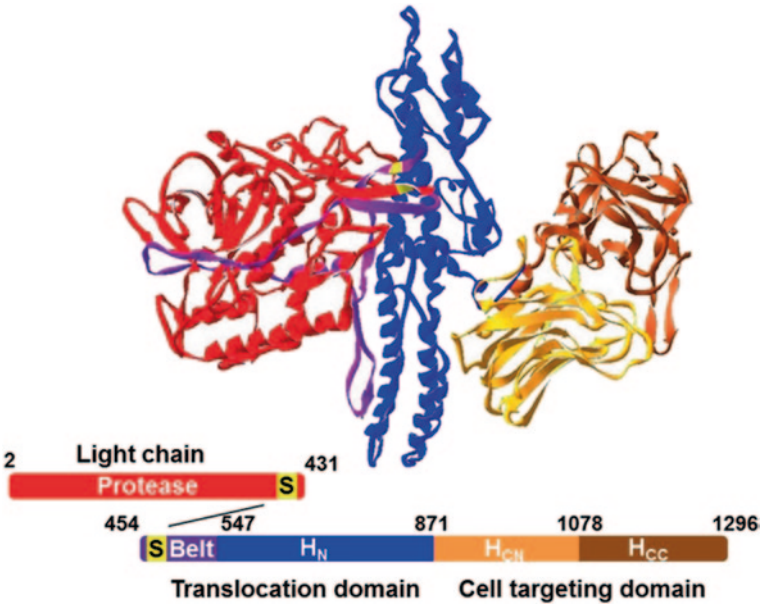

Fig. 7.1 The figure shows structural and functional domains in BoNT/A1 (UniParc sequence UPI0000ED909E, structure 3BTA) (Lacy et al. 1998). These domains are well conserved in the other serotypes (BoNT/B-G). Structural image created with DeepView/Swiss-Pdv Viewer v3.7

surrounding translocation were among the least studied and least well-understood aspects of BoNT biology. Recent progress now provides important insights into the nature and characteristics of the translocation event. Electrophysiological techniques have begun to elucidate the characteristics of protein channels formed by BoNT proteins. It is becoming apparent how the channel properties are engendered by structural features of the H_N domain. Other studies have provided insights into the pharmacology of BoNTs. Similarities and differences between various serotypes of BoNTs and between BoNTs and other bacterial toxins are also becoming apparent. This new information presents opportunities to target the translocation event with therapeutic inhibitors of toxin function [32, 75] and even the possibility to harness and adapt BoNT translocation domains for other uses, by incorporating them into engineered recombinant proteins designed to deliver new therapeutic proteins into cells [5].

7.2 Overview of Internalization and Translocation

The high affinity and specificity with which BoNTs target neurons is generated by a dual-receptor mechanism. BoNTs bind to both lipid and protein receptors on the cell surface [19, 41, 63, 64, 74, 78] (see Chapter 6 of this book). The lipid recep-

tors are complex ganglioside glycolipids, which are concentrated on neuronal cell membranes. The protein receptors are luminal domains of synaptic vesicle proteins [8, 10, 23]. This leads to a model of binding and internalization in which BoNT proteins first become selectively concentrated on neuronal cell surfaces by binding to specific lipid receptors and are then rapidly internalized by binding to synaptic vesicle proteins that are transiently displayed at the presynaptic active site during neurotransmitter release. This mechanism targets BoNTs specifically into the recycling synaptic vesicle compartment of active synapses.

Endocytosed recycling synaptic vesicles become acidified by the action of V-type H-ATPase2 transporters, which pump protons into the lumen of the vesicle. This causes a drop in pH (acidification) and generates a membrane potential (positive inside). The neuron uses this electrochemical gradient as a source of energy to refill vesicles with neurotransmitter [4, 89]. BoNT proteins take advantage of the electrochemical and pH gradients to unfold and translocate their light chain. Vesicle acidification triggers the insertion of the translocation domain into the vesicle membrane where it forms a channel. At the same time, the acidic pH also causes the light-chain protease domain to unfold. The unfolded light chain becomes inserted into and translocated through the H_N channel. The translocated light chain then refolds in the neutral pH environment of the cytoplasm. Because the cytoplasm is a reducing environment, the disulphide bond between the BoNT light and heavy chains becomes reduced. This releases the refolded light chain into the cytoplasm of the synaptic terminal where it can act on its substrate SNARE protein.

7.3 Receptor Binding and Internalization

BoNTs bind and internalize very rapidly into neurons, much more quickly than they act to inhibit neurotransmitter release. Indeed, experiments measuring the time courses over which inhibitors of binding and internalization lose the ability to protect cells from BoNT action were among the first to show that BoNT proteins must inhibit neurotransmitter release by a multistep mechanism [83].

Both the dual lipid and protein receptor-binding sites of BoNT proteins are located towards the C-terminal end of the H_C cell-targeting domain (Fig. 7.1). This domain is both necessary and sufficient for cell binding and internalization. Recombinant H_C domains internalize into primary mouse spinal cord neurons and compete with full-length BoNT proteins [54]. The lipid- and protein-binding sites have been mapped onto the crystal structures of all serotypes (see Chaps. 5 and 6 of this book). For example, residues between 1193–1225 and 1203–1276 of BoNT/A1 (the serotype that forms the active component of many BoNT drugs including Dysport, BoTox and Xeomin) interact with its protein (SV2) and lipid (GT1b) receptors, respectively [23, 85]. Similar lipid and protein receptor-binding sites lie in homologous positions on the other serotypes [51, 85, 86].

Lipid binding to concentrate BoNT on the neuronal cell surface is a necessary step for BoNT function because the depletion of lipid receptors from cell mem-

branes protects from the action of BoNT [11]. Known receptor lipids of various BoNT serotypes include GT1b for BoNT/A [85] and GT1b and GD1a for BoNT/F [37]. Rapid internalization of cell surface-bound BoNT is driven by protein receptor binding. Protein receptor binding is also necessary for BoNT function because cells that have been treated with a protease, to deplete protein receptors, are protected from BoNT activity [69]. All known protein receptors for BoNTs are luminal domains of synaptic vesicle proteins (see Chap. 6 of this book). These include the synaptic vesicle protein SV2 for BoNT/A, BoNT/D and BoNT/E [24]; synaptotagmin II for BoNT/B [46]; and synaptotagmin I and II for BoNT/G [69, 79]. Treatments that inhibit synaptic vesicle recycling also inhibit BoNT internalization, leaving the BoNT exposed on the cell surface. Mouse spinal cord neurons held in ice-cold buffer (to inhibit endocytosis) while they are incubated with BoNT can be protected if neutralizing anti-BoNT antibodies are added before the cultures are rewarmed to 37 °C. In contrast, cells exposed to BoNT at 37 °C are not protected even if neutralizing antibodies are added immediately after BoNT treatment [47]. This shows that internalization occurs very rapidly after binding under normal conditions. The extent of BoNT internalization can be increased by inducing neurotransmitter release (depolarization in the presence of Ca^{2+}) at the same time when the BoNT is added [47]. This further supports that BoNT is internalized into a recycling synaptic vesicle compartment.

7.4 Light-Chain Unfolding

Based on published crystal structures, the folded BoNT light chain is a relatively large globular protein approximately $5.5 \times 5.5 \times 6.2$ nm in size [52]. Some degree of unfolding is almost certainly necessary before it can be translocated through a protein-conducting membrane channel. This hypothesis, that molecular size might limit light-chain translocation, is supported by experiments where the molecular size and thermal stability (ease of unfolding) of the light chain are altered. Manipulations that increase the molecular size or that make it more resistant to unfolding also inhibit light-chain translocation. Anti-light-chain monoclonal antibody Fab fragments that bind and thereby increase the size of the light chain arrest translocation in patch-clamp translocation assays [29, 30]. Studies where recombinant BoNT proteins are expressed as fusion proteins show that fused, stably folded proteins (such as firefly luciferase or green fluorescent protein) reduce translocation to a greater extent than do fusions with less thermostable proteins (such as dihydroflate reductase or the light chains from other BoNT serotypes) [5].

Light-chain unfolding is triggered by vesicle acidification. Agents that block acidification protect neurons from the effect of BoNT [47, 94], showing that entry into an acidifying compartment is required for the mechanism of action of BoNT proteins. Various spectroscopic techniques show structural changes in the BoNT/A light chain at pH values similar to those found in acidifying synaptic vesicles [36, 48]. Lipophilic fluorescent dye-binding experiments also show light-chain

unfolding at acidic pH by measuring increased dye binding to solvent-exposed hydrophobic patches [13]. The extent to which BoNT light chains must unfold before they can be translocated is, however, not yet fully understood. The structure of BoNT/B does not change when crystals are soaked in acidic compared to neutral buffer [27]. Circular dichroism spectroscopy measurements that probe the helical content of BoNT/A light chains have given conflicting results. One study detected a large reduction in the helical secondary structure for BoNT/A light chain at pH 5.0 relative to pH 7.0 [48], but another reported only small changes in the secondary structure under similar conditions [57]. One possible explanation for these differences is that they may be related to differences in the availability of functional chaperone activities in the various systems. Little is known about whether or to what extent chaperone activity is needed to drive unfolding of BoNT light chains. Other bacterial toxins, such as diphtheria and anthrax toxins, do rely on a chaperone activity to regulate unfolding of their catalytic subunits. Anthrax toxin relies on the host-cell endosomal chaperone GRP78 to unfold its light chain, whereas diphtheria toxin uses an intramolecular chaperone activity encoded by its translocation domain [20, 90]. BoNT light-chain unfolding might similarly be regulated either by another domain in the BoNT itself or by a host-cell endosomal chaperone protein. Nevertheless, there is clear evidence that changes, at least in the tertiary structure of BoNT light chains, do occur under acidifying conditions [19]. The weight of evidence shows that BoNT light chains adopt an unfolded, molten-globule-like state at acidic pH and that this unfolding is necessary for protein translocation [92].

7.5 Channel Formation

Simultaneously with light-chain unfolding, acidification triggers the H_N translocation domain to insert into membranes and form channels. This coordination is regulated by a peptide sequence, called the belt region, located at the extreme N-terminus of the translocation domain [9, 31, 38]. At neutral pH, the H_N domain is held away from membranes by charge interactions between the phospholipid head groups of the membrane and the negatively charged amino acid side chains in the H_N belt. When the pH drops, as in acidifying recycled synaptic vesicles, the negatively charged residues become increasingly protonated. This reduces the electrostatic repulsion holding the H_N region away from the membrane. A similar mechanism has also been proposed to explain how short, negatively charged, cell-permeating peptides, such as viral envelope proteins, can associate with and destabilise endosome membranes; in this context, it has been named the proton sponge mechanism [67]. Involvement of charge interactions in regulating BoNT membrane channel insertion is supported by observations that the interaction of isolated H_N domains with lipid vesicles increases when reactions are run at low salt concentration and that it is reduced by high salt concentration [53]. Localization of this membrane interaction regulator function to the belt region is supported by experiments with truncated variants of the H_N domain. Proteins that include the belt region associate with lipid

vesicles at acidic but not at neutral pH. In contrast, H_N truncations lacking the belt region associate with lipid vesicles even at neutral pH [38].

The belt region itself is a relatively unstructured protein loop. It wraps around the light chain and occupies (e.g. in BoNT/A) or lies near to (e.g. in BoNT/B) the substrate-binding sites on the light chain. The contacts between belt and light chains also match well to those of SNAP25 bound to the BoNT/A light chain. The crystal structures all overlay closely with each other when they are superpositioned [6, 9, 52, 88]. In addition to co-coordinating channel insertion with light-chain unfolding, various other related activities have also been proposed for the belt region. These include roles as a pseudo-substrate protease inhibitor and as a light-chain chaperone to protect and guide the light chain as it unfolds, helping it to pass through the H_N channel [6, 7, 9, 22].

H_N channel formation may also be regulated by a phosphatidylinositol phosphate-binding site that lies C-terminal to the translocation domain. This region of the heavy chain is often called H_{CN} to distinguish it from the receptor-binding domain (called H_{CC} in this scheme). It is clearly identifiable as a distinct folded structural domain in crystal structures (Fig. 7.1) and shows homology to the '**concanavalin A lectin domain family'** [12]. H_{CN} may function to facilitate membrane insertion by orienting the translocation domain so as to favour channel formation [65, 93].

The pH-regulated association of BoNT/B with lipid membranes has been studied directly by electron microscopy. BoNT/B forms ordered protein coats on lipid membranes at low pH. Two electron microscopic studies showed very similar three-dimensional reconstituted visualisations of the structure [33, 80]. BoNT/B coated and reshaped the lipid into long tubules. The BoNT itself formed a regular helical array with an apparent fourfold symmetrical lattice structure and protein loops that extend approximately 10 nm (100 angstroms) out from the surface of the membrane. Under oxidizing conditions, small oval transmembrane channels (1.4×2.5 nm in diameter) were apparent at the corners between BoNT/B subunits [80]. Under reducing conditions, in the second of the two studies, BoNT/B formed a very similar lattice structure and caused similar tubulation of the membranes but no open channels were observed [33]. This might indicate that channel opening can be regulated by the redox state. The lumen of recycling synaptic vesicles, where wild-type BoNT forms channels, is a nonreducing acidifying environment, whereas the cell cytoplasm is a reducing environment that is close to neutral pH. Several lines of evidence suggest that transmembrane redox and electrochemical gradients are important for translocation (discussed later). Membrane-permeable reducing agents added to translocation reactions arrest light-chain translocation [29, 30]. It is possible that a nonreducing, acidic lumen may be necessary to drive and maintain open H_N channels.

Sequence analysis of the translocation domains from BoNTs, and the closely related tetanus toxin, identified putative channel-forming, amphipathic helix regions calculated to be thermodynamically sufficient to assemble into four-α-helical, membrane-spanning bundles (residues 659–681 in BoNT/A: GAVILLEFIPEIAIPVLGTFALV). Synthetic peptides corresponding to these regions did indeed form cation-selective channels when they were reconstituted in planar lipid bilayers

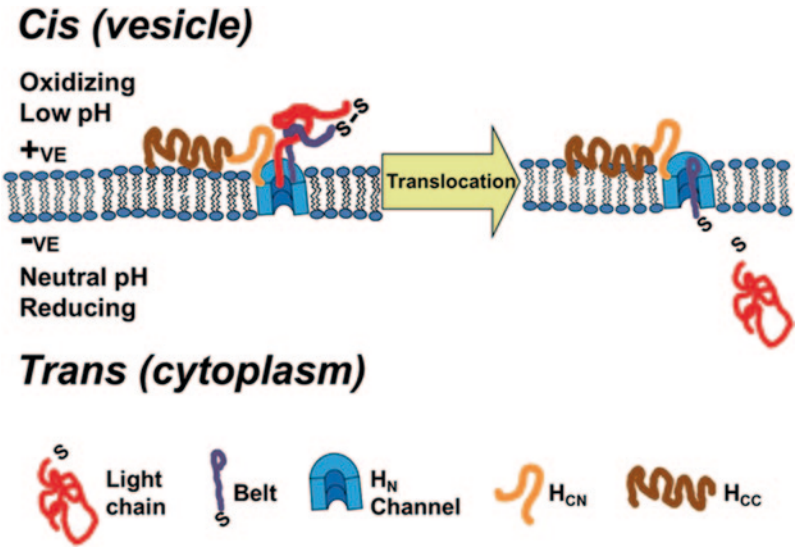

Fig. 7.2 Electrochemical and redox gradients driving translocation. The figure shows the electrochemical and redox gradients that drive botulinum neurotoxin (BoNT) light-chain translocation. Acidic, oxidizing conditions within the vesicle lumen (*cis* compartment) cause the H_N translocation domain to insert into the membrane, forming a protein-conducting channel. The light chain remains covalently linked to the heavy chain belt by a disulphide bond while it unfolds and becomes inserted into the channel. After translocation, the light chain refolds in the neutral, reducing conditions of the cytoplasm (*trans* compartment). Reduction of the disulphide bond allows the refolded light chain to dissociate

[62, 71]. The calculated fourfold stoichiometry for these bundles agrees well with the apparent fourfold symmetry observed in reconstituted three-dimensional electron micrograph visualisations of membrane-associated BoNT/B [33, 80]. A recent study using purified recombinant truncations in BoNT/A did not, however, support channel formation by this region. Site-specific labels that report if an amino acid is localized in an aqueous or lipid environment incorporated into this putative transmembrane region of BoNT/A suggest that it does not become inserted into lipid bilayers on acidification [66]. The same study did, however, identify another region in H_N (residues 805–837) as a candidate for membrane insertion. This region was identified because it becomes protected from protease digestion after acidification in the presence of lipid vesicles [66].

Electrophysiological approaches support that BoNT H_N domains form transmembrane channels on acidification. These studies monitor channel formation by taking electrical measurements across a supported membrane to detect ion currents that flow through the channels as they open. The side of the membrane to which BoNT is added (called the *cis* side) represents the lumen of a recycling synaptic vesicle. The other side (called the *trans* side) represents the cell cytoplasm (Fig. 7.2). BoNT channels are detected under conditions where the *cis* side is maintained as an acidic, oxidizing environment and the *trans* side is held as a neutral, reducing environment.

Acidification-triggered pore formation has been measured both in supported artificial lipid bilayers [44, 81] and in native membrane patches taken from Neuro-2A and other cell types [28, 48, 58]. These approaches have been very successful. They provide important insights because light-chain translocation can be monitored in real time. Distinct, identifiable stages in the translocation process are revealed as a series of characteristic intermediate conductance states that are caused because the light chain occludes the channel to different extents as it enters, passes through and finally exits on the *trans* side of the membrane [29, 30].

Various studies have set out to estimate the diameter of H_N channels. Determinations range between 1.5 and 2.4 nm. However, protein channels can be highly dynamic structures that cycle between various different open and closed conformational states [39]. Differences in the estimated average channel diameter between these studies may, therefore, simply reflect different probabilities for the channels to reside in a particular conformational state under each set of experimental conditions. Patch-clamp experiments generally lead to estimates at the lower end of the range (1.5 nm) [48], whereas approaches that measure escape of various differently sized florescent-labelled molecules lead to estimates at the higher end (2.4 nm) [35, 72, 81]. A similar question, also related to average channel diameter, is whether or not channels are predominantly open under physiological conditions. Early studies suggested that BoNT H_N channels are voltage gated and predominantly open when the membrane potential is positive in the lumen of the vesicle (*cis* side) [44]. This would suggest the channels stay open after light-chain translocation has finished under physiological conditions. More recent patch-clamp studies, however, report voltage gating in the opposite direction: Channels open at negative luminal membrane potential [31]. This would suggest the channels close after translocation finishes. The reasons for this discrepancy, or indeed the physiological significance of one scenario compared to the other, are not yet fully understood.

7.6 Translocation

Translocation relies on an ordered series of interactions between the light and heavy chains that first deliver the unfolded light chain into the H_N channel and then harness energy to drive it across the membrane. Protein translocation through H_N channels proceeds very inefficiently in the absence of the light-chain subunit. Engineered fusion proteins, where the light chain is replaced by an alternative (non-light-chain) cargo protein, do not achieve cytoplasmic delivery even though the proteins are internalized efficiently into an endosomal compartment. The translocation step is blocked [40]. In contrast to this, engineered BoNT fusion proteins where an exogenous cargo protein is fused to the N-terminus of the light chain do achieve cytoplasmic delivery of the cargoes [5, 70].

Patch-clamp experiments set up to report progression through the individual stages of a translocation event reveal an ordered series of channel states. Channel activity first becomes evident as a low-conductance blocked channel state

(approximately 13 pS). Then a series of intermediate stages (24, 47 and 55 pS) are measured before the conductance finally stabilizes at a higher (approximately 67 pS), unblocked channel state. A single channel translocation process takes approximately 240 s to complete [29, 30]. The initial and intermediate conducting states are caused because the light chain partially blocks the H_N channel while it translocates [29, 30]. These observations suggest that there is a tight contact between the light chain and the channel during translocation. The stable approximately 67 pS state represents unblocked channel left behind after the light chain has passed through and dissociated on the *trans* side of the membrane. Isolated heavy chains adopt the approximately 67 pS unblocked channel state immediately on insertion, because there is no light chain to occlude the channel [29–31, 48]. Direct evidence that these electrical signatures do indeed report productive light-chain translocation events comes from observations that SNAP25 added to the *trans* compartment is cleaved only under conditions where these characteristic channel activities are seen [31, 48]. A series of elegant studies have shown that channel formation and progression through the intermediate states to complete a productive translocation event rely on all three gradients: the pH gradient, the redox gradient and the transmembrane potential. Manipulations that disrupt any one of these gradients cause translocation to become arrested.

The pH gradient regulates both the H_N translocation domain and the light-chain subunit activities. H_N membrane insertion is triggered by acidic pH in the *cis* compartment. The channels do not open, however, unless there is also a pH gradient across the membrane (acidic *cis*, neutral *trans*). BoNT/B heavy chain does not form conducting channels at symmetrical pH, but when a pH gradient is established, either by adjusting the *cis* compartment to acidic pH (starting from symmetrical neutral) or by neutralizing the *trans* side (starting from symmetrical acidic), channel activity becomes evident within 30 s of adjustment [44]. The pH gradient is also necessary for the cycle of light-chain unfolding (allowing translocation) then refolding to restore enzymatic activity. Anti-light-chain antibody fragments block translocation and cause a persistent blocked channel conductance state [29, 30]. This is because antibody binding adds a folded domain to the complex and this stops the light chain from passing through the channel. This interpretation is supported by observations that light-chain fusions with stable (resistant to unfolding) proteins are also impaired in their ability to translocate, whilst fusions with proteins able to unfold are not [5]. Protein refolding in the *trans* compartment is necessary for the light chain to regain its enzymatic protease activity after translocation, and so it is necessary for the light chain to translocate into a neutral-pH environment. In addition to restoring the enzymatic activity of the light chain, refolding may also contribute a mechanism that allows BoNT to harness energy from the pH gradient to help drive translocation. Refolding would be expected to sterically trap light chain on the *trans* side of the membrane by making it too large to re-enter the channel. This would prevent retro-translocation, and thereby act as a Brownian ratchet, forcing unidirectional transport. If this mechanism does contribute to translocation then adding agents that promote refolding into the *trans* compartment might be expected to enhance translocation. A similar, refolding-driven, Brownian ratchet

mechanism has been proposed in the translocation of other bacterial toxins such as anthrax toxin [49].

Similar to pH, the redox gradient (oxidizing *cis* and reducing *trans*) is also implicated in several, interrelated mechanisms that could function to promote translocation. An oxidizing *cis* compartment is necessary because BoNT light and heavy chains are disulphide linked together in the *cis* compartment. A reducing *trans* is needed to allow the light chain dissociate and exit the channel after translocation. Reducing agents added to the *cis* compartment before translocation cause the H_N channel to proceed directly to an approximately 67 pS unblocked state [48]. Under these conditions, the light chain does not enter the channel and BoNT behaves as if it were an isolated heavy chain; perhaps because unlinked light chain diffuses away from the heavy chain and fails to find the channel. In experiments where the redox gradient is disrupted on the other side of the membrane, and the *trans* compartment is maintained as a nonreducing environment, the translocation becomes arrested as a persistent blocked channel state [48]. This is because the light chain enters the channel but does not dissociate on the *trans* side.

The H_N channel interacts with and shields the translocating light chain. This is shown by experiments where translocation is allowed to start under symmetrical oxidizing conditions and then reducing agent is added to the *cis* side only. This does not rescue the translocation block, but rather it leads to a stable intermediate blocked channel conducting state. The light chain does not dissociate on either side of the membrane under these conditions. This shows that, once inside the channel, light-chain disulphide bonds become inaccessible from the *cis* compartment [29, 30]. The light chain is, however, exposed on the *trans* side. Single-chain BoNT proteins that have not been activated proteolytically (such as BoNT/E) become arrested at a persistent blocked state in patch-clamp experiments. This is because the light chain remains linked to the H_N channel by a peptide bond and cannot dissociate on the *trans* side of the membrane. In this case, productive translocation can be rescued if trypsin is added to the *trans* compartment. The trypsin activates the BoNT in situ, by cleaving between the light and heavy chains, and thereby allows the light chain to dissociate from the channel [29, 30].

Redox interactions also seem to play additional roles in translocation, beyond controlling the linking and then releasing of the light and heavy chains. Shuffling of disulphide bonds between cysteine residues may contribute energy or directionality to drive the translocation process. Evidence for this comes from experiments where translocation of single-chain BoNT is initiated under symmetrical reducing conditions. In this set-up, the light and heavy chains again remain linked by a peptide bond. The light chain enters the channel and translocation arrests at an intermediate conductance state where the light chain cannot dissociate on the *trans* side of the membrane. Under these conditions, with no redox gradient, adding trypsin to the *trans* compartment does not, however, rescue translocation. This is unlike the situation in the presence of a redox gradient (described above) where adding trypsin does rescue translocation of single-chain BoNT [29, 30]. These data suggest that, although the light chain has entered the channel to a sufficient extent to occlude conductance, when there is no redox gradient, the translocation does not proceed

sufficiently to expose the activation loop on the *trans* side of the membrane. Further evidence that redox interactions within the channel are important to drive translocation comes from observations that membrane-permeable reducing agents added to the *trans* compartment after translocation of normal activated BoNT has started, but has not yet gone to completion, are sufficient to block in a low-conductance intermediate state [29, 30]. Finally, another bacterial toxin, diphtheria toxin, needs the action of a large host-cell protein complex that includes the redox enzyme thioredoxin reductase to translocate from acidified endosomes. This again suggests a role for disulphide rearrangement during the translocation of bacterial toxins [76].

The electrical membrane potential can also provide energy for light-chain translocation. Indeed, at least in the cases of electrophysiological experimental set-ups, this gradient can be sufficient to drive the process [44, 48, 49]. The precise contribution of membrane potential and its relative importance compared to other potential sources of energy in native intoxicated cells are, however, not yet established.

7.7 Light-Chain Refolding

Light-chain refolding in the cytoplasm is necessary to restore the enzymatic protease activity, but this process may also contribute to driving unidirectional translocation. It is not yet fully understood if BoNT light-chain refolding is spontaneous and unregulated or if host-cell chaperone proteins are also involved. SNAP-25 cleavage is observed in a minimal patch-clamp system where BoNT/A is added to the *cis* compartment and purified SNAP-25 is added to the *trans* [31, 48]. This shows that either host-cell cytoplasmic proteins are not essential for refolding or sufficient activity to support the function is supplied associated with the plasma membrane patch in this system. Either way, this observation does not show if host-cell proteins play a role in promoting efficient light-chain refolding in intoxicated cells. Other bacterial toxins do use host-cell chaperones to refold after translocation. Diphtheria toxin needs adenosine triphosphate (ATP) and the action of a host-cell multiprotein complex (referred to as cytosolic translocation factor) which comprises the chaperone Hsp90, thioredoxin reductase and β-COP to drive its translocation out of endosomes [76]. The interaction with β-COP at least might be conserved in BoNT because the ten-amino-acid β-COP-binding motif in diphtheria toxin is also present in BoNTs/A, /C and /D and in anthrax toxin [77]. The *C. botulinum* C2 toxin (an actin–ADP–ribosylating toxin) also uses host-cell Hsp90 chaperone activity to refold after translocation [43]. Furthermore, similar endogenous host-cell protein translocation events, such as retro-translocation of mis-folded proteins out of the endoplasmic reticulum, rely on chaperone proteins and ATP hydrolysis to drive translocation and to refold the translocated proteins in the cytoplasm [68]. Another obvious candidate chaperone protein for light-chain refolding is the BoNT protein itself. The belt region wraps around the light chain in the holotoxin structure and seems well placed to act as a chaperone, at least in the *cis* compartment prior to translocation [9]. It is also plausible that the belt could function to promote refolding in the *trans* compartment after translocation [31]. This could be tested by running experiments

to monitor light-chain translocation by recombinant BoNT proteins with truncations in their belt regions. The inserted H_N channel itself might also serve a chaperone-like function. By holding unfolded light chains anchored to the cytoplasmic face of the membrane for a time after translocation and thereby limiting their freedom to diffuse it might prevent them from aggregating before they have had an opportunity to refold. If, as seems to be the case, the disulphide bond linking light and heavy chains remains protected within the channel for most of the translocation process but the N-terminal region of the light chain becomes exposed to the cytoplasm early, then light-chain refolding might proceed to near completion before the light and heavy chains become separated.

7.8 Potential to Modify Translocation Events

Among the most exciting benefits from recent progress in unravelling the mechanisms of BoNT translocation are new possibilities to manipulate and to harness these processes and use them for medical and technological benefit. Opportunities include creating new biochemical tools to further investigate BoNTs and the cells they target, creating new medicines to protect against or to speed recovery after intoxication by BoNT, as well as opportunities to harness and adapt the protein domains that drive translocation and to incorporate them into new engineered proteins that can deliver therapeutic molecules into cells [5, 26, 70].

Small molecules that alter BoNT internalization and translocation include inhibitors of endocytosis, such as the dynamin inhibitor Dyngo-4a [42]; inhibitors of endosome acidification, such as Baflomycin and Concanamycin A [47, 94]; and direct inhibitors of the translocation processes itself, such as Toosendanin (a tetranortriterpenoid compound derived from the plant *Melia toosendan*) [1, 32, 58, 75]. These inhibitors have been used to dissect the mechanistic steps in BoNT endocytosis, channel formation and translocation (Fig. 7.3). Increasingly, they are also of interest as potential new drugs to protect against or to treat botulism. This growing interest is largely because BoNT is increasingly becoming viewed as a potential bioweapon, which could be produced and used by terrorist organizations. Translocation inhibitors in particular may have advantages over other drug candidates such as protease inhibitors that target the light chain directly. This is because protease inhibitors may prove to be either too specific (protecting against only a subset of BoNT serotypes) or not specific enough (targeting many cellular proteases in addition to the BoNT). The different BoNT serotypes are specific for different SNARE proteins; this specificity arises from extensive, subtype-specific, substrate-binding sites that lie away from the catalytic site [3, 6, 21, 22, 82]. The catalytic sites themselves are similar between serotypes but are also highly similar to other Zn metalloproteases, including mammalian cellular proteases [45]. Therefore, light-chain inhibitors are likely either to be restricted to protecting against just one or two BoNT serotypes (by inhibiting SNARE binding) or to show undesirable side effects due to inhibition of cellular proteases (by binding to the conserved active site). In contrast, the translocation activity appears to be highly similar between all serotypes of BoNT

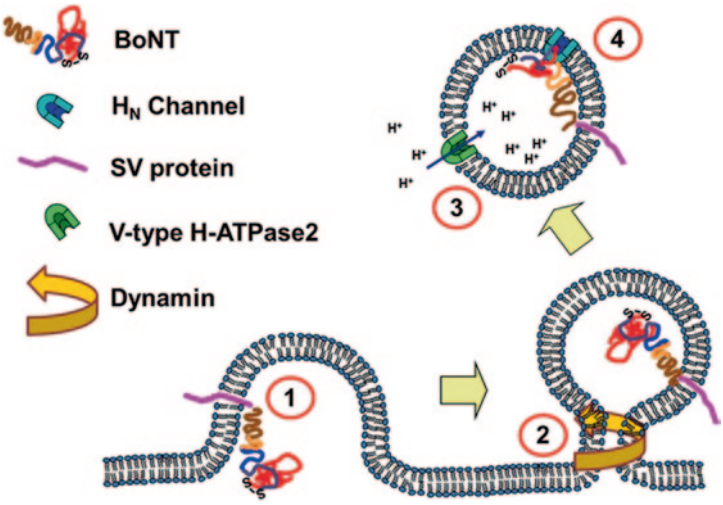

Fig. 7.3 Inhibitors of internalization and translocation. The figure shows the stages of action for various inhibitors of botulinum neurotoxin (BoNT) internalization and translocation: *1* inhibitors of cell binding, such as treatments to deplete ganglioside glycolipids or proteins from the cell surface, *2* inhibitors of endocytosis, such as low temperature or the dynamin inhibitor Dyngo-4a, *3* inhibitors of acidification, such as Baflomycin or Concanamycin, *4* direct inhibitors of H_N channel function, such as Toosendanin

and unlike mammalian physiological processes. Therefore, translocation inhibitors are good candidates to provide a source of new drugs to protect against botulism.

In addition to being an attractive target for new, protective, anti-botulinum drugs, understanding the translocation process is opening opportunities to create new biotherapeutics to treat other diseases. There are many bioactive proteins and nucleic acids that have enormous potential to become new drugs if they could be delivered efficiently into the cytoplasm of cells. Cytoplasmic delivery is, however, proving to be a major roadblock. Current approaches, such as cell-permeating peptides [61, 67, 84], lipid vesicles [56], charged polymers [25] and viral vectors [97], have all shown limited utility and even then only in a small number of cases. Harnessing the translocation activity of BoNT to deliver large soluble molecules directly into the cytoplasm of cells is a very attractive possibility. Recombinant BoNT proteins have the potential to deliver a wide range of proteins into neurons [5]. Even more excitingly, engineered BoNT proteins have been created that do not bind to neurons and are therefore nontoxic [17, 59, 60, 87]. These proteins have been retargeted to deliver their light-chain protease into and inhibit secretion from other cell types [18, 91]. They are called targeted secretion inhibitors (TSIs), and they represent a new class of protein therapeutics with potential to treat diseases where hypersecretion is a causal factor, such as endocrine disorders [14–16, 26, 34] (see Chap. 7 of the companion volume to this book, KA Foster (ed) *Clinical Applications of Botulinum Neurotoxin*, Springer, New York). Combining cell type-selective retargeting with delivery of (non-light-chain) bioactive molecules into cells opens the potential to

create an enormously diverse array of new protein therapeutics, which could relieve suffering and help patients recover from many different diseases.

7.9 Summary

Recent progress has revealed a wealth of new information about the mechanistic details of BoNT translocation into cells. Translocation is not a single event but an interconnected series of activities mediated by co-operation between all three major functional domains in BoNT. Specific targeting into recycling synaptic vesicles is mediated by H_C binding to cell surface receptors and by the belt region of H_N which prevents premature channel formation on the cell surface plasma membrane [38]. The initial internalization into cells occurs very rapidly [47]. Acidification of the internalized compartment relieves the inhibition of H_N channel formation and triggers light-chain unfolding [13, 31, 38]. It is likely that the belt region also chaperones the unfolding light chain preventing aggregation and guiding it into the H_N channel [9]. The light and heavy chains interact closely to drive translocation and at least one disulphide-bonded cysteine pair must be maintained in the correct redox state for productive translocation to occur [29, 30]. Then the translocated light chain refolds and dissociates from the channel in the neutral, reducing environment of the cytoplasm where it acts on its target SNARE proteins [31, 48, 95]. Major questions that remain unanswered include:

1. What are the mechanistic details of the interactions from the N-terminal belt region and the C-terminal (H_{CN}) phosphatidylinositol phosphate-binding site that flank the H_N channel domain and regulate its insertion into membranes?
2. How does the belt region transition from regulating channel formation to chaperoning the unfolded light chain?
3. What is the driving force for translocation in living cells?
4. Are host-cell cytoplasmic chaperone proteins recruited to refold translocated light chains in the cytoplasm?

Finally, perhaps the most important question of all is how soon will we be able to realize the potential offered by engineered and retargeted BoNT translocation domains to create new drugs that specifically deliver large therapeutic molecules into target cells and thereby provide new and better treatments for patients?

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Chapter 8
Protease Activity of the Botulinum Neurotoxins

Sheng Chen and Joseph T. Barbieri

Abstract The flaccid pathology associated with intoxication by the botulinum neurotoxins (BoNTs) is the result of the association of the toxin to neuronal-specific host receptors and the cleavage of neuronal substrates, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. Each of the seven serotypes of BoNTs (A–G) targets a specific neuronal SNARE protein(s) for cleavage. Neuronal SNARE proteins function in the binding and fusion of neurotransmitter vesicles with a host membrane, and SNARE protein cleavage by the BoNTs disrupts the fusion process leading to host paralysis. The mechanism that BoNTs utilize to bind and cleave the SNARE proteins involves recognizing an extended substrate surface to allow the BoNTs to efficiently cleave the coiled SNARE protein substrate. BoNT serotypes comprise natural variants termed subtypes, which extends the complexity and potential pathology of the BoNTs. Understanding the mechanisms of BoNT action provides tools towards the development of strategies to identify novel small-molecule inhibitors of BoNT catalysis and to extend the use of BoNTs as therapeutic agents.

Keywords Botulinum neurotoxin · SNARE proteins · v-SNARE · t-SNARE · Zinc-metalloprotease · Syntaxin · Synaptosomal-associated protein of 25 kDa (SNAP-25) · Synaptobrevin · Vesicle associated membrane protein (VAMP) · Exosites · Scissile bond · "Pocket" model · Belt region

8.1 Cleavage of SNARE Proteins by the Light Chain of the Botulinum Neurotoxins

As Category A agents and the most potent protein toxins for humans [1], the botulinum neurotoxins (BoNTs) have been subjected to considerable biochemical and cell biological analyses. The recent use of BoNTs as therapeutic agents to treat

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spastic neurological inflictions ([2, 3]. Chapters 3–6 of the companion volume to this book, KA Foster (ed) *Clinical Applications of Botulinum Neurotoxin,* Springer, New York) has also highlighted the need to understand how BoNTs cleave soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins.

Clostridium botulinum and several other species of clostridia produce BoNTs as components of a protein complex that may facilitate toxin protection from hostile host environments and contribute to the intestinal absorption by facilitating transport across the intestinal epithelium for the delivery of BoNT within the intestine ([4, 5] and Chapters 3 and 4 of this book). BoNTs are grouped into seven serotypes (A–G) based upon antisera neutralization [6, 7]. Serotypes A, B, E and F are associated with natural human intoxication, while serotype G has been occasionally associated with sudden infant death syndrome [8] and serotypes C and D are associated with natural intoxication of animals [9, 10]. While BoNTs elicit a flaccid paralysis, the related neurotoxin, tetanus toxin (TeNT), elicits spastic paralysis [11–19], although BoNT/B and TeNT have identical catalytic activities [20, 21].

BoNTs are AB toxins composed of two domains [22]. The N-terminal light chain (LC) comprises the enzymatic (zinc metalloprotease) domain. The C-terminal heavy chain (HC) includes two independent functional domains, the translocation domain (HCT) and the receptor-binding domain (HCR) [23]. While the three domains are structurally independent [24], the HC possesses several distinguishing features; HCT comprises a pair of α-helices that are ~105 Å long that are predicted to contribute to the channel-forming activity of the HC that facilitates LC translocation across the synaptic vesicle membrane [25]. In addition, the N-terminus of the HC forms a "belt" that wraps around the LC blocking the substrate-binding site and securing the LC within the holotoxin structure [26]. The HC belt appears to place the LC inaccessible to substrate (SNARE proteins) and may protect the LC from proteolysis by host enzymes and, for some serotypes, protect LC from auto-cleavage [27]. The HCR consists of two sub-domains; the N-terminal sub-domain does not have a known function and is arranged in a jelly-roll motif comprised of several β-sheets while the C-terminal sub-domain comprises the dual-receptor-binding regions that are organized into a β-trefoil structure.

BoNTs are di-chain toxins linked by a disulphide bond. Earlier studies showed that a *Clostridium*-derived endoprotease was responsible for nicking BoNT/A to generate the di-chain toxin [28] where cleavage removed ten amino acids at the junction between the LC and the HC yielding an LC of ~438 amino acids, which is considered the active form of the LC [22]. The importance of nicking was revealed by the reduction in the toxicity of several derivatives of BoNTs produced by clostridia in an unnicked form. BoNT LCs are large relative to other bacterial AB toxins. For example, the LC of BoNT/A is 448 amino acids compared to the diphtheria toxin LC, which encodes an adenosine diphosphate (ADP)-ribosyltransferase domain, which is 193 amino acids. The large size of the BoNT LCs is due in part to the extended complexity of mechanism for SNARE protein recognition utilized by these endoproteases. The LC structures of the seven BoNT serotypes have been solved [26, 29–34]. While the overall LC structures are conserved, the regions surrounding the conserved zinc-binding motif show considerable divergence which contributes to the unique SNARE protein specificity of the individual serotypes.

8.2 SNARE Proteins

SNARE proteins are membrane associated and comprise a large family of proteins that function in the binding and fusion of vesicles with a host membrane. The target membrane can be internal, such as the Golgi apparatus, or external, such as the plasma membrane. There are two classes of SNARE proteins associated with exocytosis: vesicle SNARE (v-SNARE) proteins that are resident on vesicles and target SNARE (t-SNARE) proteins that are associated with the target membrane. SNARE proteins possess a cytosolic SNARE motif composed of ~60 amino acids that forms reversible helical bundles which anchor the vesicle to the target membrane [35, 36]. During exocytosis, the coiled, cytoplasmic domains of v-SNARE and t-SNARE proteins align to facilitate fusion of the vesicle with the target membrane. In neurons, two t-SNARE proteins (syntaxin and synaptosomal-associated protein of 25 kDa (SNAP-25)) form a pre-core SNARE protein complex that fuses with the v-SNARE protein (synaptobrevin or vesicle-associated membrane protein (VAMP)) on the incoming neurotransmitter vesicle. The basis for the fusion of vesicles with target membrane follows [37, 38]. v-SNARE and t-SNARE proteins assemble into a complex starting at the N-terminal region of the SNARE proteins and extend towards the C-terminal region of the SNARE proteins. This assembly brings the vesicle into close contact with the target membrane to facilitate binding of the vesicle with the target membrane to release the contents of the vesicle at the site of fusion. The exact steps involved in the fusion and release step of exocytosis are not completely understood and are the topic of considerable investigation.

SNARE proteins that mediate the exocytosis of neurotransmitter vesicles with the plasma membrane of neurons are the substrates for cleavage by the BoNTs. Each of the BoNT serotypes cleaves the cytosolic domain of one or more SNARE proteins. BoNT/A, C, and E cleave unique sites on the C-terminal end of SNAP-25. In addition, BoNT/C cleaves syntaxin 1(a and b), 2, and 3. BoNT/B, D, F, G, and TeNT cleave unique sites on VAMP-1, 2, and 3. BoNT/B and TeNT cleave VAMP-2 at the same site, showing that properties distinct from catalysis are responsible for the unique pathologies elicited by these two neurotoxins. Thus, the neuropathology associated with these neurotoxins is facilitated by the neuronal-specific SNARE substrate that is targeted for cleavage, in addition to the neuron-specific receptors. The utility of BoNTs for therapeutic intervention beyond neurological spasticity will allow the development of BoNT LCs that have extended substrate specificity beyond neuronal SNARE proteins.

8.3 Mechanism for BoNT LC Cleavage of SNARE Proteins

As stated earlier, the exquisite specificity of BoNTs as neurotoxins is based on the ability of LC to cleave neuronal SNARE proteins. Cleavage of SNARE proteins can be partitioned into two steps: an early step that involves the high-affinity

recognition of the SNARE protein outside the active site (AS) of the LC and the cleavage reaction that follows a general acid–base reaction mechanism. Using thermolysin as the prototype protease [39], a mechanism for SNARE protein cleavage by BoNTs can be described, where BoNT LC utilizes a hydrolytic water molecule to cleave a specific peptide bond within each SNARE protein. In this reaction, histidines within the LC HEXXH motif and a downstream glutamic acid, along with the hydrolytic water molecule, coordinate the Zn^{2+} ion located within the interior of each LC. The proposed mechanism for LC cleavage of the peptide bond within SNARE proteins follows. First, the hydrolytic water molecule attacks the carbonyl carbon of the scissile peptide bond and a proton is extracted from the hydrolytic water and shuttled to the scissile peptide bond nitrogen. The negative charge on the carbonyl oxygen atom of the scissile peptide bond is stabilized by hydrogen bonding. A coordinated histidine is then protonated by hydrogen bonding with an aspartic acid, which breaks the scissile bond using a proton derived from the hydrolytic water molecule. Several investigators have provided data to support this reaction mechanism, including Binz and coworkers [40, 41].

The alignment of a SNARE protein within a specific LC is serotype specific with respect to the residues within the LC that contribute to SNARE protein specificity and cleavage and to the sites along the SNARE protein that are recognized for the high-affinity binding to the LC. An important finding to elucidate these unique aspects of LC–SNARE protein interactions was made by Brunger and coworkers [26, 42] who generated a co-crystal of a non-catalytic LC/A bound to a truncated form of SNAP-25(146–206). The mutated LC/A did not possess catalytic activity and therefore allowed visualization of bound SNAP-25. The study identified “exosites” that represented interactions between LC/A and SNAP-25 located upstream and downstream of the LC AS, which provided a basis for the recognition and orientation of the coiled SNARE protein substrate for efficient cleavage. In addition, this study identified for the first time, a structural change within the AS when the LC bound the SNARE protein. The author proposed that this structural change placed the LC in a conformation that was now competent for cleavage of the SNARE protein. The observation that the LC undergoes a conformational change upon binding the substrate also has implications for the development of small-molecule inhibitors of LC catalysis.

In the holotoxin BoNT/A structure, the N-terminal HC loop forms a belt that wraps around the SNARE protein-binding cleft of the LC. Unexpectedly, the region of the HC loop that bound LC/A aligned well with the region of SNAP-25 that bound to LC/A (Fig. 8.1), where SNAP-25 and the HC loop of BoNT/A show spatial and sequence homology. The similarities extend along a stretch of 38 amino acids of SNAP-25 that abruptly ends at the P5 residue of SNAP-25. Importantly, the residues that play an important role in SNARE protein binding and recognition align with HC loop residues that interact with LC/A. This suggests that the recognition of SNAP-25 with LC/A mimics the binding of the HC loop within the binding cleft of LC/A in the native holotoxin. The biological function of the HC loop in BoNT/A may be as a pseudo-substrate that blocks the AS of LC/A to prevent premature substrate cleavage and to protect against auto-cleavage of the LC.

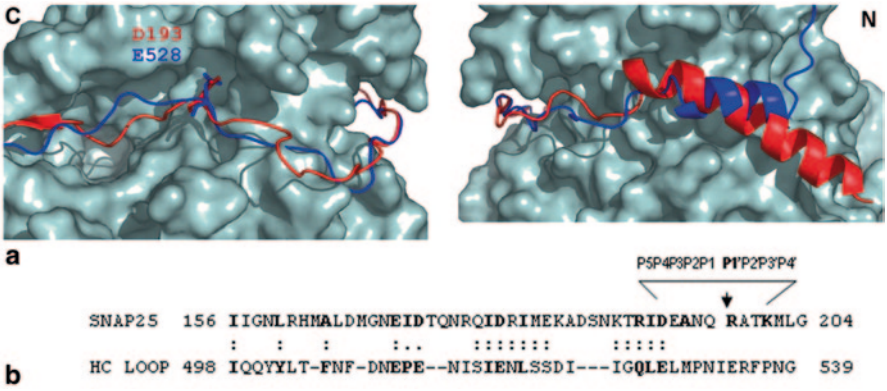

Fig. 8.1 Structural alignment of BoNT/A heavy chain loop with SNAP-25. **a** Structural alignment of the interactions of the HC loop of BoNT/A and SNAP-25 with LC/A. The *left panel* shows the alignment from the C terminus of the HC loop of BoNT/A and SNAP-25 on the face of LC/A that continues in the *right panel* along the back of LC/A toward the N termini. The *blue ribbon* represents the HC loop; the *red ribbon* represents SNAP-25. Asp193 of SNAP-25 and Glu528 of the HC loop interactions with LC/A S5 pocket ends the similarity between SNAP-25 and the HC loop of BoNT/A. **b** Sequence and spatial similarity of HC loop of BoNT/A and SNAP-25 interacting with LC/A. The spatial overlap of the HC loop of BoNT/A and residues of SNAP-25 that interact with LC/A is indicated by (:) or partial overlap (.), which was determined by manual alignment of the two structures. The *highlighted* residues in SNAP-25 contribute to the substrate cleavage. Note the structural overlap between the HC loop of BoNT/A and SNAP-25 ends at Asp193. This research was originally published in Journal of Biological Chemistry. Chen, S., J.J. Kim, and J.T. Barbieri, *Mechanism of substrate recognition by botulinum neurotoxin serotype A*. J Biol Chem, 2007. 282(13): p. 9621-7. © the American Society for Biochemistry and Molecular Biology

8.4 Cleavage of SNAP-25 by BoNT LC/A and LC/E

Comparative studies on the interaction of SNAP-25 with LC/A and LC/E provided an assessment of how LCs recognized and properly aligned the coiled substrate for efficient cleavage, since both LC/A and LC/E efficiently cleaved SNAP-25 but at different sites along the molecule. LC/A cleaves SNAP-25 between Gln197 and Arg198 while LC/E cleaves between Arg180 and Iso181 [13]. The K_m/k_{cat} of LC/A and LC/E for SNAP-25 were similar [43]. Thus, the primary difference between LC/A and LC/E was the site of cleavage. Early studies resolved residues 141–202 as the minimal region of SNAP-25 (206 residues) that can be efficiently cleaved by LC/A [44]. Subsequent studies showed that deletion of the SNARE motif (residues 145–154) within SNAP-25 had a fivefold lower rate for cleavage by LC/A, while SNAP-25(167–202) was efficiently cleaved by LC/E. Thus, LC/A had a longer SNAP-25 recognition region than LC/E. Saturation mutagenesis of SNAP-25 showed a differential effect of representative single amino acid mutations on the cleavage capacities of LC/A and LC/E [43]. Note, the nomenclature for the orientation of amino acids within the SNARE proteins that surround the scissile

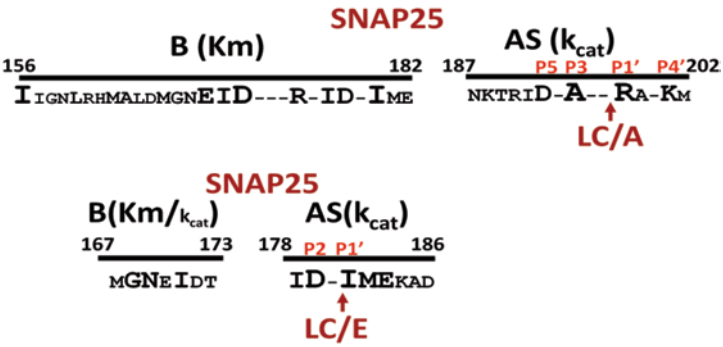

Fig. 8.2 Functional organization of BoNT LC/A and LC/E. Saturation mutagenesis and kinetic analysis of single point mutated derivatives of *LC/A* and *LC/E* identified two domains recognized by the LC. The binding (***B***) domain consisted of amino acid residues that when mutated reduced the affinity of the LC for SNAP-25. The active site (***AS***) mutations consisted of amino acid residues that when mutated reduced the catalytic rate for the cleavage of *SNAP-25*. Note the ***AS*** residues were organized in discontinuous (Pocket) alignment

bond (the P1–P1′ site) is designated with increasing integers as residues are distanced from the site of cleavage, such as P3, P2, P1–P1′, P2′, P3′.

For cleavage by LC/A, mutations within two regions of SNAP-25 showed the greatest inhibitory action, residues 193–202, termed the AS domain, and residues 156–181, termed the binding (B) domain (Fig. 8.2). Mutations in the B region affected the affinity of LC/A for SNAP-25, while mutations within the AS (Asp193 (P5), Ala195 (P3), Arg198 (P1′), and Lys201 (P4′)) were required for efficient cleavage by LC/A. The hydrophobic and charge properties of the AS residues implicate roles for both hydrophobic and ionic interactions for the optimal alignment of the scissile bond for cleavage, where the primary effect of mutations within the AS residues was to reduce the k_{cat} of scissile bond cleavage. LC/E showed a similar mechanism for SNAP-25 recognition, where mutation of amino acids N-terminal to the LC/E site of scissile bond cleavage influenced SNAP-25 cleavage with two residues, Iso178 (P3), Asp179 (P2), and Iso181 (P1′), within the AS, being required for efficient cleavage of SNAP-25 by LC/E [45]. Like LC/A, the primary effect of these mutations within the AS was to reduce the k_{cat} of scissile bond cleavage. Thus, a series of discontinuous residues upstream of the site of cleavage as well as amino acids immediately surrounding the cleavage site contribute to effective cleavage of SNAP-25. There was no detectable cleavage of SNAP-25(I181E) (P1′) by LC/E, whereas a more conserved mutation, SNAP-25(I181A), had a primary defect in k_{cat} LC/E cleaved SNAP-25(D179A) (P2) and SNAP-25(I178N) (P3) with a lower k_{cat} than wild-type SNAP-25, also implicating a role for the P2 and P3 sites in SNAP-25 recognition by LC/E. In contrast, SNAP-25(I171A), a B region residue, had an increased K_m, which indicated that residues within the B region contributed to SNAP-25 binding to LC/E. Thus, the AS domain residues contributed to catalysis while the B domain residues contributed to the affinity of SNAP-25 by LC/A and LC/E.

While both LC/A and LC/E recognize a B and AS region, which are organized in a similar N-terminal → C-terminal orientation, the complexity of the interactions between LC/A and SNAP-25 appear more complex than those of LC/E and SNAP-25. This may be reflected by recent observations that LC/A has a higher affinity for the cleavage product of SNAP-25, SNAP-25(1–197), than LC/E with the SNAP-25 cleavage product, SNAP-25(1–180). This differential affinity appears to contribute to the observed localization of intracellular LC/A which is found bound to the plasma membrane of neurons, while LC/E appear to localize more diffusely within neurons [46, 47].

With respect to SNAP-25 isotopes, LC/A does not cleave human SNAP-23 and cleaves mouse SNAP-23 poorly [40]. While AS recognition of LC/A is identical between human SNAP-25 and these isoforms, residues within the B domain of human SNAP-25 vary with respect to human SNAP-23 and mouse SNAP-23. The higher homology within the B domains of mouse SNAP-23 and human SNAP-25 than human SNAP-23 and SNAP-25 may be responsible for the relatively better cleavage of mouse SNAP-23 by LC/A than human SNAP-23.

The extended recognition of the B regions of SNAP-25 by LC/A and LC/E is physically and possibly functionally analogous to the association of the SNAP-25/syntaxin protein complex with VAMP during the fusion of neurotransmitter vesicles with the plasma membrane [43, 48]. In the case of v-SNARE and t-SNARE recognition, the N-termini of the proteins appear to initiate recognition and continue to associate by a "zipper"-like mechanism to contribute to the movement of the neurotransmitter vesicle towards the plasma membrane for fusion. Thus, LC/A and LC/E may utilize a similar mechanism to associate with the substrate SNAP-25.

8.5 Minimal Domain of SNAP-25 that is Cleaved by LC/A and LC/E

The AS interaction with LC/A and LC/E contributes to the catalytic capacity and substrate specificity of SNAP-25. Utilizing fusion protein analysis, LC/A was found to cleave 192DEANQRATK200, but not 194ANQRATK200, indicating that the P5–P4′ region of SNAP-25 defined a minimal substrate for LC/A. LC/E cleaved 178IDRIMEKAD186 more efficiently than 178IDRIME183, but cleavage of IDRIME was detected, making this peptide a minimal substrate domain for LC/E. Mass spectrometry analysis detected two peptide products that showed that LC/E cleaved the peptide NRQIDRIMEK at the scissile bond, between Arg180 and Ile181. Thus, interactions between the AS regions of LC/A and LC/E with SNAP-25 define the substrate specificity of the cleavage reaction. The smaller peptide that LC/E recognized for cleavage is consistent with LC/E possessing a smaller AS region than LC/A. These results indicate that while the LC/A and LC/E utilize similar mechanisms for substrate recognition, the simpler organization of the interaction between LC/E and SNAP-25 makes this an attractive LC to develop strategies to engineer mutated forms of the LC that display modified substrate specificity [49].

8.6 Pocket Model for Substrate Recognition by BoNT LCs

Utilizing the co-crystal of the non-catalytic LC/A bound to the truncated SNAP-25 [26], along with molecular modeling of the LC/A AS domain and saturation mutagenesis [50], the organization and recognition of SNAP-25 by LC/A was defined where the AS region defined substrate cleavage and a B region that was distanced from the AS defined high-affinity binding. The analysis of molecular models predicted a series of discontinuous interactions between the B region of SNAP-25 and LC/A that aligned the AS of LC/A to optimize scissile bond cleavage through a series of interactions that involve four pockets (S5, S3, S1′, S4′) that comprise the LC/A AS.

The multistep mechanism for recognition and cleavage of SNAP-25 by BoNT/A (Fig. 8.3) [51] appears to start with SNAP-25 binding along the "belt" region of BoNT/A, which allows alignment of the P5 residue to the S5 pocket at the periphery of the AS. The P5–S5 interactions subsequently orients the P4′ residue to form a salt bridge with the S4′ residue, which opens the AS allowing the P1′ residue access to the S1′ pocket. Subsequent hydrophobic interactions between the P3 residue of SNAP-25 and the S3 LC/A pocket residues optimize the alignment of the scissile bond for cleavage. The details of these interactions between LC/A and SNAP-25 follow.

- *Interactions That Align the P5 Residue of SNAP-25 to the LC/A AS:* The LC/A–SNAP-25 co-crystal revealed that LC/A residues Ile115, Lys41, Cys134, and Val129 directly interacted with residues within region B of SNAP-25, and subsequent studies showed that Ala mutations to Ile115, Lys41, Cys134, and Val129 had increased K_m for the cleavage of SNAP-25. The B region of SNAP-25 performs two functions: as the initial site of LC/A recognition and as the first AS interaction between the P5 residue of SNAP-25 and the S5 pocket of the LC/A AS. The analysis of the LC/A-SNAP-25 structure shows that Leu175, Thr176, and Arg177 of LC/A are organized as a pocket that surrounds Asp193, the P5 residue of SNAP-25, and that mutation of Leu175, Thr176, and Arg177 to Ala reduced LC/A catalytic activity with a greater effect on k_{cat} than K_m. Thus, the S5 pocket contributes to the proper alignment of the scissile bond for cleavage rather than contributing to substrate affinity. While P5 Asp193 is recognized by S5 pocket residues, electrostatic interactions between the basic S5 pocket and negatively charged Asp193 contribute to this interaction. The S5 pocket residues appear specific, since LC/A mutations at residues adjacent to the P5 residue of SNAP-25 did not affect catalytic activity. Thus, interactions between the S5 pocket residues of LC/A and the P5 Asp193 residue of SNAP-25 orient the next step in SNAP-25 recognition: the binding of the P4′ residue of SNAP-25 to the S4 pocket LC/A AS.
- *Recognition of the SNAP-25 P4′ residue (Lys201) by the LC/A S4′ residue (Glu257):* A loop within LC/A that has been termed the 250 Loop (residues 242–259) is the site of LC/A auto-cleavage [52] and subsequently was observed to pack next to LC/A loop370 (residues 359–370) when LC/A binds SNAP-25 [26].

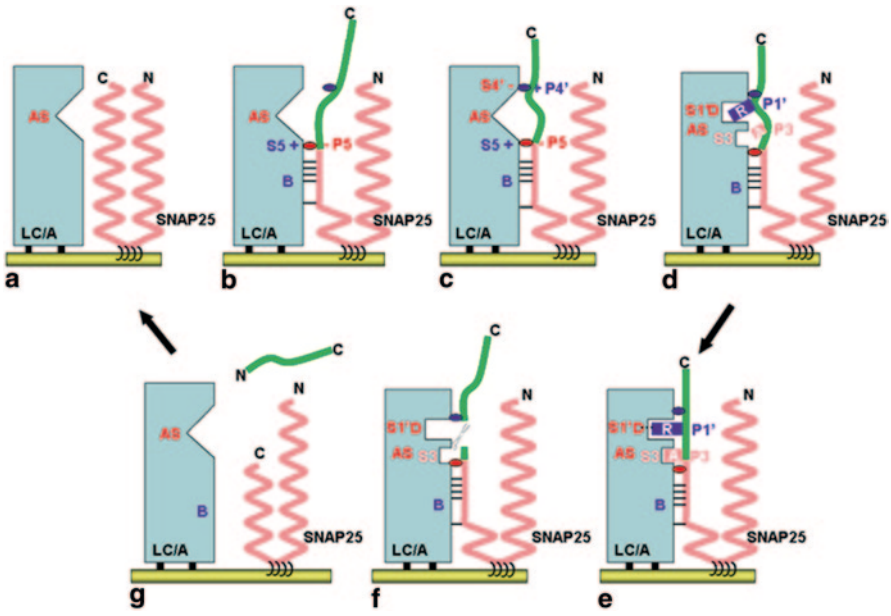

Fig. 8.3 Multistep "Pocket" model for recognition and cleavage of SNAP-25 by LC/A. At the plasma membrane LC/A initially binds to SNAP-25 through discontiguous surface interactions between residues within the belt region of LC/A and the B region residues of SNAP-25 (a). The zipper-like binding of SNAP-25 to LC/A (b) aligns the P5 residue Asp193 to form a salt bridge with Arg177, an S5 pocket residue, at the periphery of one side of the active site. This orients SNAP-25 for the formation of a salt bridge between the P4′-residue Lys201 and the S4′-residue LC/A(Asp257) (c). These interactions broaden the LC/A active site cavity and dock Arg198, the P1′-residue, via electrostatic interactions with Asp370 within the S1′-pocket (d). The fine-tuning of the alignment of Arg198 into the S1′-pocket is facilitated by the binding of SNAP-25-Ala195 to P3 residues in the hydrophobic S3 pocket of LC/A. The proper alignment of the P1′-P3 sites into the Zn2 + active motif (e) facilitates the substrate cleavage (f). After the cleavage, the C-terminal product dissociates from LC/A, which returns the AS to the original conformation (g). This research was originally published in Journal of Biological Chemistry. Chen, S., J.J. Kim, and J.T. Barbieri, Mechanism of substrate recognition by botulinum neurotoxin serotype A. J Biol Chem, 2007. 282(13): p. 9621–7. © the American Society for Biochemistry and Molecular Biology

Whereas earlier studies proposed that the loop250 residue Met202 represented the primary contact of SNAP-25 to LC/A, mutations of SNAP-25-Met202 have a limited effect on catalysis, but mutations at SNAP-25-Lys201 yielded poor substrates for cleavage by LC/A [53]. Examination of the LC/A–SNAP-25 structure showed a potential salt bridge between SNAP-25-Lys201 and LC/A-Glu257 [26]. Supporting a role of Glu257 in SNAP-25 recognition/cleavage was the determination that mutations at Glu257 of LC/A had a lower k_{cat} for SNAP-25 cleavage than WT-LC/A. Thus, in this model, the sequential binding of LC/A to the P5 and P4′ sites of SNAP-25 aligns the LC to the substrate such that the P1′ residue of SNAP-25, Arg198, can interact with the S1′ pocket of LC/A. Importantly, the co-crystal of LC/A bound to SNAP-25 shows a conformational change

in the AS region of LC/A upon SNAP-25 binding that could be mediated by the P5–S5 and P4–S4′ interactions between SNAP-25 and LC/A [26].

- *Recognition of the P1′-Site Residue of SNAP-25 by S1′ Pocket Residues of LC/A:* The LC/A residue Asp370 forms a salt bridge with the inhibitor l-ArgHX [54], where the LC/A–I-ArgHX complex shows an additional LC/A residue (Phe163) contact with the inhibitor and several residues, Phe194 and Thr220, that also align near the inhibitor. Since LC/A-Asp370Ala shows reduced cleavage of SNAP-25 and LC/A-Phe163Ala, LC/A-Phe194Ala, and LC/A-Thr220Ala each have reduced catalytic efficiency for the cleavage of SNAP-25, in addition to a salt bridge of the guanidinium group of Arg198 with the Asp370, there appears to be hydrophobic interactions between the aliphatic portion of the side chain of Arg198 and the hydrophobic S1′ pocket residues. Of note, the LC/A–l-ArgHX complex formed a bidentate ligand with the zinc ion and the guanidinium group of Arg370, suggesting that the inhibitor-LC/A structure mimicked a catalytic intermediate for the P1′ residue, Arg198, in the AS [55].
- Mutagenesis studies are consistent with the predicted role of the P1′ residue in substrate recognition by BoNT LCs. Within each BoNT serotype LC, zinc is coordinated by the HEXXH motif and a conserved Glu that lies downstream of the HEXXH motif. However, conservation of amino acids within the AS domain of each BoNT serotype LC is not extended to the S1′ pocket. This is consistent with the diverse chemical nature of P1′ residues as substrates of various BoNT serotypes, where the S1′ pocket residues within a specific LC correlate in size and hydrophobicity with the cognate P1′ residue within the SNARE protein substrate. For example, in LC/A, the four residues that form the S1′ pocket play different roles in substrate recognition, where Asp370 forms a salt bridge with the guanidinium group of Arg198, while Phe194, Phe163, and Thr220 have hydrophobic interactions with the aliphatic chain of the Arg198 side chain.
- *Alignment of the P3-residue Ala193 of SNAP-25 with the S3 Pocket residues of LC/A:* The mutation of the P3 residue, SNAP-25(Ala195Ser), yields a reduction in catalysis relative to the cleavage of SNAP-25 by LC/A. The co-crystal structure of LC/A with SNAP-25 explains this phenotype since the methyl side chain of the SNAP-25 P3 residue, Ala195, fits into the S3 pocket of LC/A without a space to accommodate the –OH of Ser [26]. Thus, spatial constraints on S3 pocket main chain interactions predicts that there are no conserved substitutions that will maintain the cavity of the S3 pocket. The S3 pocket residues of LC/A provide an optimal orientation for interactions of the P1′ residue of SNAP-25 and the S1 pocket, which results in a precise alignment of the scissile bond in the AS for cleavage.

These data establish a multistep “pocket” model for SNAP-25 cleavage by LC/A (Fig. 8.3). In this model, there are initial interactions between the “belt” region of LC/A and the B region of SNAP-25 (a/b). The “zipper”-like binding of SNAP-25 to LC/A aligns the P5-residue Asp193 to form a salt bridge with the S5 pocket residue Arg177 of LC/A at the periphery of the AS. These interactions orient SNAP-25 to form a salt bridge between the P4′-residue Lys201 and the S4′-residue Asp257 of LC/A (c), which broadens the LC/A AS cavity and allows the P1′ residue to dock

in the AS with the S1′ pocket residues of LC/A via electrostatic and hydrophobic interactions (d). The P3 residue, Ala195, via hydrophobic S3 pocket interactions (e), allows optimal docking at the "AS" to initiate SNAP-25 cleavage (f). After cleavage, the P4′ residue of SNAP-25 dissociates from the S4′ residue of LC/A (g), which returns the "AS" to an unbound conformation, facilitating dissociation of the P1′ residue from the "AS".

8.7 LC/B and LC/TeNT Utilize Similar, but Unique Strategies to Cleave VAMP-2

One of the remarkable observations in the biology of neurotoxin pathology is the determination that while BoNT/B and TeNT yield unique pathologies, the two LCs cleave the same site (Q76/F77) within VAMP-2 to elicit spasticity. Therefore, the basis for how these LC recognize their substrate provides a unique opportunity to continue to elucidate the basis for substrate recognition at a short evolutionary distance [20]. LC/B demonstrated a slightly different mechanism of recognition and catalysis where amino acids N-terminal to the cleavage site (Q76/F77) affected binding and catalysis when mutated. TeNT, which has ~40% primary amino acid homology with BoNT/B, showed similar substrate recognition, but included an extension of the B domain upstream of the cleavage site. These amino acid interactions were involved in binding and catalysis as a mechanism for substrate recognition and they explain the specificity of neurotoxin substrate recognition and cleavage site specificity among BoNT serotypes and TeNT despite a highly identical tertiary structure.

8.8 BoNT Subtypes

BoNT serotypes include subtypes that can vary between 3 and 32% at the primary amino acid level [56] (see Chap. 10 of this book). BoNT/A includes five subtypes (A1–A5). Earlier studies showed that BoNT/A1 and BoNT/A2 possessed ~95% primary amino acid homology and had similar capacity to cleave SNAP-25 [57]. BoNT/A5 also has high sequence homology with BoNT/A1 [58, 59]. DNA sequence analyses revealed two additional A subtypes: BoNT/A3 and BoNT/A4 [6, 57]. Clostridia producing BoNT/A3 were isolated from an outbreak of botulism in Scotland in 1922, while clostridia producing BoNT/A4 were isolated from an infant with botulism in 1988 (reviewed in [60]). At the primary amino acid level, BoNT/A3 shows ~80% identity to BoNT/A1, while BoNT/A4 shows ~90% identity to BoNT/A1 [57]. A phylogenetic analysis of BoNT/A1–A4 showed that LC/A3 and LC/A4 are potentially different from LC/A1 [6, 57], while the BoNT/A subtypes possess common and unique catalytic properties. For example, LC/A2 and LC/A3 have similar kinetic properties as LC/A1, but LC/A4 shows an ~80-fold decrease in

the capacity to cleave SNAP-25 [27]. This effect on catalysis appears to be due to a single amino acid substitution that causes an indirect loss of zinc coordination at the LC/A4 AS. A point mutation to LC/A4 that restores the LC/A1 sequence restores catalytic activity to LC/A1 levels [27]. While LC/A1 and LC/A2 show autoproteolysis, LC/A3 and LC/A4 did not demonstrate this activity and, unexpectedly, each of the LC/A subtypes shows a unique capacity to cleave a synthetic SNAP-25 peptide, SNAPtide™. This indicates an intrinsic difference in cleavage activity between the subtypes [27]. Thus, while LC/A subtypes retain the capacity to cleave SNAP-25, the efficiency and mechanisms of the cleavage reactions differ. These unique properties may influence the ability to generate small-molecule serotype-specific inhibitors of LC catalysis and challenges the ability to establish common steps in catalysis that can be targeted for inactivation. Continued characterization of the BoNTs serotypes may provide useful information to develop strategies to generate vaccines and therapies against botulism and to develop novel BoNT derivatives that can extend the therapeutic utility.

8.9 Intracellular Trafficking of BoNT LCs

In addition to substrate recognition to facilitate neuronal intoxication, BoNTs bind neurons via host receptors that are specific to neurons. The BoNT/A–receptor complex is endocytosed upon the cycling of synaptic vesicle proteins from the plasma membrane and as the synaptic vesicle acidifies the translocation domain of the HC inserts into the synaptic vesicle membrane and facilitates LC translocation into the cytosol. While earlier studies recognized the intracellular localization of LC/A to the host plasma membrane [61, 62], recently a new interaction between LC/A and SNAP-25 was described, which facilitates the high-affinity binding of LC/A to SNAP-25 on the plasma membrane of neurons [46]. Intracellular LC/A directly binds SNAP-25 on the plasma membrane via the N-terminal residues of LC/A with residues 80–110 of SNAP-25. These interactions were observed in vitro and in cultured neurons. The association of the N-terminus of LC/A and SNAP-25 also enhanced substrate cleavage. A recent finding that an N-terminal deletion to BoNT/A has reduced toxicity supports a role of the N-terminus in substrate recognition [63].

These findings explain how LC/A associates with SNAP-25 on the plasma membrane and provides a basis for LC/A cleavage of SNAP-25 within the SNARE complex (Fig. 8.4) [64]. In neurons, SNAP-25 is associated with syntaxin 1a forming a pre-SNARE complex for VAMP-2 recruitment [65, 66], and the C-terminus of SNAP-25 may not be accessible to LC/A since two regions of SNAP-25, residues 1–87 and 131–206, form a double helix complex with syntaxin 1a [67]. Thus, interactions of LC/A with the non-SNARE complex region of SNAP-25, residues 80–110, may represent an initial access of LC/A to SNAP-25 and allow LC/A to compete with syntaxin 1a for SNAP-25 binding. The finding that LC/A localizes to the plasma membrane through direct interactions with SNAP-25 also provides

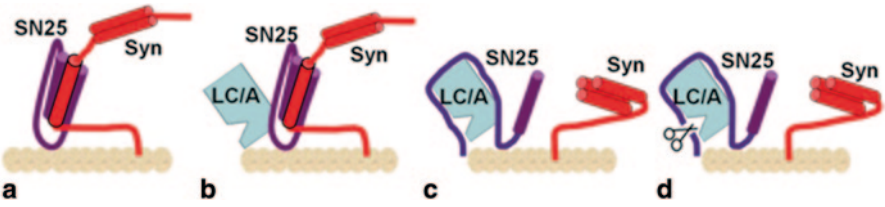

Fig. 8.4 High affinity binding of LC/A to SNAP-25 Schematic of LC/A interactions with membrane bound SNAP-25. **a** At the cell plasma membrane, syntaxin 1a (*Syn*) and SNAP-25 (*SN25*) form a SNARE complex. **b** The N terminus of *LC/A* binds residues 80–110 of SNAP-25, which **c** facilitates substrate binding in competition with syntaxin 1a. **d** After SNAP-25 cleavage, *LC/A* has a high affinity for membrane bound SNAP-25(1–197). "This research was originally published in the Journal of Biological Chemistry. Chen, S. and J.T. Barbieri, Association of Botulinum Neurotoxin Serotype A Light Chain with Plasma Membrane-bound SNAP-25. J Biol Chem, 2011. 286(17): p. 15067–72. © the American Society for Biochemistry and Molecular Biology"

a basis for the potency of BoNT/A within neurons. Intracellular targeting to the plasma membrane does not influence LC half-life within a neuron, consistent with the recent determination that recognition by the proteasome contributes to LC stability within neurons ([68] and Chap. 9 of this book).

8.10 Small-Molecule Inhibitors of BoNT LC Action

As described earlier, LC/A cleaves SNAP-25 between residues Gln197 and Arg198, but unlike thermolysin, LC/A recognizes an extended region of SNAP-25 for cleavage. The structure of LC/A(1–425) treated with ethylenediaminetetraacetic acid (EDTA) was determined to be similar to that reported for the LC/A domain within the holotoxin, indicating that the role of Zn is catalytic, not structural [54]. The structures of a non-catalytic LC/A complexed with and without an inhibitor, I-ArgHX, were also determined to be identical, showing that AS conformation was not perturbed by inhibitor occupancy. When the inhibitor bound to the AS, the carbonyl and N-hydroxyl groups formed a bidentate ligand to the Zn ion and the arginine moiety bound to Asp370, suggesting that the inhibitor-bound structure mimicked a catalytic intermediate with Arg at the P1′ site. These results provided new information on the reaction mechanism and insight into the development of strategies for small-molecule inhibitors of BoNTs that have been developed by Stevens and coworkers [69]. Recently, several lead molecules have been identified for inhibitors of LC cleavage that utilize different approaches to identify high-affinity small molecules that inhibit LC/A catalysis.

One approach identified a new class of BoNT/A protease inhibitors, chicoric acid, from a natural product. Chicoric acid's mechanism of inhibition was through interaction with an exosite of the LC, which showed noncompetitive inhibition.

Interestingly, this inhibitor was competitive when used in combination with an AS inhibitor of LC/A catalysis [70]. This shows the advantage of utilizing diverse environments as sources of protein inhibitors. Another approach combined in silico screening and biochemical assays to identify small-molecule inhibitors of BoNT/A. In this study, in silico screening was performed by the computational docking of compounds from the National Cancer Institute database into LC/A. Lead compounds were evaluated in an in vitro cleavage assay to identify functional inhibitors of LC/A protease activity. The best candidate was (7-((4-nitro-anilino)(phenyl) methyl)-8-quinolinol (NSC 1010)). Analogs of NSC 1010 were synthesized and several NSC 1010 derivatives were screened in a neuroblastoma cell-based and tissue-based mouse phrenic nerve hemidiaphragm assay where one compound, CB 7969312, showed protection at 0.5 M. This study demonstrates the utility of a multidisciplinary approach to identify small-molecule LC/A inhibitors [71]. Brunger and coworkers [72] recently designed peptide-like inhibitor molecules, using information derived from the co-crystal structure of LC/A–SNAP-25. The peptide-like molecules were competitive inhibitors of LC/A catalysis and inhibited substrate cleavage with nM K(i)s. Structural studies showed that one inhibitor had ionic interactions with the P1 residue of SNAP-25 and hydrophobic contacts with the aromatic ring of the P2′ residue of SNAP-25. These studies show the utility of iterative structure-based design to develop LC/A inhibitors. Using a co-crystal of LC/A in complex with inhibitory substrate analog tetrapeptides including ArgArgGlyCys, ArgArgGlyLeu, ArgArgGlyIle, and ArgArgGlyMet interactions between substrate and LC/A defined residues that contribute to substrate stabilization and catalysis. This study showed that the hydrolytic water was replaced by the amino group of the N-terminal residue of the tetrapeptide and identified interactions at the S1′ site to generate a tetrapeptide with an nM K(i). These are several approaches that show the progress that has been made in elucidating strategies to identify small-molecule inhibitors of LC catalysis.

8.11 Development of Strategies to Modify LC Substrate Specificity to Extend LC Therapeutic Applications

The reversible nature of muscle function after BoNT intoxication that replaces toxin-affected nerves with new nerves has turned the BoNTs from deadly agents to therapies for neuromuscular conditions. As early as 1989, BoNT/A was approved by the Food and Drug Administration (FDA) to treat strabismus, blepharospasm, and hemifacial spasm and then for cervical dystonia, cosmetic use, glabellar facial lines and axillary hyperhidrosis [7, 60]. BoNT/A efficacy in these disorders correlated to modulation of involuntary skeletal muscle activity and, coupled with an excellent safety profile, prompted off-label use of BoNT in disorders related to secretions, pain and cosmetic origin [73–75].

The clinical use of BoNTs is limited to targeting afflictions affecting neuromuscular and autonomic cholinergic activity [7]. Upon defining the structure–function

relationship of BoNTs with their SNARE protein substrates, attempts followed to design therapies that retarget BoNT to unique neuronal and non-neuronal cells. One productive approach has been to replace the BoNT HCR domain with the nerve growth factor or with lectin from *Erythrina crista-galli* to retarget BoNT/A to neurons or non-neuronal cells such as nociceptive afferents and airway epithelium cells [76, 77]. However, the selective cleavage of neuronal SNARE proteins by BoNT has limited development of therapies in these non-neuronal systems. A prerequisite to develop these therapies is the requirement to retarget the catalytic LC domain to non-neuronal SNARE isoforms.

Understanding SNARE protein specificity of BoNTs enabled the engineering of a LC derivative of BoNT/E possessing extended substrate specificity. LC/E recognizes the B region and AS region of SNAP-25 [43], including the P3 residue of SNAP-25 that facilitates alignment of the P2 and P1′ residues of SNAP-25 into the LC/E AS. The S1′ pocket of LC/E is formed by Phe191, The159, and The208 with hydrophobic interactions between Phe191 of LC/E and the P1′-residue Iso181 of SNAP-25. The basic S2 pocket contains Lys224, which recognizes the P2 residue, Asp179, through a predicted salt bridge. Docking the P2 and P1′ residues of SNAP-25 into the AS pockets of LC/E aligns the scissile bond for cleavage [43, 78].

SNAP-23 is a non-neuronal SNARE protein involved in cell secretion, including mucus, gastric acid and antibody secretion [79]. Cleavage of SNAP-23 by a substrate-modified BoNT may reduce the secretion of hypersecretion syndromes. A SNAP-23-targeting BoNT may also have therapeutic applications that include diabetes and inflammation, which include a hypersecretory component [80, 81]. The overall homology between SNAP-23 and SNAP-25 is high, except that the P2, P2′, and P3′ residues are unique. One difference is at the P2 residue where SNAP-25 is an Asp and SNAP-23 is a Lys. The inability of LC/E to cleave SNAP-23 may be due to electrostatic repulsion between the P2 Lys within SNAP-23 and Lys224 of LC/E, which could indirectly affect the alignment of P1′ Iso with the S1′ pocket of LC/E. This possibility prompted an analysis of the role of Lys224 of LC/E in the cleavage of SNAP-23.

LC/E(Lys224Asp) was engineered and found to cleave human SNAP-23 with an ~twofold higher K_m and ~fivefold lower k_{cat} than LC/E for the cleavage of human SNAP-25 [49]. LC/E(Lys224Asp) cleaved SNAP-23 between Arg186 and Iso187. LC/E(Lys224Asp) cleaved SNAP-23, and the natural substrate, SNAP-25, but did not cleave SNAP-29 or SNAP-47. Ectopic-expressed or protein-delivered LC/E (lys224Asp) into cultured human epithelial cells cleaved endogenous SNAP-23 and inhibited secretion of mucin and interleukin 8 (IL-8). These studies showed the feasibility of genetically modifying LCs to target a non-neuronal SNARE protein that may extend therapeutic potential for treatment of human hypersecretion diseases. SNARE proteins participate in membrane fusion and vesicle trafficking within neuronal and non-neuronal secretory pathways [82]. The use of BoNT has contributed to the understanding of vesicle fusion and neurotransmitter release mechanisms in neuronal cells. The ability of a BoNT derivative to cleave non-neuronal SNARE proteins may provide useful tools to investigate intracellular vesicular trafficking and the mechanism of membrane fusion in non-neuronal systems. The successful

delivery of LC/E(Lys224Asp) into non-neuronal cells to inhibit IL-8 and mucin secretion shows the potential for therapy to regulate human hypersecretion diseases such as asthma and inflammatory diseases. The therapeutic specificity of LC/E(Lys224Asp) and similar LC derivatives would be based upon the receptor-binding component, as described for bacterial toxin chimeras, such as diphtheria toxin A fragment-IL2 [83] and Exotoxin A fragment-IgG-targeting proteins [84]. Future approaches will seek to extend the utility of LC/A that possesses extended substrate specificity for other SNARE proteins beyond SNAP-25 or to address the issue of immunoresistance of BoNT therapy.

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Chapter 9
Molecular Basis for Persistence of Botulinum Neurotoxin: The Role of Intracellular Protein Degradation Pathways

Yien Che Tsai, Brian E. Moller, Michael Adler and George A. Oyler

Abstract A key aspect of botulinum neurotoxin biology, which underpins both the nature of botulism and the clinical success of therapeutic neurotoxin preparations, is the duration of effect of the neurotoxin on neurotransmitter release. There are seven different distinct serotypes of botulinum neurotoxins which exhibit a wide range in the duration of action or "persistence" after intoxications. The biological basis of persistence is beginning to be understood. One mechanism which underpins the duration of neurotoxin activity is survival of the light chain within the presynaptic terminal of the intoxicated neuron. For the neurotoxin light chain to remain in the presynaptic terminal maintaining the intoxication state, the bacterial protein must evade the two major pathways for cellular protein degradation, the ubiquitin–proteasome system degradation and the lysosomal/autophagy mechanism. A role for substrate cleavage products in persistence has also been suggested in perpetuating the intoxication state. These various ideas and the evidence for and against them are reviewed. The opportunity to modify the persistence of the neurotoxin and its therapeutic potential is also considered.

Keywords Bioterrorism · Botulinum neurotoxin · Ubiquitination · Deubiquitination · Persistence

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9.1 Introduction

The botulinum neurotoxins (BoNTs) represent several noteworthy features of evolutionary adaption. BoNTs, along with their toxic cousin tetanus neurotoxin (TeNT), are the most lethal substances known to man. BoNTs possess a remarkable degree of thermal or environmental stability, allowing the toxin to remain active after months, perhaps years, of exposure to unfavorable temperatures and environmental conditions. As part of their intoxication life cycle, BoNTs are able to avoid denaturation and proteolysis in the presence of stomach acidity and digestive enzymes and transit through the intestinal walls into the bloodstream structurally intact ([19], Chap. 3, this volume). Once in the bloodstream, the heavy chain (HC) portion of the holotoxin molecule targets BoNT to cholinergic terminals of motorneurons, preganglionic autonomic fibers and postganglionic parasympathetic nerves [48]. BoNT binds to a dual protein–ganglioside receptor on the extracellular surface and rides the endocytotic machinery into the endosomal compartment [10, 11].

Upon acidification of the endosome, the N-terminal half of the HC (translocation domain) creates a pore in the endosomal membrane to convey the partially unfolded light chain (LC) through the membrane and into the cytosol [16]. Once in the cytosol, the LC targets a single soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein (except for BoNT/C1, which targets two SNARE proteins) and cleaves the target at a single specific peptide bond. The SNARE protein cleavage results in blockade of Ca^{2+}-dependent release of neurotransmitter, culminating in flaccid paralysis and autonomic dysfunction. For BoNT/a, /B and /C1, paralysis can last for months [2, 26, 38, 54].

This latter feature of BoNT, that we define here as *persistence*, is the subject of this chapter. Persistence must be distinguished from *stability*, which is the ability of BoNTs to remain toxic while enduring extremes in their environment before entering the host organism. While persistence is desirable for providing long-term relief of muscle hyperactivity [23], it is a major problem for the management of BoNT intoxication [50]. In addition to their extremely high potency, the extraordinary persistence of BoNT/A, /B and /C1 makes treatment of intoxication by these serotypes especially challenging, since procedures such as nasogastric tube feeding and assisted ventilation are required for weeks to months until the natural processes of recovery can occur.

Currently, there are no approved pharmaceutical treatments for reversal of BoNT intoxication (see Chap. 13, this volume), and the physiological and cellular mechanisms mediating persistence are not well understood. Therefore, a significant effort will be needed to elucidate the mechanisms underlying LC persistence and to develop effective medical countermeasures for the treatment of BoNT intoxication.

9.2 Dual Identity of BoNT

The BoNTs comprise a family of seven distinct neurotoxic proteins (A–G) produced by immunologically discrete strains of anaerobic bacteria, chiefly *Clostridium botulinum* [44, 48]. These spore-forming, gram-positive bacteria secrete the most

lethal toxins known to mankind with an estimated human median lethal dose (LD50) of 1–3 ng/kg [3, 49]. Due to their extraordinary high potency, ease of production and previous history of weaponization, the BoNTs have been designated as Tier 1 select agents by the US Department of Health and Human Services. Intoxication by BoNT leads to inhibition of acetylcholine (ACh) release at all peripheral cholinergic synapses, culminating in bilateral flaccid paralysis of skeletal muscle and in impaired function of tissues innervated by postganglionic parasympathetic fibers [20, 44, 9].

The specific target for BoNT/A and /E is the 206-amino-acid, 25-kDa vesicle-docking protein, synaptosomal-associated protein of 25 kDa (SNAP-25); BoNT/A cleaves the last nine residues to generate SNAP-25 Δ9, whereas BoNT/E cleaves 26 residues from the C-terminus of this protein giving rise to SNAP25 Δ26 [5, 42, 52]. The target of BoNT/B is the transmembrane protein synaptobrevin/vesicle-associated membrane protein (VAMP), located on the surface of small synaptic vesicles. The enzymatically active portion of the 150-kDa BoNT is the 50-kDa LC; the role of the 100-kDa HC resides in binding to cholinergic nerve endings and in delivering the LC into the cytosol [9, 46, 48].

The duration of muscle paralysis following intoxication by BoNT/A exceeds that resulting from exposure to other BoNT serotypes [2, 12, 35, 38]. The remarkable persistence of BoNT/A action has led to its widespread use for treatment of conditions stemming from imbalance in cholinergic tone such as dystonia, hyperhidrosis, overactive bladder, and wound healing ([6, 23, 40, 43]; Chaps. 3–6 of the companion volume to this book, KA Foster (ed.) *Clinical Applications of Botulinum Neurotoxin*, Springer, New York). Although a long duration is desirable in clinical use, the prolonged action of BoNT/A also makes intoxication by this serotype difficult to treat, particularly if it were employed as an agent of bioterrorism [3, 18]. The duration of intoxication by BoNT/E is relatively brief (several weeks), whereas BoNT/B is of intermediate duration [2, 17, 27, 38, 54, 55].

9.3 Models of Botulinum Neurotoxin Persistence

We will briefly describe three models of persistence in this section, although it should be noted that the models are not mutually exclusive, and each likely makes a contribution to the duration of BoNT paralysis in vivo.

9.3.1 Hidden Physiological Reservoir Model

This hypothesis of BoNT persistence stems from the idea that the toxin, once it enters the intestinal mucosa or the systemic circulation, evades circulating antibodies by "hiding" in some unknown physiological compartment isolated from the immune system [30]. BoNT would be released from this compartment into circulation over the course of intoxication. Evidence in favor of this hypothesis is the observation that even several days after exposure, when there is little or no *measurable* level

of circulating BoNT, antibody treatment is still capable of reducing the severity of intoxication [37]. However, 1–2 weeks after intoxication, this reservoir is likely to be depleted, since antibody administered after this time period does not lead to improvement of symptoms [30].

The persistence of BoNT/A action extends well beyond the time when measureable levels of the toxins would remain in circulation, however, and experiments with primary spinal cord cells indicate that BoNT/A is able to cleave SNAP-25 for months after a single exposure [27]. Thus, it is unlikely that the *hidden physiological reservoir model* can account for more than a small fraction of BoNT/A persistence. Delineation of the cellular mechanisms mediating the intracellular persistence of BoNT/A is critical to the development of treatment paradigms for enhancing the rate of intracellular degradation of BoNT/A LC.

9.3.2 Persistent Cleavage Product Model

The seven BoNTs collectively target just three SNARE proteins: SNAP-25 by BoNT/A, /C1 and /E; syntaxin by BoNT/C1; and synaptobrevin/VAMP by BoNT/B, /D, /F and /G. However, exposure to these serotypes results in markedly different durations of paralysis. The duration is dramatically different even for BoNT serotypes cleaving a common substrate [17, 27, 55]. Particular attention has been paid to BoNT/A and /E, both of which cleave SNAP-25 but diverge markedly in their duration of action. It was noted by [32] that 4-aminopyridine, a K^+ channel blocker, could relieve symptoms of paralysis in rat *extensor digitorum longus* (EDL) muscles injected with BoNT/A as well as from intraperitoneal injections of lethal doses of BoNT/A. However, additional studies demonstrated that both 4-aminopyridine and 3,4-diaminopyridine were ineffective in reversing the actions of BoNT/B, /E, /F and TeNT [1, 45, 47]. It was later revealed that despite sharing a common substrate, BoNT/A and /E cleaved SNAP-25 at distinct sites [41], and that muscle paralysis induced by BoNT/A, but not by BoNT/E, resulted in preterminal sprouting leading to transient formation of new synaptic contacts [8, 33, 38]. These findings led to the hypothesis that the differences in susceptibility to aminopyridines and in the duration of paralysis might arise from different abilities of BoNT/A-generated SNAP-25 Δ9 and BoNT/E-generated SNAP-25 Δ26 to participate in the formation of the SNARE complex [12].

In support of this hypothesis, co-injection of BoNT/A and /E in human volunteers resulted in a shorter duration of local muscle paralysis, similar to muscles injected with BoNT/E alone [12]. In contrast, sequential injection of BoNT/A and /E in rat EDL muscle in either order [2] or co-injection of the two serotypes (Adler et al. 2010) culminated in long-lasting paralysis. The observation that the order of injection had no significant effect on recovery of muscle tension is not consistent with the hypothesis that divergent effects of the SNAP-25 proteolytic fragments mediate persistence of the toxins. In a detailed study by [34], mouse EDL muscles were injected with BoNT/A, /E or /F, and the duration of paralysis was correlated with the formation of nerve sprouts and the presence of truncated SNARE proteins in nerve terminals.

Single injections of standardized effective doses (EDs) of BoNT/A, /E or /F led to paralysis times of 28, 5 and 7–8 days, respectively. Co-injection of 0.5 ED of BoNT/A with 0.5 ED of BoNT/E resulted in a paralysis time of 12.5 days, significantly shorter than paralytic times observed after injection of BoNT/A alone. Sequential injections of 0.5 ED of BoNT/A followed 3 days later by 0.63 ED, 0.44 ED or 0.31 ED of BoNT/E led to paralysis times of 15, 17, and 28 days. The data were used to support the hypothesis that BoNT/E converted the presumably long-lasting SNAP-25 Δ9 to the less stable SNAP-25 Δ26, resulting in shorter paralytic times.

A problem with this conclusion is that the paralysis times of the 0.5-ED BoNT/A + 0.5-ED BoNT/E co-injection and sequential injections were compared to a 1-ED injection of BoNT/A. The appropriate control would be a single 0.5 ED of BoNT/A, but these data were not provided. Relative to a 1-ED dose, a 0.5-ED injection of BoNT/A could lead to a shorter paralysis time regardless of the cellular mechanism of persistence.

The study by [34] also demonstrated that in muscles injected sequentially with 0.5 ED BoNT/A followed 3 days later by 0.5 ED BoNT/E, the SNAP-25 Δ9 fragment appears to peak at 11 days whereas the SNAP-25 Δ26 peaks at 5 days. If BoNT/E-mediated cleavage of the SNAP-25 Δ9 fragment were leading to its more rapid clearance, then the peak time of SNAP-25 Δ9 should be the same as that for SNAP-25 Δ26. The most likely mechanism for a continuation of the appearance of additional SNAP-25 Δ9 is, in fact, that BoNT/A-mediated proteolytic activity persists in the intoxicated neuron after BoNT/E activity has ceased.

9.4 Persistent Light Chain Protease Model

An alternative to the *persistent cleavage product model* is one which assumes that differences in the duration of paralysis and in functional exocytosis result directly from differences in the duration of action of the BoNT LCs within the presynaptic terminal. According to this model, the persistent BoNT serotypes (BoNT/A, /B and /C1) would be resistant to intracellular protein inactivation and degradation mechanisms, allowing them to continue cleaving their respective substrates over an extended time course of weeks to months. The short-duration serotypes (BoNT/D, /E, /F and /G) would be inactivated in hours to days by these same intracellular pathways. BoNT persistence would therefore arise from differences in the individual LCs and their interactions with host cell proteins, independent of the respective cleavage products or any downstream effects of the cleavage products on transmitter release.

Several lines of evidence suggest that the intracellular half-lives of the different BoNT LCs determine the duration of intoxication [17, 27]. In cultured embryonic mouse spinal cord neurons, SNAP-25 Δ9 was detected for greater than 11 weeks after a 24-h toxin exposure, whereas BoNT/E-truncated SNAP-25 was present for less than 3 weeks [27]. Incubating BoNT/A-intoxicated cells with BoNT/E 25 or 60 days after BoNT/A did not increase the rate of recovery of full-length SNAP-25, suggest-

ing that the continued presence of SNAP-25 Δ9 for more than 11 weeks following exposure to BoNT/A was the result of continued proteolytic activity of BoNT/A LC. Parallel experiments performed in rat EDL muscles injected with BoNT/A, BoNT/E or sequentially with BoNT/A then BoNT/E clearly demonstrated a significantly longer duration of action of BoNT/A compared to BoNT/E [2]. Furthermore, sequential treatment with BoNT/E after BoNT/A did not increase the recovery rate of BoNT/A-injected muscles. This provides further support for the hypothesis that the long duration of BoNT/A toxicity is associated with the persistence of active BoNT/A LC within cells. Finally, pulse-chase experiments in cerebellar granule neurons indicated that the half-time for removal of SNAP-25 Δ26, SNAP-25 Δ9 or SNAP-25 Δ8 (product of BoNT/C1 cleavage) was < 1 day and did not differ significantly among serotypes [17]. Therefore, replacement of truncated SNAP-25 cannot be rate limiting for determining the duration of BoNT intoxication.

Direct evidence for the persistent light chain model is currently unavailable. It has not been possible to detect the presence of BoNT LCs in neurons under realistic exposure conditions due to the exceedingly small copy numbers of LC molecules that are required for intoxication. This is especially the case for motoneuron terminals, where even the presence of truncated SNAP-25 is difficult to demonstrate in the absence of extremely high levels of BoNT [24, 25]. Consequently, determination of the continued presence of active intraneuronal BoNT LCs must be inferred from the persistence of cleaved SNARE proteins.

9.5 Mechanisms of Cellular Persistence of BoNT LC

There are several possible mechanisms to explain divergent degrees of intracellular persistence among BoNT LCs, none of which is necessarily exclusive of the others. The three general classes for long-duration BoNT LCs are summarized as follows:

Class I: Distinct BoNT LC domains facilitate interaction with protective cellular proteins or, alternatively, facilitate blockade of interaction with degradative cellular proteins. In either case, the LC evades cellular protein degradation pathways.

Class II: Distinct LC domains target BoNT/A LC for sequestration in a subcellular compartment or location. This subcellular compartment is shielded from protein degradation pathway surveillance or, alternatively, retains the LC within the terminal region, close to its substrate, and prevents inactivation arising from diffusional dilution.

Class III: The BoNT/A LC is active at vanishingly small copy numbers within cells and maintains paralytic activity at numbers too low for efficient degradative pathway targeting. The long duration of BoNT action may reflect the inability of the protein degradation pathways to remove all copies of the LC.

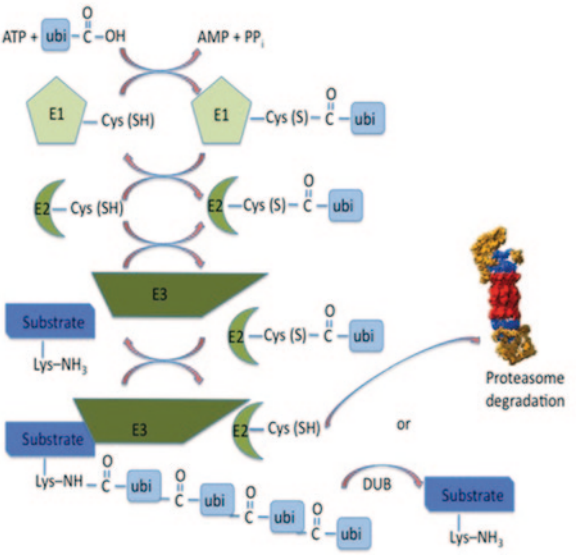

Fig. 9.1 The ubiquitin–proteasome system (*UPS*). *ATP* adenosine triphosphate, *AMP* adenosine monophosphate, *DUB* deubiquitinating enzyme

9.5.1 Class I: Ubiquitin–Proteasome System vs. Lysosomal–Autophagy System

To review potential mechanisms for class I persistence described earlier, there are two principal pathways for the elimination of proteins from cells: the lysosomal–autophagy system (LAP) [39] and the ubiquitin–proteasome system (UPS) (Fig. 9.1) [58]. These systems are both highly regulated and can degrade proteins in a targeted fashion; however, the UPS is generally considered to be the more important pathway for the specific regulation of protein degradation.

9.5.1.1 Role of LAP in BoNT Persistence

There are three primary forms of autophagy: chaperone-mediated, macroautophagy and microautophagy [28]. Chaperone-mediated autophagy is primarily associated with responses to starvation. Microautophagy is distinguished by sequestering portions of the cytosol via invagination/septation of the lysosomal membrane, whereas macroautophagy involves the formation of cytosolic double-membrane vesicles that sequester large portions of the cytosol. The LAP is primarily responsible for the removal and digestion of cellular organelles and large macromolecular aggregates within the cell, entering the cell either via phagocytosis or as aggregates of cellular protein debris. Since the LAP pathway functions by forming large endosomes around portions of the cytosol or by invagination of the endosomes, the LAP could play a role in the nonspecific clearance of BoNT LC from the cells. Early studies us-

ing codon-optimized recombinant BoNT LCs suggest that BoNT/A LC is localized to the plasma membrane whereas BoNT/E LC is mostly cytosolic [14, 15]. BoNT/A LC, by virtue of being largely sequestered to the internal plasma membrane, may in this way evade the LAP system, whereas BoNT/E LC, residing primarily in the cytosol, may be more accessible to the LAP system.

9.5.1.2 Role of UPS in BoNT Persistence

The essential elements of the UPS consist of ubiquitin (a highly conserved 76-amino-acid peptide), a cascade of enzymes to attach ubiquitin to a specific target protein, and the proteasome, which is a large macromolecular complex [22, 57]. Ubiquitin serves a number of cellular functions, but the most widely investigated is its role in targeting proteins to the proteasome for degradation. The signature for designating a protein for degradation is the attachment of a polyubiquitin chain to the protein. Ubiquitin is attached to a specific protein through the action of a cascade of enzymes designated E1, E2 and E3, which sequentially relay ubiquitin, culminating in the covalent ligation of the ubiquitin carboxyl terminus to a Lys side chain of the target protein. The covalent attachment of ubiquitin is highly regulated, and the selection of the protein target for ubiquitination resides primarily in the function of E3 ubiquitin ligases. A specific E3 may have a single or very small number of protein targets that it will recognize to bring about ubiquitination of that target (Fig. 9.1). Examples of such specific relationships are the recognition and ubiquitination of the proteins important in cancer [31].

Using recombinant BoNT LCs, we have shown that BoNT/A LC is stable whereas BoNT/E LC is rapidly degraded in cells [51]. The rapid degradation of BoNT/E LC is associated with its more extensive polyubiquitination in cells. These results suggest that the susceptibility of BoNT LCs to UPS-mediated proteolysis is the key determinant of serotype persistence. Consequently, it should be possible to design E3 ubiquitin ligases that accelerate BoNT/A LC degradation via the UPS, thereby accelerating recovery from BoNT/A intoxication. E3 ubiquitin ligases are generally modular, with one domain specifically binding and recognizing the target protein and another domain (E3 ligase catalytic domain) facilitating the attachment of ubiquitin to the target protein Lys residue. Such a modular design allows for the generation of chimeric recombinant proteins, which contain a combination of a recognition domain for novel targets together with an E3 ligase catalytic domain to attach ubiquitin to this novel target. This approach of generating chimeric recombinant “designer” ubiquitin ligases is being applied to a number of fields, including cancer therapy [21, 36, 59, 60]. The possibility of designating a target protein for degradation may be applicable to the development of therapeutics for BoNT intoxication and is being actively pursued.

As proof of concept, we have designed ubiquitin ligases based on a BoNT-resistant SNAP-25 as the targeting domain. These SNAP-25-based ubiquitin ligases accelerate the degradation of recombinant BoNT/A LC, supporting the idea that

designer ubiquitin ligases could be of value in reducing the duration of BoNT intoxication [51]. Since SNAP-25 forms part of the SNARE complex at the nerve terminal, a SNAP-25-based designer ubiquitin ligase may also target other components of the SNARE complex for degradation. An alternative design takes advantage of a single-chain antibody that specifically recognizes BoNT/A LC. This nanobody approach has shown some success in accelerating the recovery of M17 neuroblastoma cells from BoNT intoxication [29]. The finding that accelerating the degradation of BoNT/A LC can hasten recovery from BoNT/A intoxication provides strong evidence that the continued presence of BoNT protease activity, rather than cleavage products, mediates BoNT persistence.

9.5.2 Class II: Sequestration of Persistent BoNT LCs

BoNT/A is thought to enter nerve terminals near transmitter release sites, since these have the highest concentration of the cell surface receptor synaptic vesicle protein 2 (SV2) [10]. With time, it may be expected that the concentration of BoNT LC in the nerve terminal cytosol would diminish by diffusion and redistribution within the much larger volume of the axon and soma. However, if the LC were actually sequestered in the nerve terminal, diffusion may be hindered, leading to an increased duration of LC action. The findings of Baldwin and Barbieri [4] that the LCs of BoNT/A and BoNT/B are co-localized with synaptic vesicle proteins suggest that sequestration of LCs may indeed be the case.

A potential mechanism for sequestration would be anchoring the LC to the cytoplasmic surface of the presynaptic membrane. Confocal microscopy of nerve growth factor-differentiated PC12 cells transiently transfected with green fluorescent protein (GFP)–BoNT LC fusion proteins demonstrates a divergence in localization between the LCs of BoNT/A and of BoNT/E [14]. Under the conditions described, GFP–BoNT/A LC was trafficked primarily to the plasma membrane and processes, where it was co-localized with SNAP-25 Δ9. In contrast, GFP–BoNT/E LC was found throughout the cell, excluding the nucleus, and was co-localized with SNAP25 Δ26.

Truncation of 22 C-terminal residues or mutation of vicinal Leu within this region to Ala reduced the degree of membrane localization, and deletion of the 8 N-terminal residues resulted in a generalized cytoplasmic expression similar to that of GFP–BoNT/E LC-transfected cells. Similar studies on BoNT/B LC indicate it can be found throughout the cell including the nucleus [15]. These experiments utilized transient transfection, resulting in intracellular levels of LCs that are likely to be orders of magnitude higher than would be found in a true intoxication; furthermore, the LC protein is synthesized from within the cell and thus passes through cellular compartments that it would not normally be exposed to during a typical intoxication. Nevertheless, the data support the possibility of divergent subcellular localization among BoNT LCs. More recent studies using fluorescent protein-labeled BoNT/A

and BoNT/E LCs in other neuroblastoma cells show that the LCs are co-localized, suggesting that localization of BoNT LCs may be cell specific [51].

The preferential localization of BoNT/A LC to the plasma membrane appears to depend on a stretch of nine amino acids at its N-terminus and a di-Leu motif near the C-terminus [7, 15]. The N-terminus of BoNT/A LC contributes to its plasma membrane localization by interacting with SNAP-25, which binds to the membrane by palmitoylation of several Cys residues [53]. Consistent with this proposal, BoNT/A LC can be detected in the cytosol when co-expressed with a SNAP-25 mutant lacking residues required for palmitoylation [7]. Thus, a deletion mutant of BoNT/A LC missing the nine-amino-acid N-terminal is not localized to the plasma membrane. The deletion mutant retains persistence, however, showing that plasma membrane localization is not required for LC persistence [7, 54].

To investigate the contributions of the N- and C-terminal regions in greater detail, Wang et al. [54] created a series of recombinant full-length toxins in which the N-terminal residues were deleted and the C-terminal di-Leu residues were mutated to di-Ala. The results, although complex, provided important insights regarding the molecular basis for toxin potency and duration. First, the N-terminal and di-Leu modifications resulted in a significant loss in lethality of 1,000- and 10,000-fold, respectively. The N-terminal deletion mutant also exhibited lower potency in a SNAP-25 cleavage assay in cerebellar granule neurons and was less effective in blocking tension in phrenic nerve–hemidiaphragm preparations, but it produced paralysis in mouse gastrocnemius muscle of long duration resembling that of wild-type recombinant BoNT/A. The N-terminal region has also been demonstrated to make direct contacts with SNAP-25 within the plasma membrane [7]. Conversely, the di-Leu to di-Ala mutation resulted in potencies in the cerebellar granule SNAP-25 cleavage and hemidiaphragm assays that were comparable to wild-type BoNT/A, but, strikingly, a significantly reduced duration of action in the gastrocnemius SNAP-25 cleavage and paralysis assays. Furthermore, fusion of BoNT/E LC to the N-terminus of an inactive mutant of BoNT/A (termed LC_E-$BoTIM_A$) resulted in a toxin with SNAP-25 cleavage properties of BoNT/E but with the duration similar to BoNT/A. This latter finding supports the persistent LC model of BoNT action, while strongly arguing against the hypothesis that SNAP-25 cleavage products account for toxin persistence [12, 34, 55] by clearly demonstrating that the presence of SNAP-25 Δ26 does not lead inevitably to a reduced duration of BoNT action.

Despite the role of the di-Leu motif in the persistence of BoNT/A, no data are offered indicating the motif directs subcellular localization of the LC to the plasma membrane as previously described [14, 15]. In fact, in an addendum to the paper, the authors indicate their results may be attributable to altered clearance of the mutant LC by the UPS pathway described in Tsai et al. [51]. Thus, the di-Leu motif may not mediate a class II but rather a class I mechanism as described earlier (see Sect. 9.4.1). Finally, only BoNT/A possesses this motif; the other long- or moderate-duration serotypes, namely BoNT/C1 and BoNT/B, lack the motif, indicating that the conclusion cannot be generalized to the other BoNT serotypes.

9.5.3 Class III: Evasion of Removal Due to Low Copy Numbers of BoNT LCs

This last potential mechanistic class of BoNT persistence has little data to support it and experiments to prove its importance would be difficult to perform. Copy numbers of all BoNTs are vanishingly small under standard intoxication conditions. It would first be necessary to show that among BoNT serotypes, the copy number necessary to support paralysis is low for long-duration serotypes and high for short-duration serotypes. However, it may serve to complement class I and II mechanisms; as BoNT LCs are removed from the presynaptic terminal, their numbers are reduced to a point that they are no longer attacked by the cellular degradation machinery, yet retain enough activity to support paralysis.

9.6 Conclusions and Future Directions

As stated previously, one of the key features of the toxic effects of BoNT/A, /B and /C1 is the persistence of these toxins within their target cells following intoxication. There has been significant effort placed into the investigation of small molecule inhibitors (SMIs) of BoNTs with limited success. The structure of the BoNT LC itself may preclude development of an SMI of sufficiently high affinity to block the activity of the protease to a therapeutically useful level. As a complementary treatment strategy to the development of SMIs, alternative methods for postexposure treatment of BoNT intoxication are needed. If the intoxication time can be shortened from several months to a few days of intensive care and assisted ventilation, it would greatly reduce the impact on the health-care system for both the civilian population in the event of a terrorist attack on food, milk or water supplies, as well as the war fighter in the event of the toxin being released in the war theater [51]. Thus, given the lack of effective SMI options, exploiting the potential to shorten the paralysis time by targeted degradation of BoNT LC can provide significant positive outcomes.

We have pioneered the designer ubiquitin ligase approach to hasten recovery from BoNT intoxication. In addition to the development of designer ubiquitin ligases for accelerating the elimination of BoNT LC from the presynaptic terminal, studies to determine the role of the endogenous neuronal UPS in regulating differences in persistence between BoNT/A and BoNT/E have been undertaken. Recent efforts have focused on the identification of cellular protein-binding partners for the LCs of BoNT/A and BoNT/E; future studies should aim to identify elements of the UPS that may preferentially recognize one of the two serotypes of BoNT LC to account for the greater rate of UPS degradation of BoNT/E.

The precise mechanisms employed by BoNT/A LC to evade the UPS remain unknown but two possibilities are emerging. In the first, cellular E3 enzymes may interact more extensively with BoNT/E LC to accelerate its degradation; in the second, BoNT/A may recruit a cofactor that shields it from the ubiquitin-dependent degradation. The first possibility is supported by the identification of tumor necrosis

factor receptor-associated protein 2 (TRAF2) as a ubiquitin ligase that selectively recognizes BoNT/E LC [51]. A simple proposition is that BoNT/A LC is not recognized by endogenous ubiquitin ligases in the cell. This scenario is unlikely since ubiquitination of recombinant BoNT/A LC was detected [51]. Nevertheless, it is possible that the specific ubiquitin ligase for BoNT/A LC is absent or poorly expressed in motorneurons.

In the second possibility, BoNT/A LC is stabilized by association with a cofactor. The cofactor could stabilize the LC by preventing exposure of certain motifs important for substrate recognition by the UPS, or by recruiting deubiquitinating enzymes (DUBs) to reverse the effects of ubiquitination. Both alternatives have implications for therapy. Thus, development of a designer E3 ubiquitin ligase directed to the BoNT/A LC and the delivery of this designer E3 to affected neurons may shorten the duration of intoxication. Alternatively, identifying DUBs that remove ubiquitin from BoNT/A LC and inhibiting these DUBs with SMIs could selectively target BoNT/A LC for degradation within cells.

DUBs may be a more suitable target for intervention because the BoNT/A LC degradation rate would be *increased* by *decreasing* the activity of the putative DUB with an SMI. The design of an SMI to a specific target is potentially more direct than attempting to engineer increased activity of the E3 ligase pathway. In addition, if inhibition of DUBs leads to elimination of BoNT/A LC, a brief encounter with the SMI may be sufficient to reverse intoxication whereas targeting the LC protease activity may require months of sustained inhibitor therapy. The era of therapeutics directed towards regulation of protein degradation by the UPS is emerging and holds promise in the evolution of novel therapeutics addressing the persistence of BoNT intoxication.

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Chapter 10
Clostridium botulinum Genomes and Genetic Diversity

Theresa J. Smith

Abstract Botulinum neurotoxins (BoNTs) are produced by multiple members of the clostridial genus. Seven neurotoxin serotypes and 35 distinct toxin subtypes/genetic variants have been identified based on immunological characteristics and sequence diversity. Analysis of neurotoxin and genome sequences provides evidence that horizontal gene transfer may be a primary mechanism for the movement of neurotoxin genes within these bacteria and that genetic mobility among different bacterial species is facilitated by their phage or plasmid locations. Bacterial and neurotoxin relationships are described based on historic parameters and recent genomic sequencing efforts. Examination of the factors that provide genetic mobility for neurotoxin genes contributes to understanding the diversity resident in the neurotoxins, the toxin complexes and the bacteria that produce them.

Keywords Botulinum neurotoxin · *C. botulinum* groups · 16s *rrn* genes · BoNT-producing clostridial species · *C. botulinum* genome · Genomic islands · Pathogenicity islands · Gene clusters · Chromosomal synteny · Proteolytic strains · Nonproteolytic strains · Clostridial genomes · Gene clusters · Neurotoxin serotypes · Toxin subtypes · Recombination

10.1 Introduction

In 1897, Professor Emile van Ermengem demonstrated that an outbreak of botulism in humans was caused by a potent toxin generated by an anaerobic bacterium [1]. Bacterial characteristics included the presence of large spore-bearing bacilli, growth only under anaerobic conditions, a growth temperature optimum of 20–30 °C and increased growth in the presence of glucose. These organisms were designated *Bacillus botulinus*.

The opinions, interpretations, and recommendations expressed in this chapter are those of the author and are not necessarily those of the US Army.

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The van Ermengem isolate, commonly referred to as the Ellezelles strain, was later compared with a second strain, the Darmstadt strain, isolated in connection with an outbreak of botulism in 1904 due to bean salad [2]. The Darmstadt strain had a wider growth tolerance, higher optimum growth temperature and different growth medium requirements than the Ellezelles strain. These observations provided the first evidence that *B. botulinus* organisms have variable growth characteristics and medium requirements.

In 1917, the committee on classification of the Society of American Bacteriologists standardized bacterial nomenclature. They recommended that the genus *Bacillus* should be subdivided into *Bacillus* for aerobic forms and *Clostridium* for anaerobic forms [3]. Thus, *Bacillus botulinus* was formally changed to *Clostridium botulinum*.

A rapid presumptive identification method for *C. botulinum* using reactions on egg yolk agar was developed in the 1940s [4]. Identification was based on two separate reactions. *C. botulinum* colonies growing on agar plates containing egg yolk produce an oily iridescent sheen due to lipase activity. They do not produce lecithinase, which causes opacity within the egg yolk agar. Anaerobic growth habits, characteristic Gram's stain and lipase-positive-/lecithinase-negative characteristics, coupled with demonstration of toxicity, continue to be essential traits relied on for identification of botulinum neurotoxin (BoNT)-producing clostridia in many laboratories worldwide.

The first organisms that were isolated in association with botulism were divided into proteolytic and nonproteolytic groups. As additional *C. botulinum* organisms were isolated and examined, variations from these standard characteristics began to appear. A third set of organisms were discovered in 1922 that displayed subtle cultural differences from other nonproteolytic strains [5, 6]. Additional strains exhibiting various growth optima, cultural morphologies and Gram's stain characteristics were observed. Exceptions to the lipase-positive-/lecithinase-negative rule were discovered, along with other variations from standard biochemical characteristics. Eventually, four *C. botulinum* groups (I–IV) were identified based on dissimilarities in standard cultural, biochemical, and ecological characteristics [7–10].

While the organisms were differentiated by their cultural morphology, growth characteristics and biochemical reactions, the neurotoxins that they produced were distinguished through neutralization bioassays using toxin-specific antisera. Antiserum produced using the well-known Ellezelles strain as the antigen was unable to neutralize the Darmstadt toxin, and vice versa, providing the first evidence that the toxins, like the bacteria that produce them, are diverse [11]. These were the first two toxin serotypes to be described.

From 1922 to 1966, five additional toxin serotypes were identified using antisera developed by repeatedly injecting rabbits, goats and other animals with small amounts of toxin over time. Each serotype was determined to be immunologically distinct, with only negligible cross-reactivity [12].

With the advent of DNA sequencing, BoNTs and the organisms that produce them began to be characterized using genetically based methods. Toxin subtypes emerged that were serologically indistinguishable but genetically diverse. Data

Table 10.1 Biochemical/biophysical characteristics of BoNT (botulinum neurotoxin)-producing clostridia. (Data from [10–12])

	Group					
	I	II	III	IV	*C. baratii*	*C. butyricum*
Toxin types produced	A, B, F; Ab, Af, Ba, Bf	B, E, F	C, D	G	F	E
Toxin gene locations	chr, plasmid	chr, plasmid	phage	plasmid	?	chr
Proteolysis[a]	+	−	±	+	−	−
Lipase	+	+	+	−	−	−
Lecithinase	−	−	±	−	+	−
Fermentation of:						
Glucose	+	+	+	−	+	+
Mannose	−	+	+	−	+	+
Sucrose	−	+	−	−	+	+
Trehalose	−	+	−	−	+	+
Optimal growth temperature	35–37 °C	18–30 °C	40 °C	37 °C	30–37 °C	30–45 °C
Minimal growth temperature	10 + °C	3 °C	15 °C			10 °C
Related species	*C. sporogenes*		*C. novyi*	*C. subterminale*		

Chr chromosome [a] Proteolysis of meat, milk, egg

from these genetic analyses have confirmed earlier delineations and characterizations and expanded our understanding of the complexities that are inherent in BoNT-producing clostridia.

10.2 Organisms

The four *C. botulinum* groups differ in ecological and biochemical/biophysical characteristics, in the toxins they produce and in their genomic organization. *C. botulinum* group I bacteria are proteolytic organisms that produce neurotoxin serotypes responsible for the majority of human botulism cases worldwide [13, 14]. They are lipase positive and lecithinase negative, with growth optima ranging from 35 to 37 °C. They may produce one or more of several toxin serotypes (A, B, F; Ab, Ba, Af, and Bf) and they are closely related to *C. sporogenes* [8–10, 15]. BoNT/A1 strains that carry a mutant *bont/B* gene which does not produce active BoNT/B toxin (A1(B) strains) have also been identified. Group I is the most studied and best characterized of the *C. botulinum* groups. Table 10.1 summarizes the biochemical and biophysical properties of the BoNT-producing clostridia [8–10].

Group I strains, especially those producing BoNT/A1, BoNT/A2, BoNT/B1 and BoNT/B2 subtypes, are particularly prevalent. In the USA, BoNT/A- and proteolytic BoNT/B-producing strains are almost equally responsible for botulism cases [13]. The toxin subtypes involved are overwhelmingly A1 and B1. BoNT/A1 strains have been identified from North America, Europe and Asia [16–19]. A single BoNT/A1-producing isolate from Venezuela has also been identified [20], and BoNT/A and /B strains of unknown subtype have been isolated in connection with botulism cases in South America, Russia, Africa, Australia, Indonesia and China [14, 21–23].

Recent studies indicate that BoNT/A2 strains are widespread in South America, particularly in Argentina [24] (R. Fernandez, personal communication). Multiple early infant botulism cases in Japan associated with BoNT/A2 strains were connected with the ingestion of honey that might have been imported from South America; infant botulism cases dropped precipitously after a public health campaign warning against giving honey to infants under the age of 1 year [25]. Subtyping studies have shown that at least 7/12 isolates from Japanese infant cases during 1986–1989 were of subtype A2, while 3/12 and 0/12 strains isolated after the public health campaign were of subtype A1 and A2, respectively [19]. Further, a comparison of the toxin sequences from three of these Japanese A2 infant strains with six A2 Argentinean strains shows them to be identical.

BoNT/A2 is the most commonly isolated BoNT/A subtype in Italy [26]. In contrast to Japan, the majority of Italian BoNT/A2 cases have been food-borne. BoNT/A2-producing strains were also isolated from an infant botulism case in the USA [27] and from food-borne cases in Puerto Rico [26, 27] and the island of Mauritius, near the eastern coast of Africa [28]; both of the food-borne cases involved fish. Altogether, this links the A2 subtype to at least four continents.

C. botulinum strains that produce BoNT/B2 are the most commonly isolated strains in Europe. Their range extends from Finland [29] to Italy [27], but they are particularly prevalent in France. BoNT/B2-producing strains have been isolated in Japan [30] and Korea [18] as well. Botulism cases due to BoNT/B of unspecified toxin subtype have also been reported in Russia and China [14, 23]

Group II bacteria are similar to group I organisms, but they are nonproteolytic (they do not digest meat, milk, or egg). They have lower temperature optima for growth (18–30 °C) and they are known to ferment a wider variety of carbohydrates than their group I counterparts. They also produce multiple toxin serotypes (B, E or F) [8–10]

Group II strains are rarer than group I organisms, judging from the numbers of isolations and reported botulism cases attributed to this group. They have been isolated however from food-borne, infant and wound botulism cases. BoNT/E-producing strains are the most prevalent of the group II strains. They have been associated with many food-borne cases involving fish and marine mammals in colder climates, such as Canada, Alaska, the Baltic Sea area, northern Russia and northern Japan [31, 32]. Currently, only one infant case [33] and one outbreak of drug-related wound botulism [34] associated with BoNT/E-producing *C. botulinum* strains have been reported. A recent review of type E botulism pointed out several differences between *C. botulinum* type E in China versus other locales [23]. While type E botu-

lism in other parts of the world is due to fish and marine mammals, in China it is associated with the consumption of raw or undercooked meat. Cases due to type E botulism predominate in two northwestern landlocked provinces (Qinghai and Tibet) that are between 30 and 40 degrees north latitude, as opposed to the marine-associated cases that are found in other parts of the world above the 40th parallel. Type E toxin has been detected, however, from 60% of soil and fish samples collected at three coastal locations representing middle, northeastern and southeastern China [23]. Foods and food preparation methods in China differ from those in other countries where botulism due to BoNT/E is a hazard, and this may account for an abundance of bacterial spores in these marine regions but an absence of cases.

Botulism outbreaks involving group II BoNT/B and BoNT/F producers are exceedingly rare. Only one botulism case attributed to nonproteolytic BoNT/B4 and one case associated with nonproteolytic BoNT/F6 have been reported [35, 36]. However, cases due to BoNT/B2 produced by group I or group II organisms have been recorded [27, 37]. This is the only toxin subtype known that is produced by organisms from more than one *C. botulinum* group. It is possible that many of the European food-borne botulism cases linked to nonproteolytic BoNT/B-producing strains of unknown subtype, such as the Ellezelles strain, may involve BoNT/B2 instead of the classic nonproteolytic B4 toxin.

Group III organisms are weakly proteolytic or nonproteolytic and are generally lecithinase-positive. They produce type C or D toxins, which commonly cause botulism in animals, but very rarely in humans. Despite their nonproteolytic nature, they differ from the nonproteolytic group II in having a relatively high optimum growth temperature of approximately 40 °C [8, 9]. This may be a factor in the ecological range of these groups. Group II BoNT/E-producing strains are abundant in colder climates, such as Alaska, Canada, the Baltic Sea region, northern Japan and northern Russia [31, 32], while group III strains tend to be isolated in warmer areas, such as the southern USA [38], Africa [39], Taiwan [40] and parts of South America [41]. Exceptions have been noted, however, such as outbreaks of botulism due to BoNT/C in fur animals in Finland and Norway [42, 43] and the isolation of BoNT/E strains in the Gulf of Mexico and southern China [23, 44].

Group IV organisms are proteolytic but negative for both lipase and lecithinase, and they do not ferment carbohydrates. They produce type G toxin [9]. BoNT/G-producing *C. botulinum* strains were confirmed to be a distinct species, *C. argentinense,* by examining DNA relatedness among these strains and some strains of *C. subterminale* and *C. hastiforme* [45]. BoNT/G-producing strains are rare and have only been isolated in Argentina and Switzerland [46, 47].

Additional BoNT-producing clostridia were discovered, beginning with an organism that produced BoNT type F but was clearly identified as *C. baratii* [48]. Shortly after this discovery, several infant botulism cases caused by BoNT/E-producing *C. butyricum* strains were found [49]. These species designations were confirmed using DNA hybridization studies [50].

BoNT/F-producing *C. baratii* strains have sometimes been designated as group V. They are nonproteolytic and saccharolytic but, unlike group II, they are not psy-

chrotropic. Their lipase and lecithinase reactions differ from *C. botulinum*, being negative for the former and positive for the latter.

The first BoNT/F infant botulism case recorded was in 1979 in New Mexico [51]. Several years later, it was discovered that the toxigenic bacteria involved was *C. baratii*, not *C. botulinum* [48]. As of 2006, five infant botulism cases and 13 adult-onset botulism cases due to BoNT/F-producing *C. baratii* have been reported [52, 53]. While several of the adult cases were initially suspected to be food-borne in origin, there is growing evidence that these cases may actually be adult toxicoinfections [53–55]. Several of these cases can be linked to recent surgical interventions or the use of broad-spectrum antibiotics. In one case, toxigenic *C. baratii* was isolated from a patient and from contaminated food; several family members had eaten the implicated food, but only the member with a history of recent antibiotic use became ill [56]. In all other cases, none of the suspected food samples were positive for BoNT/F or *C. baratii* organisms [53].

BoNT/E-producing *C. butyricum* strains, sometimes known as group VI strains, are nonproteolytic, but they differ in several ways from their group II counterparts. They are negative for both lipase and lecithinase, and they have a higher growth temperature optimum (30–37 °C) than their group II counterparts. Their ecological ranges are also more temperate than group II; perhaps, this is a reflection of their differing growth ranges and optima. The BoNT/E-producing *C. butyricum* strains are divided into two groups geographically and ecologically. The Italian *C. butyricum* strains were the first to be identified in connection with infant botulism cases near Venice [57]. Four cases of infant botulism and two cases of adult toxicoinfections due to Italian *C. butyricum* strains have been reported. As with *C. baratii* type F strains, the Italian *C. butyricum* type E strains appear to be associated with toxicoinfections [54].

Chinese researchers investigating food-borne outbreaks in the Lake Weishan area discovered a second type of BoNT/E-producing *C. butyricum* strain. In contrast to the Italian *C. butyricum*, these strains have exclusively been associated with food-borne botulism. The causative foods included soybean and wax gourd paste [23]. In addition to differences in the type of botulism seen, the two *C. butyricum* type E neurotoxins differ by 5.0% at the amino acid level, in contrast to the 2.7–4.4% differences seen between the type E toxins produced by group II *C. botulinum* and either of the *C. butyricum* strains.

An early use of DNA in bacterial identifications involved sequencing of 16s *rrn* genes [15]. These genes code for 16s ribosomal RNAs (rRNAs), which are relatively conserved within species. Thus, species-level identifications can be simplified using comparisons of 16s *rrn* genes. When this type of analysis was used with *C. botulinum* strains, the four biochemical groups listed above were separated into distinct species [15, 27].

Genomic backgrounds and relationships among clostridia were also uncovered through analysis of 16s *rrn* genes. Group I appears to be the most conserved group overall. Comparison of *C. sporogenes* 16s *rrn* genes shows several to be identical with the genes from multiple group I *C. botulinum* strains; others are very closely related [27, 58]. Group II strains form their own distinct cluster, but they are some-

what related to *C. butyricum* and *C. baratii* as well as several other clostridial species. Group III strains align closely with *C. haemolyticum* and *C. novyi*. Group IV organisms are closely related to non-neurotoxin-producing *C. argentinense* and *C. subterminale*. These close relationships based on 16s *rrn* gene analysis may complicate strain identifications using ribotyping assays, especially if the material is complex, such as wound or stool material [59]. Therefore, identifications should always be based on both toxin detection and 16s *rrn* results.

It is now widely recognized that BoNTs may be produced by one of at least six different clostridial species. The level of diversity among BoNT-producing organisms contrasts sharply with *C. tetani*, a homogeneous species that produces only one toxin type, tetanus.

10.3 Genomes

In the 1970s, the ability to reliably sequence DNA using chain-terminating methods allowed the first genetically based analyses of individual genes and viral genomes [60, 61]. The introduction of fluorescently labeled dideoxynucleoside triphosphates (ddNTPs) and primers allowed for the development of automated, high-throughput DNA sequencing [62]. In 1995, this technology was used to sequence the first complete genome of a free-living organism, the bacterium *Haemophilus influenzae* [63].

The first *C. botulinum* genome was published 12 years later [64]. This was quickly followed by the posting of 27 genomes from BoNT-producing clostridial strains. GenBank accession numbers for these genomes are listed in Table 10.2. Thirteen of these are complete and 12 are whole genome shotgun sequences. In addition to the chromosomal sequences, there are 17 distinct plasmid sequences present within these genomes. Several of the plasmid sequences are prophage sequences, which code for bacteriophages. The 27 organisms that were sequenced include members of three of the four *C. botulinum* groups plus BoNT/E-producing *C. butyricum* strains, and 17 of the currently recognized 35 toxin variant/subtypes are also represented. Table 10.2 lists basic characteristics of the available BoNT-producing clostridial genomes. A repeat genome sequence of *C. botulinum* strain Eklund 17B has recently been posted by the Sanger Center, enabling comparisons of two genomes from the same strain [65]. In addition, two genome projects, *C. botulinum* 4411 and 5311, have been listed by the J. Craig Venter Institute and one genome, Af84, has been listed by the Los Alamos National Laboratory. The sequencing of the last three genomes are in progress and no sequence information on them is currently available.

The genomes range in size from approximately 2.4 to 4.7 megabases. The group III genomes are the smallest and the BoNT/E-producing *C. butyricum* strains have the largest genomes. Most genomic sequences contain none to two plasmids; however, it has recently been discovered that some BoNT/C strains may contain up to six plasmid or prophage sequences. Neurotoxin gene sequences reside within ten of these plasmids/prophages; the adenosine diphosphate (ADP)-ribosylating C2 toxin

Table 10.2 Characteristics of published BoNT (botulinum neurotoxin)-producing clostridial genomes

Species	Group	Subtype	Strain/plasmid	Size (kb)	GC (%)	Gen bank accession
C. botulinum	I	A1	ATCC 3502	3,887	28	AM412317
			pBOT3502	16	26	AM412317
C. botulinum	I	A1	ATCC 19397	3,863	28	CP000726
C. botulinum	I	A1	Hall	3,761	28	CP000727
C. botulinum	I	A1(B)	NCTC 2916	4,031	28	ABDO02000001–49
C. botulinum	I	A1(B)	CFSAN001627	4,077	27	AMXI01000001–1743
C. botulinum	I	A2	Kyoto-F	4,155	28	CP001581
C. botulinum	I	A3	Loch Maree	3,993	28	CP000962
			pCLK	267	25	CP000963
C. botulinum	I	Ba4	strain 657	3,978	28	CP001083
			pCLJ	270	25	CP001081
			pCLJ2	10	24	CP001082
C. botulinum	I	A5	H04402 065	3,920	28	FR773526
C. botulinum	I	B1	Okra	3,958	28	CP000939
			pCLD	149	25	CP000940
C. botulinum	I	B	CFSAN001628	4,014	28	AMXJ02000001–246
C. botulinum	I	Bf	–	4,200	28	ABDP01000001–70
C. botulinum	I	F1	Langeland	3,995	28	CP000728
			pCLI	18	26	CP000729
C. botulinum	I	F1	230613	3,993	28	CP002011
			pCBF	18	26	CP002012
C. botulinum	II	B4	Eklund 17B	3,800	27	CP001056
			pCLL	48	24	CP001057
C. botulinum	II	E1	Beluga	4,000	27	ACSC01000001–16
C. botulinum	II	E3	Alaska E43	3,660	27	CP001078
C. botulinum	II	E9	CDC66177	3,852	27	ALYJ01000001–119
C. botulinum	II	E	CB11/1–1	3,823	27	AORM01000001–171
C. botulinum	III	C	Stockholm	2,660	27	AESA01000001–588
			p6CSt	55	27	AESA01000588
			phage	186	26	AP008983
C. botulinum	III	C/D	Eklund	2,961	28	ABDQ01000001–76
C. botulinum	III	C/D	BKT015925	2,773	28	CP002410
			p1BKT015925	203	27	CP002411
			P2BKT015925	99	26	CP002412
			P3BKT015925	80	27	CP002413
			P4BKT015925	40	28	CP002414
			P5BKT015925	12	26	CP002415

Table 10.2 (continued)

Species	Group	Subtype	Strain/plasmid	Size (kb)	GC (%)	Gen bank accession
C. botulinum	III	C/D	BKT028387	2,834	28	AESB01000001–237
C. botulinum	III	C/D	V891	3,139	28	AESC01000001–301
C. botulinum	III	D	1873	2,379	27	ACSJ01000001–19
			pCLG1	108	26	CP001659
			pCLG2	54	25	CP001660
C. butyricum	VI	E4	BL5262	4,758	28	ACOM01000001–13
C. butyricum	VI	E4	BL5521	4,541	28	ABDT01000001–123

genes are also located within a plasmid [66]. The G+C content of the plasmid and phage DNA is slightly lower (24–26%) than that of chromosomal DNA (27–28%).

Comprehensive analysis of genomic data from a variety of bacteria has enabled the identification of regions containing mobile genetic elements, such as insertion sequences, transposons, plasmid sequences, prophages and large unstable regions designated as genomic islands. These mobile genetic regions typically have distinguishing G+C content. Genomic islands are often referred to as "pathogenicity islands" when they include genes or gene clusters that encode pathogenic functions [67]. Because regions within *C. botulinum* genomes containing BoNT genes/gene clusters also contain many of the basic elements of pathogenicity islands, they could be defined as such. However, the distinctive overall low G+C content throughout these genomes hampers the ability to identify these pathogenicity islands on the basis of G+C content differences.

These genomes provide valuable information on diversity at the organism and neurotoxin levels. Chromosomal synteny comparisons and comparative genome hybridization studies of group I organisms producing BoNT/A1, /A1(B), /A2, /A3, /A5, /Ba4, /Bf, /B1 and /F1 show them to be very similar in gene organization and content [17, 29, 64, 68].

There is also a very high degree of similarity with *C. sporogenes* [15]. There appears to be an exceptionally close relationship between proteolytic BoNT/B-producing *C. botulinum* strains and some *C. sporogenes* strains. When compared using genomic hybridization microarrays, several of these strains clustered together in a single clade [17]. Comparison of the genomic synteny plots between a *C. sporogenes* strain and group I *C. botulinum,* however, revealed the presence of a large (701 kb) inversion, indicating that a single large-scale genetic rearrangement had occurred in this strain [68].

Neurotoxin gene-containing plasmids are a common feature in the group I strains. Plasmid locations for neurotoxin gene clusters from group I strains were identified using pulsed field gel electrophoresis (PFGE)/Southern hybridization techniques [36, 69] and analysis of whole genome sequences [70] from strains producing BoNT/A3, /Ba4, /Bf and /B1 toxins. A large study of BoNT/B-producing

strains using PFGE indicated that plasmid locations for these genes were not rare or unusual events. Of the 58 group I BoNT/B-producing strains that were examined, 30 (52%) were found to have plasmid locations for their neurotoxin genes. Genes coding for BoNT/B1-producing strains, which are the predominant B subtype found in North America, were found almost exclusively within large plasmids. In contrast, genes coding for European BoNT/B2-producing strains were mainly confined to the chromosome. Overall, 28/34 neurotoxin genes from US BoNT/B strains were found within plasmids, while neurotoxin genes from 22/26 Italian BoNT/B strains were located within the chromosome [36].

Among the seven known BoNT/B toxin subtypes/variants is BoNT/B5, the toxin subtype associated with bivalent toxin-producing strains. BoNT/B5-producing strains may contain *boNT/A1* or *boNT/A2* genes in addition to the *bont/B5* gene; on rare occasions, they contain only *bont/B5* genes [36]. New bivalent toxin combinations A2b1 and A2b3 have been found in addition to the classic A1b5 and A2b5 strains. Strains containing *bont/A1* plus *bont/B5* genes or the similar "silent B" genes, which do not produce active full-length BoNT/B toxin, were always located within the chromosome in these studies. However, when *bont/A2* or bivalent *bont/F2* genes were involved, or when *bont/B5* genes were found without a second neurotoxin gene cluster, these genes were always located within large, highly conserved plasmids ranging from approximately 150 to 270 kb [36, 70].

A common factor among BoNT/A1, BoNT/A2 and BoNT/B2 subtypes, in addition to their ubiquitous nature and their group classification, is the chromosomal location of their toxin genes. With the exception of BoNT/B1 strains, *C. botulinum* group I strains that contain neurotoxin genes within plasmids, such as BoNT/A3 or bivalent BoNT/Ab, /Ba or /Bf strains, are relatively rare. Their toxin gene sequences also tend to show more variability, especially when compared to the extremely highly conserved *bont/A1* gene [68].

Synteny comparisons of group II strains indicate a high level of chromosomal synteny within this group, but they are very different from group I strains in organization and content [68]. Their genomes are too different to allow comparative genome hybridization analysis using microarrays based on group I strain ATCC3502 [17, 64]. While the genes for two BoNT/E-producing *C. botulinum* strains were located within the chromosome, the genes for nonproteolytic BoNT/B-producing strains were found within a small unique plasmid [36]. With the exception of the toxin gene cluster, the 48-kb plasmid showed no identity or synteny with the larger group I plasmids [68]. Interestingly, a novel group II genome has recently been sequenced that produces BoNT E9 but whose genome is nearly identical to a nonproteolytic type B genome. This strain is unique in its location (Argentina) and neurotoxin sequence (E9); additionally, its genomic structure is unexpected for a BoNT E-producing strain [71].

A recent study of the genomes of several BoNT C strains has revealed a highly conserved chromosomal structure with minimal redundancy that contrasts with a richly varied plasmidome [66]. Up to six different plasmid or prophage sequences have been found, with abundant evidence that plasmids can be readily exchanged among group III strains and that segments of DNA have been exchanged among

the plasmids as well. The majority of these mobile genetic elements carry virulence factors, such as toxins (BoNTs, C2 toxin, C3 toxins; alpha and epsilon toxin homologs), bacteriocins and antibiotic resistance genes. Basic Local Alignment Search Tool (BLAST) analysis of phage sequences for D-1873 showed 87–100% identity with the phage from BoNT/C1 Stockholm. The regions containing the neurotoxin gene clusters and exoenzyme C3 showed 97 and 99% identity, respectively. The genes for C2 toxin, an actin-ADP-ribosylating toxin found in BoNT/C and BoNT/D strains, reside on a 107-kb plasmid [66]. When plasmid sequences pC2C203U28 (a non-neurotoxigenic variant of strain C203) and D-1873 pCLG1 (from strain D1873) were compared, they were found to be identical. This high level of identity indicates that the same mobile elements are able to freely move among group III bacteria. This has been confirmed by experiments where phages were lost and/or transferred among BoNT/C1- and BoNT/D-producing group III *C. botulinum* strains [72].

If one assumes that in order for strains to exchange genetic information they must germinate near each other, then it would follow that the ranges of these bacteria and/or their spores must be widespread and overlapping. The association of BoNT C/D strains with wild birds provides a convenient method for dissemination of spores during migration. There is some evidence that fish may carry spores and/or toxins, and their journeys may also spread these strains. Interactions of wild birds with domestic fowl or mammals may provide further opportunities for genetic transfer among BoNT C-, C/D-, D- and D/C-producing strains.

Genomic sequences from group IV strains are not currently available. However, it is known from DNA sequencing of neurotoxin clusters and other studies that BoNT G gene clusters are similar to those of group I and group III but show a unique rearrangement of their hemagglutinin genes [73]. It is also known that these gene clusters are found within large (80–100 kb) plasmids [74, 75].

BoNT/E4-producing Italian *C. butyricum* strains are the fourth species/group for which comparative genomic sequence information is available. As would be expected with different species, there is little synteny seen between BoNT/E-producing group II genomes and these genomes [68]. There is no genomic information available on Chinese *C. butyricum* strains, which may or may not cluster with their Italian counterparts.

Two distinctive BoNT gene clusters have been identified. One cluster contains genes (*ha* genes) encoding three hemagglutinin proteins that form part of the BoNT cluster. The second cluster does not contain *ha genes,* but instead contains three genes designated *orfX*1, *orfX*2 and *orfX*3. There have been a few recent reports that proteins matching those that would be encoded by the *orf* genes may be found in association with *ha–* toxin complexes, but their significance or potential functions are unknown. The cluster types are designated as *ha+/orfX–* or *ha–/orfX+*, respectively [20].

With the exception of type A strains, the cluster types are related to toxin serotype. BoNT/B, /C1, /D and /G strains all contain *ha+/orfX–* gene clusters, while BoNT/E and /F strain gene clusters are of the *ha–/orfX+* type [15, 73 , 76]. Type A strains may contain either gene cluster; however, the *ha+/orfX–* A1-producing strains are more widespread than *ha–/orfX+* A1, A2 and A3 strains. With the bivalent strains that

have been studied, the BoNT/B component has always been within a *ha+/orf*X– cluster, while the alternative toxin cluster (A or F) has been *ha–/orfX+*.

Analysis of the regions surrounding neurotoxin gene clusters shows that these genes are not inserted at random within genomes, but rather appear to be placed within discrete locations throughout the genome and within various plasmids and phages [68]. BoNT toxin gene clusters in group I strains are found at two discrete locations within the chromosome and two discrete locations within highly conserved plasmids. The two chromosomal locations are: (a) among the two to seven genes that compose the arsC operon (two gene clusters) and (b) at the oppA/brnQ operon (six gene clusters). The distance between these two sites is approximately 50 kb. Toxin gene clusters containing *ha* genes (*ha+/orfX–* A1 and BoNT/B strains) are located at the oppA/brnQ operon, while the genes from *ha–/orfX+* +strains, such as HA- A1, A2 and F1 strains, are at the arsC location [68, 73].

Group I strain neurotoxin gene clusters have also been found in two distinct locations on large, highly conserved plasmids. Bivalent Ba4 and Bf strains have A4 or F gene clusters located approximately 120 kb upstream from BoNT/B genes. Strains that produce only one toxin type, such as A3 or B1, have gene clusters located according to toxin type, with A3 at the A/F location and B1 at the B location [68, 70]. The identical location of *bont/A* and group I *bont/F* genes both within chromosomes and plasmids is an indication of a close relationship between strains containing these two serotypes.

The neurotoxin genes from group II strains may be located within the chromosome (type E strains) or within plasmids (nonproteolytic B4 strains). Interestingly, the toxin gene clusters for two *C. botulinum* type E and one *C. butyricum* type E strain are located at the same position within the chromosome, despite the limited genomic synteny among these strains. All three clusters are positioned such that they bisect the same gene, the *rarA* gene, in the same place [68].

A recent article has described an unusual *C. botulinum* strain having three distinct neurotoxin gene clusters that contain BoNT/A2, /F4 and /F5 genes [77]. The A2 gene cluster is located at the *arsC* location within the chromosome, and the F5 gene cluster is located at the A/F position within a large, 246-kb plasmid showing significant homology with the plasmids containing A3, B1, Ba4 and Bf gene clusters. The BoNT/F4 gene cluster is found at a unique location within the chromosome (*pulE*). The toxin cluster is inserted into the chromosome by splitting the *pulE* gene in a manner that is nearly identical with BoNT/E gene clusters within the *rarA* gene. It will be of interest to investigate whether these locations are common among type F4 and F5 gene clusters or if, in contrast to other toxin types, they are inserted at random.

The finding of highly similar or identical neurotoxin gene clusters within different clostridial groups and species, coupled with their association with mobile genetic elements such as plasmids, phages and various insertion sequence elements, indicates that a primary mechanism for diversity among these pathogenic bacteria is horizontal gene transfer [78].

The mechanisms that determine horizontal gene transfer of pathogenic genes among *C. botulinum* and related clostridia have not been completely elucidated.

BoNT/E gene clusters have been found within unrelated group II *C. botulinum* and *C. butyricum* strains, and BoNT/F gene clusters have been found within group I and group II *C. botulinum* strains, as well as *C. baratii*. The factors that may limit or target such transfers remain unknown.

10.4 Neurotoxins

Botulism is an intoxication that is caused by BoNTs. Thus, identification and characterization of these proteins and/or their genes provide the definitive confirmation of botulism. The earliest studies of toxins produced by *C. botulinum* involved observation of classic signs and mortality after injection of sample filtrates in animals [1]. Neurotoxin serotypes were differentiated based on protection by specific antisera. These serotypes were identified using alphabetic characters (A–G).

Georgina Burke was the first scientist to use an alphabetical nomenclature for the BoNT toxin serotypes [79]. In her initial studies, she differentiated 12 strains from California and Washington into serotypes A and B. The antisera and the system for differentiating strains she developed were then used to characterize toxin serotypes for more than 733 positive cultures isolated from samples collected in North America, Europe, and Asia [80–83]. These laborious studies provided initial evidence of the ubiquitous nature and worldwide distribution of BoNT/A- and /B-producing *C. botulinum* strains.

In 1922, several *C. botulinum* strains were isolated from independent laboratories in the USA and Australia that were not neutralized by type A or B antitoxins [5, 84]. The toxins produced by these strains were designated as serotype C. Several immunological differences were seen between the strains isolated in the USA and Australia, leading to subdesignations of Cα and Cβ [85]. The Cα strains produce toxins (BoNT C/D) that are mosaics, where the initial two thirds of the sequence is identical to BoNT/C1 and the final one third closely resembles BoNT/D [86]. In 1927, serotype D was identified from isolates associated with disease in cattle in South Africa [87]. A mosaic BoNT D/C toxin type similar in composition to BoNT C/D but reversed in sequence identities (two thirds BoNT/D and one third BoNT/C) has also been identified [86].

The first description of a type E strain associated with an outbreak of botulism was provided in 1937 [88]. This was followed by identification of type F in 1958 [89] and of type G in 1966 [46]. All identifications were based on reactivity to serotype-specific antitoxins.

Serotyping involves neutralization by specific antisera, typically using mice, and this continues to be the most common method for toxin differentiation. Concerns about excessive animal use and issues related to assay sensitivity, relatively large sample needs, and timing for completion of such assays are, however, prompting the development of alternative assays for the identification of BoNTs.

In 1990, the first complete BoNT sequences were published almost simultaneously [90, 91]. Other sequences followed in rapid succession. By 1995, sequences repre-

senting at least one member of each of the seven neurotoxin serotypes were publicly available [92–102]. In addition to enabling the first genetic comparisons of the BoNT serotypes, these initial sequences also provided evidence of underlying genetic differences between proteolytic and nonproteolytic organisms expressing distinct type B and type F toxins, differences in BoNT/E from *C. botulinum* and *C. butyricum*, and differences among food-borne BoNT/A1 and BoNT/A1(B) and infant BoNT/A2.

The DNA sequences and their translated amino acid sequences expanded knowledge about the nature and diversity of these large proteins. Discoveries related to the mechanisms of action of these toxins followed, as well as further studies involving the nature of the nontoxic components that form the BoNT complexes. Detection and characterization assays based on genetic sequences have been developed that can rapidly and sensitively detect and identify neurotoxin genes from a variety of clinical and environmental samples. Details about these developments are covered in the next chapter in this book. Some *C. botulinum* strains that contain more than one set of toxin genes have been reported [103] and it has also been noted that toxin genes can be "lost," rendering formerly toxic organisms nontoxic [104].

Differences in toxin sequence have led to the designation of toxin "subtypes," or perhaps more appropriately, toxin genetic variants. In the past, toxin subtypes were identified by differences in growth or biochemical characteristics, immunological recognition, or function. Proteolytic and nonproteolytic BoNT/B and BoNT/F strains were differentiated by their ability or inability to digest meat, egg or milk proteins. BoNT/A1 and /A2 were distinguished by differential binding to monoclonal antibodies [105]. With BoNT/B1 and /B2, differences in antibody binding and interactions with neuronal cell receptors were noted [106]. Some of these differences were actually associated with the organisms producing the toxins instead of the neurotoxins themselves; nevertheless, these subtype distinctions were confirmed by neurotoxin sequence comparisons.

Recently, toxin subtypes/genetic variants have been differentiated exclusively through comparison of nucleotide or amino acid sequences. Initially, toxin subtypes/genetic variants were defined as having amino acid sequence differences of approximately 2% or more when compared to existing strains within that serotype [70, 107]. Presently, subtype classification relies on identification of distinct phylogenetic clades [27, 73, 108, 109]. Large subtype sequence differences that were readily identified with BoNT/A, BoNT/C and D, and BoNT/F subtypes contrast with the more subtle differences seen in BoNT/B and BoNT/E strains (Fig. 10.1; Table 10.3). Currently, 35 toxin subtypes/genetic variants have been published that differ by as little as 1.5% to as much as 32.6% at the amino acid level (Table 10.3) [17, 27, 73, 108, 109].

Figure 10.1 illustrates the close relationships of several toxin subtypes/genetic variants. BoNT/A1 and /A5, BoNT/B2, /B3 and /B6, BoNT/E1, /E2 and /E3 and BoNT E7 and E8 all vary by less than 3.0% at the amino acid level. In contrast, BoNT/C1-C/D and BoNT/D-D/C differ from each other by approximately 24%, and BoNT/F5 diverges from other BoNT/F subtypes/genetic variants by 26–36.2% (Table 10.2). In the highly conserved BoNT/E serotype, BoNT/E9 is clearly very different from all other subtypes/genetic variants. While the overall range for all other subtypes is 6% or less, E9 differs from the others by ~10–12% [71].

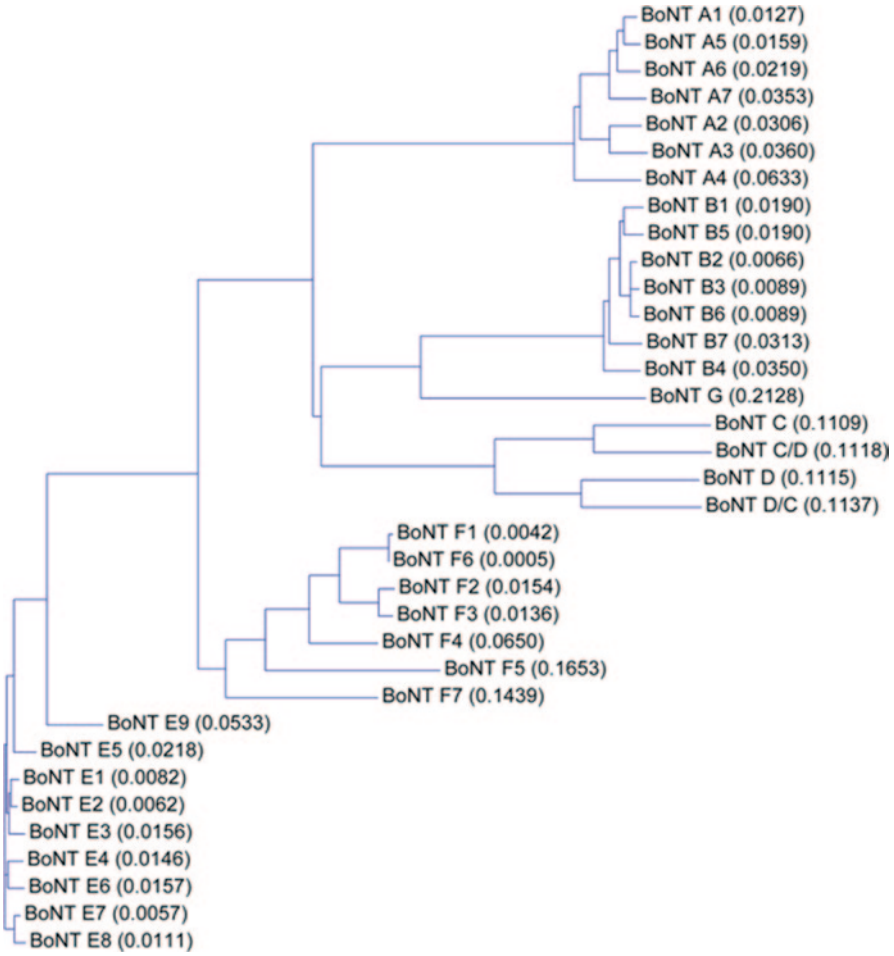

Fig. 10.1 Dendrogram showing the relationships of botulinum neurotoxin serotypes and subtypes. Amino acid sequences were compared using ClustalW alignment software

Differences in the underlying genetic sequences of these toxins reveal possible mechanisms for the diversity that is seen in these toxin protein sequences. Random mutations can be identified, but a major factor in genetic variation among the toxin subtypes is more likely recombination [68, 110]. On its grandest scale, the mosaic BoNT C/D and D/C subtypes might be considered the result of single large recombination events between BoNT/C1 and BoNT/D. An analysis of BoNT/C1-C/D and D-D/C gene sequence alignments indicates 96.9–97.8 % identity from nucleotides 1 to 2,649, but only 60.5–60.8 % identity from nucleotides 2,650 to 3,901. Conversely, BoNT/C1-D/C and D-C/D gene alignments show only 71.8–72.4 % identity for the first 2,649 nucleotides, but 85.6–97.8 % identity over the final 1,251 nucleotides.

Another example of a large-scale recombination event might be within the BoNT/F5 subtype, whose heavy chain sequence has 84.0–92.6 % identity at the nucleotide

Table 10.3 Amino acid identities among BoNT/A, /B, /E and /F subtypes. Percent identities were generated using a representative sequence for each subtype. Comparisons with ≥95 % identity are shown in red font and comparisons with less than 80 % identity are shown in blue font. The color coding illustrates the high degree of identity among BoNT/B and /E strains

	A2	A3	A4	A5	A6	A7
A1	89.9%	84.6%	89.3%	97.1%	95.6%	93.7%
A2	-----	93.0%	88.3%	90.3%	91.7%	89.7%
A3		-----	84.4%	85.0%	86.2%	84.8%
A4			-----	87.4%	87.8%	86.7%
A5				-----	95.8%	94.4%
A6					-----	93.0%

	B2	B3	B4	B5	B6	B7
B1	95.6%	96.0%	93.2%	96.1%	96.1%	94.7%
B2	-----	98.4%	93.9%	95.0%	98.4%	95.8%
B3		-----	93.7%	95.4%	98.1%	95.7%
B4			-----	92.7%	93.1%	93.6%
B5				-----	95.4%	94.3%
B6					-----	95.1%

	E2	E3	E4	E5	E6	E7	E8	E9
E1	99.0%	98.2%	97.3%	96.8%	96.9%	97.8%	96.2%	89.0%
E2	-----	97.4%	97.0%	96.3%	96.7%	97.0%	97.0%	89.2%
E3		-----	95.6%	95.1%	95.8%	97.3%	95.6%	88.7%
E4			-----	95.0%	96.8%	96.2%	96.1%	89.9%
E5				-----	94.7%	94.7%	94.0%	89.4%
E6					-----	96.2%	96.6%	88.1%
E7						-----	98.2%	89.1%
E8							-----	89.3%

	F2	F3	F4	F5	F6	F7
F1	83.4%	83.9%	92.2%	69.8%	87.4%	73.7%
F2	-----	97.0%	83.5%	74.0%	89.8%	68.6%
F3		-----	83.8%	74.0%	89.8%	68.9%
F4			-----	69.4%	86.9%	71.9%
F5				-----	73.6%	63.8%
F6					-----	69.8%

level with BoNT/F1–F4 and BoNT/F6 and 74.7 % identity with BoNT/F7 from *C. baratii*. However, the sequence of the light chain and the beginning of the heavy chain (amino acids 15–20) is unique, differing from all other BoNT/F subtypes by 51.2–53.1 %. This light chain sequence also differs from all other known BoNT serotypes and subtypes by 58.7–79.8 % [109].

Smaller scale recombination events have also been identified. For example, BoNT/A1 and /A2 genes show 99.1 % identity for their initial 1,050 nucleotides, but only 93.0 % identity thereafter. In contrast, BoNT/A2 and /A3 genes show only

90.8% identity for the first 1,205 nucleotides, but 99.0% identity over the remaining 2,686 nucleotides. Recombination analysis has indicated that the A2 variant is a recent recombinant of A1 and A3 lineages [27]. Other toxin subtypes show similar nucleotide differences that might be attributed to recombination events. A similar relationship between the BoNT/B2 and /B6 lineages can be seen. These two subtypes show 97–100% identity in nucleotide sequence until the final 390 nucleotides, where the identity drops to 93.9%. A third example of possible recombination may be found within the BoNT/E1, /E2 and /E3 subtype genes. BoNT/E1 and /E2 genes are identical in sequence for the initial 3,180 nucleotides, but a difference of 3.6% is seen from nucleotides 3,181–3,750. A similar pattern is seen with BoNT/E1 and /E3 genes, where sequences are nearly identical except from nucleotides 501 and 1,200 respectively, where a 3.6% difference is also seen. These examples appear to be relatively simple recombination events, which indicate they may be recent occurrences.

New toxin variants continue to be discovered, some with widely varying sequences and characteristics. It is remarkable that despite the genetic diversity seen among these toxins, their mechanism of action is highly conserved, resulting in the distinctive characteristic signs and symptoms that define botulism.

Some clostridial species, such as *C. tetani*, produce only one homogeneous toxin and others, such as *C. perfringens* and *C. difficile*, produce multiple toxins having a range of pathogenic effects. BoNT-producing *C. botulinum* species are unique in that they produce neurotoxins that are quite diverse in sequence but uniform in mechanism of action. These contrasts provide interesting examples of bacterial evolution.

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Chapter 11
Botulinum Neurotoxin Risks and Detection in Environment, Agriculture and Food Chains

Miia Lindström, Riikka Keto-Timonen and Hannu Korkeala

Abstract Botulinum neurotoxin (BoNT) is produced by the anaerobic bacterium *Clostridium botulinum* during its vegetative growth. *C. botulinum* strains are commonly found in the environment, animals and in food raw materials. The bacterium forms dormant endospores, which do not produce toxins. However, the spores are highly resistant to environmental stress and thus maintain the toxic potential in environmental and agricultural systems and the food chains that occasionally provide conditions favourable for spore germination and outgrowth into a toxic culture. Besides causing botulism to man and animals, BoNTs are widely exploited by medical industries in treatment of spastic diseases. Thoroughly validated, sensitive and specific assays for neurotoxin detection are thus needed not only for laboratory diagnostics of botulism outbreaks but also for potency testing of the pharmaceutical neurotoxin products.

Keywords Botulinum neurotoxin · Anaerobic · Vegetative growth · Endospores · Germination · Toxicoinfectious botulism · Food poisoning botulism · Toxin assays · Bioassay · Immunoassay · Sandwich · ELISA · Endopeptidase assays · Lab-on-a-chip

11.1 Botulinum Neurotoxin (BoNT) Producing Clostridia

Based on their serological properties, botulinum neurotoxins (BoNTs) are divided into seven types, A–G. BoNTs are synthesized by *Clostridium botulinum* groups I–IV and some strains of *C. butyricum* and *C. baratii* (Table 11.1 and Chap. 10 of this book). All these anaerobic bacteria are frequently found in the environment, the gastrointestinal tract of animals and in raw foods. BoNTs are produced by vegetative cultures of these bacteria, whereas the bacterial spores are dormant and do not produce toxin. Strains representing different groups or species produce different

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Table 11.1 Factors affecting the growth and toxin production by BoNT-producing clostridia

Property	*C. botulinum*				*C. butyricum*	*C. baratii*	
	Group I	Group II	Group III	Group IV			
BoNT types	A, B, F	B, E, F	C, D	G	E	F	[190]
Minimum growth tempertature	12–17	3–9 °C	15 °C	12–15 °C	12 °C	10–15 °C	[47, 55, 77, 190]
Minimum pH	4.6	5.0	5.1	Not known	4.8	Not known	[10, 190]
Spore heat resistance	High	Moderate	High	High	High	Not known	[108, 120, 123]

BoNT types (Table 11.1). BoNT types A, B, E and F are pathogenic to humans and some animals, while types C and D cause disease only to animals. Type G toxin has not been associated with illness.

Growth and toxin production by BoNT-producing clostridia require anaerobiosis but strictly reducing conditions are not needed, and growth in anoxic media with positive redox potential of up to 300 mV has been reported [124]. Other growth-limiting conditions vary by the group of bacteria and by strain. Generally, groups I, III and IV *C. botulinum* and BoNT-producing strains of *C. butyricum* and *C. baratii* are mesophilic with their optimum growth temperatures being around 37 °C, while group II *C. botulinum* strains are psychrotrophic with their generally accepted optimum growth temperatures being 28–30 °C (Table 11.1). Our recent findings suggest, however, that some type E strains grow faster at 37 °C than at 30 °C and thus resemble mesophilic bacteria in their growth [47]. Depending on the strain, growth of the mesophilic strains is inhibited by temperatures below 12–17 °C and above 41–48 °C [92, 152, 181]. By contrast, the psychrotrophic group II strains are inhibited by temperatures below 3–9 °C [47, 56, 77] and above 35–40 °C [47]. Large strain variation in growth temperature requirements challenges growth models and prediction of BoNT formation in different food, environmental and agricultural matrices. This is further complicated by the effect of intrinsic factors, such as pH and water activity. Growth-limiting pH for most BoNT-producing clostridia is in the range of 4.5–5.1 and water activity is 0.94–0.97 (Table 11.1). Growth-limiting factors for groups III and IV *C. botulinum* and BoNT-producing *C. baratii* have been poorly characterized and existing literature data are based on a limited number of strains (Table 11.1).

11.2 Botulism Risks in Agriculture and Foods

11.2.1 BoNT-Producing Clostridia in Environment

BoNT-producing clostridia are frequently found in the environment. Spores have been isolated from soils, sea and freshwater sediments, animals (including wild and domesticated mammals, birds, insects and fish) and plants all over the world

(Table 11.2). In general, all group I, part of group III and all group IV *C. botulinum* strains predominate in soils, whereas group II and some group III strains are associated with aquatic environments (Table 11.2). Particularly high prevalence of group II *C. botulinum* type E has been found in sea and freshwater sediments in the Baltic Sea area, coastal waters of USA and Canada and in Japan (Table 11.2). Type B botulism cases due to group II *C. botulinum* in meat and meat products in Poland [84], however, suggest a terrestrial origin for part of group II strains.

BoNT-producing *C. butyricum* has been isolated in soils of China [134], but strains apparently also exist in Italy where human botulism due to this species is relatively common [60]. BoNT-producing *C. baratii* has not been reported in environmental samples.

In most environmental niches, BoNT-producing clostridia are supposed to occur as dormant spores. Spores are stable against environmental stress factors, such as low water activity, lack of nutrients and extreme pH and temperatures, and they can persist for decades. Germination and outgrowth to form toxic cultures require anoxic conditions, organic material and water. Certain amino acids have been reported to trigger germination [7, 161]. Individual strains and spores respond to these germinants with different rates [198, 199], depending on their history and preceding sporulation conditions [212]. Other environmental factors enhancing germination include the presence of lytic enzymes [8, 156], sodium bicarbonate, sodium thioglycollate and heat shock [161].

Temperature requirements for germination are less stringent than for growth; germination of group II *C. botulinum* strain Eklund 17B has been reported at a temperature range of 1–40 °C [161] while growth of this strain has been reported at a temperature range of 4 °C [56] to 35 °C [47]. Moreover, germination from type A spores was reported at 70 °C [168], a temperature eliminating most vegetative cultures of clostridia.

The environmental niches assumed to favour spore germination and growth include dead animals and rotting carcasses. Cessation of blood circulation upon death of an animal creates anaerobic conditions, and animal tissues and gastric contents provide a wealth of nutrients to trigger germination and support growth. When formed in the gastrointestinal lumen of a dead animal, the neurotoxin may be diffused into the tissues of the carcass and act as a contamination source to live animals. Moreover, live animals sick with botulism may further spread the toxin. Wild birds and fish are frequently reported to suffer from botulism [82, 154], whereas carnivores are considered relatively resistant to BoNTs [119]. Potential resistance mechanisms include humoral immunity [190, 197] and differentially structured BoNT receptors in the gut or in the presynaptic neurons.

How soils and sediments support spore germination and outgrowth into toxic cultures is unclear but apparently depends on the water activity, redox potential and availability of organic matter. The markedly larger genetic diversity reported for group II *C. botulinum* strains found from the Baltic sea and freshwater sediments [90, 91] than diversity of group I *C. botulinum* isolated from soils of Finland [145, 146] may suggest aquatic sediments to support more frequent growth cycles than soils [122].

Table 11.2 Prevalence of BoNT-producing clostridia in the environment

Country	Sample material	Prevalence, mean spore count,(expected) group or species of BoNT-producing isolate, BoNT type(s)	Reference(s)
Argentina	Soil	24–38 %, NR[a], *C. botulinum* I+IV, A+B+F+G	[73, 125]
Argentina	Medicinal plants	30–8 %, 30–400/kg, *C. botulinum* I, A+B+F	[19, 20]
Australia	Soil	94 %, 400/kg, NR, B	[203]
Canada	Sediment	18 %, NR, *C. botulinum* II, E	[115]
China	Soil	10–90 %, NR, *C. botulinum* I+*C.butyricum*, A+B+E	[134, 218]
Denmark	Sediment	81 %, 80/kg, *C. botulinum* II, B+E	[94]
Faroe Islands	Sediment	2 %, 0.9/kg, *C. botulinum* II, E	[94]
Finland	Soil	31 %, 50–1050/kg, *C. botulinum* I+II, A+B+E	[146]
Finland	Sediments	68–80 %, 2.2–940/kg, *C. botulinum* II, E	[89, 90, 91]
Finland	Pollen	7–15 %, 60–690/kg, *C. botulinum* I+II, A+B+E	[146]
France	Sediment	4–9 %, 1–2/kg, *C. botulinum* II, B, E	[59, 186]
Greenland	Sediment	86 %, 50/kg, *C. botulinum* II, E	[94]
Iceland	Sediment	3 %, 3/kg, *C. botulinum* II, E	[94]
India	Soil	10 %, 39/kg, *C. botulinum* II, E	[50]
Indonesia	Sediment	2 %, NR, *C. botulinum* I+III, A+B+C+D+F	[200]
Italy	Soil	10 %, NR, *C. botulinum* I, A+B	[42]
Japan	Soil	4–82 %, NR, *C. botulinum* II, B+E	[217, 218]
Poland	Soil	31 %, 80/kg, *C. botulinum* II, E	[220]
Sweden	Sediment	64–83 %, 310–530/kg, C. *botulinum* II, B+E	[31, 101]
Switzerland	Soil	44 %, NR, *C. botulinum* I+II+III+IV, A+B+C+F+G	[194]
Paraguay	Soil	NR, NR, *C. botulinum* I+III, A+C+F	[219]
Russia	Soil and water	2–62 %, NR, *C. botulinum* I+II+III, A+B+C+E	[112, 167]
Thailand		2 %, NR, *C. botulinum* II+III, D+E	[206]
United Kingdom	Soil	4–35 %, 1–9/kg, *C. botulinum* II, III, B+C+D+E	[184, 185]

[a] *NR* not reported

11.2.2 BoNT-Producing Clostridia in Agricultural Systems

As a consequence of high prevalence in nature, BoNT-producing clostridia are intrinsically present in agricultural systems (Table 11.3) and thus pose serious, and partly yet poorly understood, public health risks for humans through the environment and food chains.

11.2.2.1 Animal Feed

Numerous human botulism outbreaks due to preserved vegetables [110, 177, 195] suggest a heavy contamination of plants with *C. botulinum* spores. This is no surprise as cultivated land often contains spores [146]. Anaerobic packaging of ensiled hay intended for feeding cattle and horses has become a common practice to pre-

Table 11.3 Prevalence of BoNT-producing clostridia in agricultural niches

Country	Sample material	Prevalence, mean spore count, (expected) group or species of BoNT-producing isolate, BoNT type(s)	Reference(s)
Denmark	Fish pond bottom	28 %, 67/kg, *C. botulinum* II, E	[96]
Finland	Fish pond bottom	68–83 %, 150–360/kg, *C. botulinum* II, E	[6, 90, 91]
Finland	Swine faeces	3 %, 1,400/kg, *C. botulinum* I, B	[139]
Finland	Swine blood	100 %, 3,100–6,400/kg, *C. botulinum* I + III, A + B + C	[141]
Finland	Untreated slaughter by-products	10–12 %, 30–100/kg, *C. botulinum* III, C	[141]
Finland	Acid-treated slaughter by-products	4–10 %, 30/kg, *C. botulinum* III, C	[141]
Finland	Animal fats	45 %, 30–140/kg, *C. botulinum* III, C	[141]
Finland	Bees	10 %, 110–510/kg, *C. botulinum* I + II, A + B + E	[146]
Germany	Cattle faeces	4 %, NR[a], NR, NR	[109]
Germany	Swine faeces	24 %, NR, *C. botulinum* II, III, B + C + E	[109]
Germany	Slaughter by-products	36 %, NR, NR, NR	[109]
Germany	Meat meal	4 %, NR, *C. botulinum* II, E	[109]
Germany	Biocompost material	54 %, NR, NR, A + B + C + D + E	[21]
Sweden	Cattle faeces	73 %, 470/kg, NR, B	[44]
Sweden	Swine faeces	62 %, 99/kg, NR, B	[43]
Sweden	Unpasteurized and pasteurized biogas material	NR, NR, *C. botulinum* I + II and *C. butyricum*, NR	[13]
The Netherlands	Cattle farms	NR, 100–3,000/kg, NR, B	[150]
The Netherlands	Cattle faeces	13–100 %, NR, NR, B	[149, 150]
UK	Fish pond bottom mud	24 %, NR, *C. botulinum* II, B	[28]
UK	Landfill	5–63 %, 2/kg, *C. botulinum* II + III, B + C + D + E	[154]

[a] *NR* not reported

vent the feed from aerobic spoilage. If the hay is not acid treated to bring the pH below 5, conditions supporting spore germination, growth and toxin production may arise [148]. Occasional incarceration of wild rodents or small pets into the feed mass serves as an additional source of botulinal spores or neurotoxins to food animals. Botulism outbreaks in food animals due to contaminated feed are frequently reported [123, 140, 155]. In addition to BoNT/C and BoNT/D, food animals may be highly sensitive to BoNT/A, BoNT/B and BoNT/E. Therefore, botulism in domestic animals also poses a potential risk to humans.

A dramatic example of feed intoxication botulism occurred in Finland in 2003 when more than 50,000 foxes and minks were affected by BoNT/C [119]. Although these animals are not intended for human consumption and BoNT/C has not been linked to human botulism, such large outbreaks pose considerable environmental risks. Isolation and destruction of such a large amount of carcasses and their

excreta, which can be considered as highly toxic material, is not straightforward, and in the possible case of other BoNT types, would be a tremendous challenge for public health protection. Similar problems faced during large type C outbreaks in broiler chickens raise additional concern of toxic aerosol formation in the closed halls where hundreds or thousands of birds and their bedding material may contain BoNTs.

Apart from intoxication botulism, a toxicoinfective form in cattle due to consumption of dormant clostridial spores followed by their germination into toxic culture in the animal's intestine seems relatively common [22, 142]. This form of botulism may be manifested by delayed, persistent and/or mild symptoms and thus diagnosis may be challenging. Such animals may spread the neurotoxin and BoNT-producing clostridia in their faeces for extended time periods and cause a risk to other animals and their caretakers, and eventually to the food chain at slaughter.

11.2.2.2 Food Chains

Meat animals frequently carry *C. botulinum* spores in their gastrointestinal tracts (Table 11.3) and most probably in their skin and claws as a result of environmental contamination. Frequent contamination of slaughter by-products with *C. botulinum* types C and D, but occasionally also type B spores (Table 11.3), suggests that the animals serve as a continuous contamination source for the meat chain. The large number of botulism outbreaks in continental Europe due to consumption of preserved meat in the past decades [84] indicates that meat is a common source of *C. botulinum* spores; however, reports on spore contamination levels in raw meats and meat products are scarce.

A human health risk may arise from slaughter of animals suffering from mild botulism. Their meat and tissues are prone to contain BoNTs. However, it is not clear how efficiently the structural forms of BoNTs present in the animal tissues can be absorbed if consumed by humans. Moreover, the toxins are relatively heat labile [179] and would thus be inactivated during meat processing.

The probability of BoNT secretion into raw milk of sick animals has been unclear but a challenge study with three dairy cows suggests secretion is unlikely or occurs at a very low level [137], at least in cows with no underlying disease. Moreover, standard pasteurization at 72 °C for 15 s was shown to inactivate at least 99.5 % of types A and B toxins [215]. A relatively low botulism risk related to consumption of milk is further supported by the small number of milk-related human botulism outbreaks reported in the last century [123]. The British Advisory Committee on the Microbiological Safety of Foods (ACMSF) advised that the consumption of meat and milk from healthy animals on farms with cattle, sheep or goat botulism needs not be restricted [3, 4]. However, the Committee stated that should toxicoinfectious botulism be found to be more common than currently considered, or should BoNT types other than C and D emerge as a common cause of disease in cattle, the risk to food safety be reassessed [3].

C. botulinum group III strains are frequently present in the poultry chain. The gastrointestinal tract of broiler chickens frequently contains spores, and broiler chickens are also susceptible to botulism. Large botulism outbreaks in poultry chickens have become common especially in the Nordic countries, causing large economic losses [180]. The reason for the increasing number of outbreaks remains unclear. Poultry litter and bedding have been often suspected as a cause of large botulism outbreaks in cattle [155]; thus, the use of poultry litter as a fertilizer has been discouraged [3]. Since the most commonly detected BoNTs in the poultry chain include types C, D and the chimaeric C/D, human health risks due to poultry botulism are thus far considered small.

Fish contain *C. botulinum* spores in their gut, gills and skin; thus, fish production systems are frequently contaminated with the organism, particularly with type E but occasionally also with group II type B strains (Tables 11.3 and 11.4). In a Finnish study, an average of 20 % of fish was contaminated [97]. The highest prevalence of 40 % was observed in the Baltic herring, which is used to feed cultivated whitefish and salmon. Consequently, nearly 20 % of these fish species were also contaminated. Fish ponds with natural mud bottom were more contaminated than those with a concrete bottom or bottom cleaning systems [28, 90, 91]. Surprisingly, also marine net cage-farmed fish were highly contaminated [90, 91]. At least salmonid fishes may also develop botulism [95] and further spread the disease to other fish and wild birds. The most significant human health risk arises from consumption of minimally processed or poorly fermented, hermetically sealed fish or fish products (reviewed by [84, 120, 208]).

The honey production chain is rich in *C. botulinum* group I spores (Table 11.4). A Finnish study showed that high numbers of spores were present in pollen and bees, and subsequently in beehives, dead bees and beeswax (Tables 11.2 and 11.3). Important steps in production to lower the *C. botulinum* contamination in honey include cleanliness of the honey extraction facility, hygienic manufacturing practices and incorporation of a heating step in the wax foundation cleaning procedures to eliminate spores [146]. Due to the low water activity, *C. botulinum* spores are not able to germinate and grow in honey. However, the use of honey in other food systems with growth-supporting conditions may introduce a risk. Moreover, small babies and adults undergoing heavy antibiotic treatments, with gastrointestinal wounds or other underlying conditions with repressed intestinal microbial populations should not consume honey due to the risk of toxicoinfectious botulism.

11.2.2.3 Sustainable Policies

Biogas production from manure, slaughter by-products, food and municipal waste by anaerobic digestion has become an essential part of sustainable energy policy. The digested residues may be further used as fertilizers for cultivation or pasture. To control the spread of pathogens, most biological biogas materials are pasteurized at 70 °C before anaerobic digestion. The pasteurization of biogas material was shown to be inefficient to destroy *C. botulinum* spores [13]. Moreover, although *C.*

Table 11.4 Prevalence of BoNT-producing clostridia in foods

Country	Sample type	Prevalence, mean spore count, (expected) group or species of BoNT-producing isolate, BoNT type(s)	Reference(s)
Argentina	Honey	1–75 %, 25–55/kg, *C. botulinum* I, A+B	[46, 143]
Australia	Honey	29 %, 36/kg, *C. botulinum* I, A+B	[143]
Canada	Liver sausage	2 %, 0.2/kg, *C. botulinum* I, A	[87]
Canada	Bacon	<1 %, 0.1/kg, *C. botulinum* I, A+B	[86]
Canada	Mushrooims in oil	NR[a], 150–410/kg, *C. botulinum* I, B	[85]
Cuba	Honey	20 %, 20/kg, *C. botulinum* I, B	[143]
Denmark	Raw honey	24–28 %, NR, *C. botulinum* I, II, A+B+E	[144]
Finland	Fresh fish	2–40 %, 100–238/kg, *C. botulinum* II, E	[90, 91, 97, 135]
Finland	Fish roe	8 %, 58/kg, *C. botulinum* II, E	[97]
Finland	Smoked fish	3–7 %, 35–160/kg, *C. botulinum* II, E	[97]
Finland	Vacuum-packaged vegetarian sausage	6 %, 24.5/kg, *C. botulinum* I+II, B+E	[118]
Finland	Canned deer meat	9 %, 1.0/kg, *C. botulinum* I, B	[118]
Finland	Honey	2–7 %, 50/kg, *C. botulinum* I, B	[143, 146]
France	Fish	8–17 %, 2–12/kg, *C. botulinum* I+II, A+B+E+F	[36, 59]
France	Meat and poultry	8 %, 2–4/kg, *C. botulinum* I, A+B	[36]
France	Honey	20 %, 40/kg, *C. botulinum* I, A+B	[143]
France	Aroma, sauce and gravy	2 %, 0.3–0.6/kg, NR	[36]
France	Thickening agents, starch	16 %, 3–7/kg, *C. botulinum* I, A+B	[36]
France	Dehydrated dairy ingredients	12 %, 2–5/kg, NR	[36]
Germany	Fish	30 %, 80/kg, *C. botulinum* II, E	[98]
Germany	Vacuum-packaged potatoes, spices	0 %	[18]
Germany	Honey	2 %, 0.8/kg, *C. botulinum* I, A	[127]
India	Raw fish and shellfish	26 %, NR, *C. botulinum* I+III, A+C+D	[113]
India	Cured fish	13 %, NR, *C. botulinum* III, C+D	[114]
Indonesia	Fresh fish	15 %, NR, *C. botulinum* I+II+III, A+B+C+D+E+F	[83]
Italy	Dairy products	3 %, <10,000/kg, *C. botulinum* I, A	[64]
Italy	Honey	50 %, 20/kg, *C. botulinum* I, A	[143]
Norway	Raw honey	9–14 %, NR, *C. botulinum* I+II, B+E+F	[144]
Russia	Vegetables	43 %, NR, *C. botulinum* I, A+B	[147]
Spain	Honey	25 %, 60/kg, *C. botulinum* I, A+B	[143]
Sweden	Honey	2 %, NR, *C. botulinum* II, E	[144]
Thailand	Fish	<1 %, NR, *C. botulinum* II+III, E+D	[206]
The Netherlands	Fresh mushrooms	0 %, <2/kg	[151]
UK	Fresh fish	11 %, NR, *C. botulinum* II+III, B+C+E+F	[35]
UK	Vacuum-packed fish	<1 %, NR, *C. botulinum* II, E	[32]
UK	Vacuum-packed bacon	14 %, 2/kg, *C. botulinum* I, A+B	[165]
UK	Refrigerated packaged foods	0 %	[71]

Table 11.4 (continued)

Country	Sample type	Prevalence, mean spore count, (expected) group or species of BoNT-producing isolate, BoNT type(s)	Reference(s)
USA	Fresh fish and seafood	13–22 %, 90–2,400/kg, *C. botulinum* I+II, A+B+E+F	[15, 41]
USA	Filleted fish	67 %, NR, *C. botulinum* I+II, A+B+E+F	[15]
USA	Smoked fish	5–20 %, NR, *C. botulinum* II, E	[88]
USA	Semi-preserved meat	2 %, NR, *C. botulinum* I, A+B	[1]
USA	Onions	7 %, NR, *C. botulinum* I, A	[192]
USA	Chopped spinach	12 %, NR, *C. botulinum* I, A+B	[147]
USA	Packaged perishable foods	2 %, NR, *C. botulinum* II, B+E	[205]
USA	Convenience foods	<1 %, NR, *C. botulinum* I, B	[100]

[a] *NR* not reported

botulinum was absent, other clostridia were detected after the digestion step [13]. Thus, further spread of BoNT-producing clostridia to cultivated land cannot be excluded.

Recycling of household biowaste and biocomposting have become highly popular in developed countries. Considering the high prevalence of BoNT-producing clostridia in foods (Table 11.4), the individual human health risks related to this trend raise increasing concern. While a properly set up biocompost creates heat and may exceed 80 °C in temperature, which kills many vegetative bacteria and most probably eliminates BoNTs, the temperature does not kill most bacterial spores. A German study showed that 50 % of marketed biocompost samples contained *C. botulinum* groups I, II and III spores or cells [21]. BoNTs were not detected, probably due to heat and/or proteolytic inactivation. Survival and potential multiplication of BoNT-producing clostridia in biocomposts, however, poses a risk for further contamination if used as organic fertilizers for cultivation or pasture.

11.2.3 BoNT-Producing Clostridia in Foods

Prerequisites for classical food poisoning botulism are that (1) food contains spores of BoNT-producing clostridia, (2) spores are not destroyed by food processing, (3) food and its storage conditions support spore germination and outgrowth into a toxic culture and (4) preformed BoNT is not destroyed by heating before consumption. Alternatively, addition of ingredients containing preformed BoNT in foods not supporting growth of BoNT-producing clostridia themselves may have detrimental consequences [153].

As a result of high prevalence in the environment and agriculture, spores of BoNT-producing clostridia are frequently present in any kind of food raw materials (Table 11.4). Particularly high prevalence has been reported for fresh seafood

and honey. Resistance of *C. botulinum* spores to treatments employed in the food industries is demonstrated by the presence of groups I and II *C. botulinum* spores in many different types of processed foods (Table 11.4). Also, group III spores are occasionally present. A study in the UK showed *C. butyricum* also to be present in 30 % of foods but no BoNT-producing isolates were recovered [70]. The prevalence of BoNT-producing *C. baratii* in foods is not known.

The heat resistance of *C. botulinum* spores has been extensively studied (reviewed by [108, 120, 123]). Group I and III spores generally require sterilization at above 100 °C for elimination, whereas group II spores are destroyed by pasteurization processes at 70–90 °C. To control botulism risks related to commercially produced foods, heat treatments targeted to eliminate 10^{12} group I spores ('botulinum cook') have been recommended for the canning industry, and those targeted to eliminate 10^6 group II spores have been recommended for minimally processed refrigerated foods [2, 53]. The time–temperature combinations required for achieving these log reductions in spore counts vary and should be determined for each product separately. High fat and protein content and low water activity protect spores from detrimental effects of heat and thus increase the apparent heat resistance of spores in complex matrices. By contrast, low pH of the heating matrix enhances spore destruction. Spore heat resistance also varies by bacterial strain [108] and the preceding sporulation temperature [17]. As discussed earlier, lytic enzymes in food matrices assist sublethally injured spores to germinate [8, 156], thus also increasing the apparent heat resistance of spores.

Prevalence studies on foods (Table 11.4) and epidemiological reports demonstrate that very different kinds of foods can support *C. botulinum* growth and BoNT production and thus serve as vehicles for human botulism. Control of growth and toxin production from *C. botulinum* spores is often a net effect of a mild heat treatment, intrinsic factors (pH, water activity, E_h) achieved by the use of preservatives, salt and packaging and extrinsic factors such as storage temperature. Formulation of homogeneous foods, such as liquids and emulsions, for safety is theoretically straightforward but consumer preferences, health concepts and storage conditions may set limitations [75, 74]. Assessment of the net risk of *C. botulinum* growth and toxin production becomes significantly more complex with the modern foods, which often include an entire meal with multiple ingredients, components and compartments with totally different intrinsic factors and microbial populations. A dramatic reminder of this was the type B botulism outbreak associated with hazelnut yogurt in the UK [153]. While the yogurt itself had a low pH and therefore was unlikely to have supported the growth and toxin production of *C. botulinum*, the formulation for safety of the hazelnut puree used to sweeten the implicated yogurt failed due to the replacement of sugar with aspartame, which has a different effect on water activity than sugar, and this eventually allowed toxin formation in the puree. The outbreak affected 27 patients with one death [153].

Bacterial strain and cell variation further complicates risk assessment [47, 92, 198, 199]. Mathematical models predicting the growth and toxin production from a certain number of spores [16, 128, 131] can be used as decision-making tools in the

food industry, but should not form the sole basis for product safety due to the risk of fail-safe predictions [99].

Foods preserved at homes or in small kitchens with poor hygienic practices have traditionally been implicated as vehicles for classical botulism. Insufficient heating, leaking containers, lack of growth inhibitory substances such as salt and acid and abused storage temperature are typical pitfalls behind outbreaks that are often restricted to a family or a small gathering. Apart from home-canned foods, commercial products are increasingly implicated in food botulism. These outbreaks may be large and challenging to identify. A recent example affected six patients, one dying, in different states of USA and Canada, and was traced to commercial pasteurized carrot juice with a neutral pH [177]. Laboratory investigations revealed BoNT/A production in five out of ten unopened juice packages of the implicated brand when stored at 35 °C, indicating a heavy initial contamination of the juice with *C. botulinum* spores or cells and abused storage temperature. Safe production and distribution of any low-acid foods should thus rely on multiple barriers controlling the risk of botulism [2, 53].

While food-borne botulism is often a severe condition requiring intensive care and respiratory support, mild cases with gastrointestinal symptoms dominating are occasionally reported in association with more severe cases [111, 121, 191]. Considering the high risk of botulism related to modern processing and packaging of foods [120], undiagnosed mild cases are probably much more common than is generally considered.

11.3 Detection of BoNT

Botulism is a rare but life-threatening disease. Rapid diagnosis is essential for successful treatment [166, 221]. Definite diagnosis is based on detection of BoNT in serum, vomitus, gastric content and/or stool [166, 221]. However, the diagnosis of botulism often rests on clinical symptoms due to the lack of rapid and sensitive laboratory diagnostic tools [30].

BoNT is the deadliest poison known, with its minimum lethal dose (MLD) being approximately 1 ng/kg in humans [72]. Due to the extreme potency and lethality of BoNT, it is considered to be a possible agent of bioterrorism [11]. Despite its extreme toxicity, this poison is also used as a potent medication in low doses for the treatment of various neuromuscular disorders [81, 178], hyperhidrosis [207], pain [24] and migraine (Hanchanale 2010). Nowadays, BoNTs are also widely used in minimally invasive aesthetic facial procedures such as the cosmetic reduction of facial wrinkles [45]. In addition, BoNTs have shown potential to serve as a tool for pharmaceutical drug delivery [76, 178]. Due to these pharmaceutical applications, toxin assays are also needed to assess the potency of preparations of therapeutic toxin.

During the last decade, toxin assay research has focused on the development of sensitive in vitro diagnostic tests that could replace the mouse bioassay. In addition,

there is a growing demand to develop fast toxin assays that have sample-to-answer analysis time of less than 1 h [78]. Although promising sensitive and fast toxin assays have been developed, only few are able to detect all seven neurotoxin types simultaneously, and their use in diverse clinical and food samples is often limited since complex biological matrices may cause interference in assays [26, 117]. The high toxicity of BoNT also challenges assay development; a practical test must be highly sensitive since too high a detection limit may result in failure to detect lethal concentrations of toxin, e.g. in food or water samples [93].

11.3.1 Mouse Bioassay

The mouse bioassay is the gold standard for detection of BoNTs in clinical and food specimens due to its high sensitivity and specificity [78, 174]. The assay involves intraperitoneal injection of the sample into mice followed by monitoring the mice periodically for 48 h for typical symptoms of botulism. In most cases, typical signs, ruffled fur, laboured breathing, muscle weakness and finally death due to respiratory failure, are observed within the first 24 h. The amount of toxin can be estimated by using serial dilutions of the sample and the toxin type can be determined by protecting the mice with monovalent antitoxins before injection of toxic sample [193]. The mouse bioassay is highly sensitive; detection limit is < 10 pg/ml [78]. It has also been successfully applied to various sample materials such as food, faeces, serum, gastric content, wound and supernatant of bacterial cultures. However, the mouse bioassay has several drawbacks: It is expensive, laborious and involves the use of a large number of laboratory animals [117]. In addition, only a limited number of laboratories perform the mouse bioassay and for clinical diagnostic purposes, the analysis time is generally too long [221].

11.3.2 Local Flaccid Paralysis, Mouse Phrenic Nerve-Hemidiaphragm and Cell-Based Assays

Nonlethal, local, flaccid paralysis assay is considered to be a humane alternative to the lethal mouse bioassay since the subcutaneous injection of BoNT causes less distress and impaired movements in the animals [103, 117]. The drawback is that the method is best suited for potency testing of toxin preparations and it is not validated for analysis of complex matrices [117]. Mouse phrenic nerve-hemidiaphragm assay is an in vitro test that can be used as a functional alternative to the mouse lethality test [162]. However, this test still sacrifices laboratory animals and the assay is mainly used for evaluating toxin and antitoxin preparations [162, 174]. Various cell-based assays, which require the use of relatively few animals or no animals, have also been used for the evaluation of toxin potency and the screening of neutralizing antibodies [157, 158, 170, 173].

11.3.3 Immunological Methods

To overcome the drawbacks of the mouse bioassay, a wealth of in vitro assays have been developed (Table 11.5). The majority of available BoNT detection kits utilize immunology [117]. These antigen–antibody-based in vitro assays rather depict the antigenicity of the sample than measure its biological activity [67]. Thus, the toxin that is inactivated, e.g. by heat treatment, may cause positive results [117]. The affinity of the antibodies to the target antigen has a large influence on the sensitivity of the immunoassay [12, 164, 172] and therefore the lack of high-quality antibodies may hamper the assay development [117]. Yet, recently, several promising high-affinity antibodies specific for BoNTs have been generated and they may prove to be beneficial in immunoassay development [164, 172, 202]. Complex sample matrices, such as food and faeces, may interfere with the antigen–antibody interaction or with the toxin complex [48, 176]. However, in general, immunological methods are technically simpler to perform, faster and easier to interpret than the mouse bioassay [117]. Total time needed to analyse samples, e.g. by enzyme-linked immunosorbent assay (ELISA) is approximately 5–6 h [174].

The early immunological assays, such as radioimmunoassay, electroimmunodiffusion and passive haemagglutination assay, suffered from poor sensitivity [25, 102, 136]. Similarly, the first protocols utilizing ELISA showed either low sensitivity or specificity, but along with developments in signal amplification, the sensitivity of ELISA nowadays approaches that of the mouse bioassay [48, 117]. Briefly, a capture antibody is immobilized in the wells of a microtitre plate. After the toxin has bound to the capture antibody, a primary detection antibody is added. A secondary detection antibody with a conjugated enzyme is used to create a signal through enzymatic cleavage of a chromogenic substrate [78, 117, 174]. Spectrophotometers can be used to quantitatively measure the colour intensity [174]. To increase the sensitivity of conventional ELISA, various improved protocols have been developed such as an enzyme-linked coagulation assay (ELCA; [51, 52]), sandwich ELISA utilizing high-affinity monoclonal antibodies [172, 196], amplified ELISA [176] and peptide–polymer-based capture ELISA [126]. Time-resolved fluorescence (TRF) assay, which employs lanthanide-labelled detector antibodies that have unique fluorescence properties, such as long fluorescence half-life, has also shown higher sensitivity compared to conventional ELISA [159].

ELISA has been used to detect BoNTs in therapeutic preparations [57], various foods [63, 172, 176] and clinical samples [49]. Each food has a unique biochemical composition, and components of the food may interfere either with the toxin complex or with the antigen–antibody interaction in the ELISA process [176]. Therefore, each food type has to be tested to prove that it is not inhibitory to the ELISA or produce false-positive results [63]. In addition, complex food matrices may require additional sample pretreatments before being assayed [176]. With these limitations in mind, the ELISA test can be used as a fast preliminary screening tool for various food products [176]. In addition to food samples, other complex matrices such as faeces have been shown to interfere with the ELISA test, resulting in decreased sensitivity [49].

Table 11.5 Diagnostic assays for the detection of BoNTs

Assay	BoNT type(s)	Detection limit	Analysis time	Application(s)	Reference(s)
Mouse bioassay	A, B, C, D, E, F	<10 pg/ml	1–4 h	Bacterial cultures, serum, gastric contents, faeces, foods, environmental samples	[78, 117]
Radioimmunoassay	A	100 MLD	8 h	Purified toxin	[25]
ELISA	B	100 fg	NR[a]	Skim, 2% and whole milk	[172]
	A, B	NR	~8 h	Faecal samples of infants with botulism	[49]
	A	4–8 pg/ml	6 h	Toxin in therapeutic preparations	[57]
	A	2 pg/ml	NR	Skim, 2% and whole milk	[196]
	A, B	0.2 ng/ml	~8 h	Human serum	[204]
	A	NR	~8 h	Chili linked to botulism outbreak	[63]
	A, B, E, F	A 60 pg/ml; B 176 pg/ml; E 163 pg/ml; F 117 pg/ml	~8 h	Toxin complex, bacterial culture supernatant, broccoli, orange juice, water, cola soft drinks, vanilla extract, oregano, potato salad, apple juice, meat products, dairy foods	[176]
	B	<2–4 ng/ml	5–18 h	Toxin complex	[79]
	A	1 pg/ml–10 ng/ml	<3 h	Assay buffer, human serum, urine, milk, honey, hotdog, canned tuna, cheese	[126]
ELISA–ELCA	A, B, E	5–10 pg/ml	~1–2 d	Purified toxin, culture filtrates	[51, 52]
Lateral flow test	A, B, E	A, B 10 ng/ml; E 20 ng/ml	15–30 min	A variety of food and seafood	[175]
Lateral flow test	A	50 ng/ml	10 min	Purified toxin, serum, broth, urine	[38]
	A	15–150 pg/ml	20 min	BoNT/A heavy chain, vegetables, seafood	[5]
	A	20–80 pM	40 min	Serum, nasal swabs, fresh milk, sugar, flour, talcum	[12]
Immunoaffinity column assay	A, B, E, F	A 0.5 ng; B, E, F 5–50 ng	15–30 min	Milk, bottled water, grape juice with peach juice	[27]
	C, D	0.5 MLD/ml	4–16 h	Bacterial culture supernatant	[67]

Table 11.5 (continued)

Assay	BoNT type(s)	Detection limit	Analysis time	Application(s)	Reference(s)
	A	0.09–1.82 pM	40 min–1 h	Serum, nasal swabs, fresh milk, sugar, flour, talcum	[12]
Immunomagnetic beads assay	C, D	0.3 MLD/ml	< 1 d	Purified toxin, bacterial culture supernatant	[68]
Immuno-PCR	A	5 pg	8 h	Purified toxin	[216]
	A	50 fg	8 h	Purified toxin	[37]
Liposome-PCR	A	0.02 fg/ml	3 h	Purified toxin	[130]
Time-resolved fluorescence assay	A	20 pg	2 h	Purified toxin	[159]
Chemiluminescent slot blot immunoassay	E	4 MLD	< 6 h	Bacterial culture supernatant, fish, environmental samples	[29]
Electrochemiluminescence	B	< 1–2 ng/ml	1–2.5 h	Toxin complex	[79]
	A, B, E, F	A, E, F 1 ng/ml; B 5 ng/ml	< 1 h	Purified toxin, liquid egg products, ground beef, green beans	[160]
	A, B, E, F	50–400 pg/ml in clinical samples; 50–100 pg/ml in food samples	1–2 h	Purified toxin, toxin complex, human serum, urine, whole milk, ground beef, apple juice, pastry, raw eggs	[164]
Endopeptidase assay	B	5 pg/ml	5–6 h	Cheese, cod, minced beef, pate, sausage, yogurt	[213, 214]
	A	0.2–1.0 MLD_{50}/ml	~8 h	Purified toxin, therapeutic preparations	[58]
	A, B	0.1–4.5 ng/ml	~8 h	Purified toxin, bacterial culture supernatant	[80]
	C	25 pg/ml	24 h	Purified toxin	[105]
	A, E	A 40 fg/ml; E 4.8 pg/ml	~1 d	Purified toxin	[104]
	A	10 pg/ml	24 h	Carrot juice, beef	[163]
	A, B, D, F	10–100 ng/ml	~8 h	Purified toxin	[171]
BoNT/A ALISSA	A	0.5 fg/ml	2.5 h	Serum, milk, carrot juice, gelatin phosphate-diluent	[14]
Endopep-MS	A, B, C, D, E, F, G	A, B, E, F < 1 MLD_{50}/ml	4–17 h	Toxin complex	[26]

Table 11.5 (continued)

Assay	BoNT type(s)	Detection limit	Analysis time	Application(s)	Reference(s)
	A, B, E, F	0.1–10 MLD_{50}	4–7.5 h	Serum and stool samples	[106]
	A	0.5 MLD_{50}	NR	Stool samples	[211]
Biosensor	A	1 ng/ml	<10 min	Buffer	[209, 210]
	A, B	0.01 LD_{50}/ml	10 min–5 h	Buffer, carrot juice, apple juice, milk, serum from humans with botulism	[62, 129]
SAM-based microfluidic sensor	A	3 pg/ml	3 h	Purified toxin, canned vegetable soup	[65]
Magnetic bead-based microfluidic sensor	B	0.4–312 ng/ml	3 h	Buffer, milk, orange juice, peach juice	[66]
Lab-on-a-chip	A	0.5 nM (LCA)	2 h	Toxin light chain	[201]
	A	1 ng/ml	15 min	Toxin light chain	[116]

ALISSA assay with large immunosorbent surface area, *BoNT* botulinum neurotoxin, *ELCA* enzyme-linked coagulation assay, *ELISA* enzyme-linked immunosorbent assay, *LCA* light chain of BoNT/A, *MLD* minimum lethal dose, *PCR* polymerase chain reaction, *SAM* self-assembled monolayer

[a] *NR* not reported

Lateral flow tests, also called immunochromatographic strip tests, are inexpensive, easy to use, rapid to perform and suitable for field use since no additional equipment is required [30]. The test kit has a long-term stability over a wide range of climates [38]. Commercial kits have been developed for detection of BoNT on site from environmental samples for biosecurity purposes [69]. Briefly, the liquid sample containing BoNT binds with antibody conjugated to coloured particle and the formed toxin–antibody complex flows along a solid substrate via capillary action. In the sample window, the complex reacts with antibody, immobilized as a thin stripe in the nitrocellulose membrane, and a coloured line develops on the test strip [175]. Despite the user-friendly format and the speed, the use of lateral flow tests may be limited due to their low sensitivity and the lack of quantitative results in most set-ups [69, 93, 175]. In addition, poor filtration and migration resulting in false-negative results have been reported for some high-fat-content food samples [5, 175]. The lateral flow test may be best suited for initial screening of samples in suspected cases of botulism and for screening bacterial cultures for *C. botulinum* [69, 117, 174]. However, due to the limitations of the test, unclear and negative results should be confirmed by the mouse bioassay [117].

Immunoaffinity column (IAC) assays are fast and suitable for field use since no sophisticated equipment is needed, and the use of the assay requires minimal training [12, 27]. Briefly, BoNT is captured and concentrated by an antibody-coated filter as the liquid sample flows through. Captured toxin is detected using a labelled second antibody and as a result, a visual band is observed [12]. Since the antigen–antibody complex is trapped by a filter out of the sample matrix, larger sample

volumes can be tested compared to conventional ELISA test and thus the sensitivity of IAC assay is increased. Moreover, sample components that might cross-react or inhibit the immunological reaction are washed out if they are small enough to pass the filter [67]. The IAC assays have been successfully applied for the detection of BoNTs in various foods, and in general, no significant loss of sensitivity was detected in these complex matrices [12, 27]. In addition, the IAC has proved to be a sensitive assay for the detection of BoNT/A in human serum and since the sample-to-result analysis time was less than 1 h, this method might be suitable for rapid diagnosis of botulism [12]. However, further research is warranted to study the suitability of IAC assays for different sample matrices.

Immunomagnetic beads can also be used to capture and concentrate toxin from the sample matrix. This method has been used for simultaneous detection of toxin types C and D in bacterial cultures. However, suitability for complex sample matrices has not been tested [68].

In immuno-polymerase chain reaction (PCR), a reporter DNA is covalently linked to an antibody. The amount of antigen in the sample is quantified by PCR amplification of the conjugated reporter DNA [216]. Due to the enormous amplification capacity and specificity of PCR, the detection limit of immuno-PCR can be 10^2- to 10^5-fold lower than that of the conventional ELISA [37, 216]. Indirect immuno-PCR and indirect sandwich immuno-PCR have been shown to detect very low quantities of BoNT/A. However, the highest sensitivity has been reported for liposome-PCR assay which uses liposomes with encapsulated DNA reporters and ganglioside receptors incorporated in the bilayer as a detection reagent [130]. Briefly, the toxin is immobilized by a capture antibody and liposome detection reagent is added. Unencapsulated contaminating DNA is degraded using DNase solution. Liposomes are ruptured to release encapsulated reporters and the released DNA is quantified using real-time PCR, which improves the quantitative accuracy of the assay compared to conventional immuno-PCR. In addition, contaminants can be washed out and the degradation of any contaminating DNA present in the sample reduces the likelihood of false-positive results. Since the encapsulated, reporter DNA is protected by liposomes from chemical or enzymatic degradation by impurities of the sample, the possibility of false-negative results is also reduced [130].

Chemiluminescent slot blot immunoassay has been used to detect BoNT/E in bacterial cultures. The method is inexpensive, simpler than many other immunoassays and the sensitivity approaches that of the mouse bioassay. However, false-negative results were obtained with fish and environmental samples [29].

In paramagnetic bead-based electrochemiluminescence (ECL) technique, the toxin is captured by antibodies bound to paramagnetic beads. The detector antibody is labelled with ruthenium (Ru) chelate. The formed immunocomplexes are collected by a magnet onto the surface of an electrode within the ECL analyser. Reactions involving the Ru chelate lead to an electrochemiluminescent signal that is detected and quantitated by the analyser [78, 164]. Commercially available ECL assay has successfully been used to detect neurotoxin types A, B, E and F in human serum and urine and in a selection of food samples. Since the assay is fast, it may prove to be beneficial in screening of clinical and food samples for botulinum toxins [160, 164]. When commercial colorimetric ELISA and ECL assays for detection of BoNT/B

were compared, the ECL assay was also found to be faster and more sensitive than the colorimetric ELISA [79]. The ECL assay is a high-throughput assay, and it also requires less hands-on time and thus the likelihood of human error is decreased compared to conventional ELISA test [160].

11.4 Functional Assays

BoNT light chains act as zinc-dependent, sequence-specific endoproteases that specifically cleave components of the synaptic vesicle docking-fusion complex known as the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex [138]. Several in vitro assays utilizing the endopeptidase activity of BoNT have been developed. Since these assays detect only biologically active toxins, they resemble the mouse bioassay more closely than conventional immunoassays which can also detect inactive toxins with little tertiary structure [80].

11.4.1 Endopeptidase Assays

Endopeptidase assays are based on the specific cleavage of SNARE complex proteins, followed by detection of the peptide cleavage product, e.g. by immunoassay or by using fluorescence sensors to detect fluorescence resonance energy transfer-based reactions [58, 163, 214]. Endopeptidase assays have been developed for BoNT types A [58, 80, 104, 163, 171], B [80, 171, 213, 214], C [105], D [171], E [104] and F [171]. The first-generation endopeptidase assays showed higher detection limits than the mouse bioassay [80] and therefore different protocols have been developed to increase the sensitivity. Both immunoaffinity column and immunomagnetic bead technology have been used to capture and concentrate toxins from sample and to remove interference by sample matrix endopeptidases and protease inhibitors [163, 213, 214].

In BoNT/A assay with large immunosorbent surface area (ALISSA), the toxin is captured and enriched on a bead-based immuno-affinity matrix followed by stringent washing steps. The enzymatic activity of the immobilized BoNT/A is detected by cleavage of a specific fluorogenic peptide substrate. The assay is able to capture a low number of toxins, and the use of stringent washing steps and BoNT/A-specific substrate diminish interference with other sample components [14].

Multiplexed detection of endopeptidase activity of BoNT by mass spectrometry (endopep-MS) was the first in vitro assay that was able to detect all seven toxin types in a single reaction [26]. Briefly, each toxin type has its unique target site of endoproteinase activity, and thus the cleavage of peptide substrate results in formation of two peptide products. The specific peptide products are detected by MS, which is able to distinguish between peptides differing by only one amino acid [26]. The detection limit has been reported to be one MLD or less [26, 211]. However, analysis of clinical, environmental, faecal and food samples may drastically reduce

the sensitivity of the assay since endogenous proteases may degrade the substrate peptide [26]. Endogenous proteases may also cause false-positive results if they cleave at the same site as the BoNT [211]. Further research is needed to establish pretreatment protocols, such as the use of protease inhibitors both alone and in mixtures, or antibody-based methods to purify the BoNT from the matrix, to overcome this problem [26, 30, 107, 211]. Sample pretreatments that improve the limit of detection have been developed, e.g. for serum and stool samples [211]. Despite the high sensitivity and specificity, the high-throughput nature of endopep-MS is questionable and the high cost of a MS may also limit the widespread use of the assay [26, 160].

11.4.2 Biosensors

Biosensors are analytical devices that contain a biological recognition element, such as antibody, which can specifically interact with an analyte. The recognition element is coupled to a transducer, which transforms the rate of the biochemical reaction that takes place during biological recognition into a measurable response [9, 61, 132]. Immunocapture of membrane-associated SNARE proteins monitored by surface plasmon resonance has been used to measure activity of botulinum toxins A and B [61, 62, 129]. This protein chip assay was able to detect picomolar concentrations of BoNT/B in various food samples, although the sensitivity was lower in these complex matrices than in an optimized buffer. A retrospective study of serum samples from patients with botulism type B and controls also revealed that only serum samples of patients with botulism gave positive results [62]. This sensor chip assay may prove to be a suitable alternative to mouse bioassay with clinical serum samples.

Electronic biological sensors based on antibody-functionalized, AlGaN/GaN high-electron mobility transistors have been used for real-time detection of BoNT/A [209, 210]. The current change showed rapid response of less than 5 s when the toxin was added to the surface of the gate area [209]. Electronic, biological sensors have the potential to serve as field-deployed sensor chips which can be integrated with commercially available wireless transmitters to allow real-time and sensitive detection of BoNT [209, 210].

11.4.3 Microfluidic Technology

Microfluidic biosensors are fast, requiring less than 1 h hands-on time, consume a small amount of sample and reagents and show potential for on-site usage. A self-assembled monolayer (SAM)-based microfluidic sensor has been developed for BoNT/A [65]. Briefly, the assay takes place in a single microfluidic channel and can be completed in 3 h. Sample is added at the input port and incubated on SAMs that display immobilized fluorescent synthetic peptides. BoNT/A recognizes and

cleaves the surface substrates releasing fluorescently labelled fragments which are concentrated at a detection port via evaporative flow and quantified using fluorescence microscopy for signal measurement. Recently, a magnetic bead-based capture assay was used for detection of BoNT/B in microchannels [66]. The use of magnetic beads concentrates the toxin for downstream detection and allows efficient washing of any interfering agents. Further research is needed to evaluate the performance of microfluidic biosensors with complex matrices [65, 66].

Lab-on-a-chip (LOC) is a novel technology that can be used for various analytical purposes, including detection of BoNT [116, 133, 201]. LOC devices integrate and scale down one or several laboratory functions to a miniaturized chip format. Since LOC devices utilize microfluidic technology, very small quantities of sample and reagents are required. In addition, LOC devices are, in general, low cost, rapid and suitable for on-site monitoring since no laboratory or complex equipment is needed [116, 201]. The LOC assays are easy to perform and require minimal sample handling and thus protect the user from the toxin [201]. Although LOC is a promising technology, it needs further improvements before it becomes suitable for routine detection of BoNTs in clinical, environmental and food samples.

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Chapter 12
Botulism as a Disease of Humans

Susan E. Maslanka

Abstract Human botulism presents in several different forms which provide unique challenges to public health. Foodborne botulism, while still an issue with home-processed foods, is sometimes associated with restaurants and commercially produced (particularly chilled) foods. The diversity of food products available to consumers, which are widely distributed, requires an integrated, multiagency response approach to contain, evaluate and develop appropriate prevention measures. Wound and adult intestinal toxemia cases are difficult to confirm, and so the total illness burden is likely unknown. Infant botulism highlights the diversity of the neurotoxin-producing *Clostridium* sp. and associated toxins which can cause botulism worldwide. Finally, an astounding number of adverse events associated with toxin injections remind us of the hazards of botulinum toxin even as its therapeutic benefits expand.

Keywords Botulinum neurotoxin · Botulism · Foodborne botulism · Wound botulism · Infant botulism · Iatrogenic · Toxinfection

12.1 Introduction

Botulism, since its discovery in the eighteenth century, has resulted in dramatic public health response efforts to understand, identify, and control this rare but potentially fatal disease. Between 1735 and 1802, the number of outbreaks associated with blood sausage in Württemberg, Germany caused the government to provide instructions on safe preparation methods; by 1820, botulism was a mandatory reportable disease [1]. Originally thought to occur only with meat products, botulism caused by vegetables caught the world by surprise in 1904 when beans caused an outbreak in Darmstadt, Germany. While largely ignored initially, botulism due to consumption of preserved vegetables commonly occurs worldwide. Although botulism cases occurred with regularity in California, a multistate outbreak in 1919 caused by contaminated, commercially processed and packed olives brought national attention to the risk of botulinum toxin-contaminated foods. These events

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represented a turning point in regulated food protection in the USA and resulted in coordination between federal, state and university researchers to develop commercial canning standards to protect consumers from this deadly illness. As a result, large outbreaks of botulism due to commercial food products declined, but small outbreaks began to increase during World War I and World War II as home-canning practices increased [1]. Even as public health officials began to develop educational material for those who prepare home-processed food, *Clostridium botulinum*, the causative organism that produces the botulinum toxin, still had a few tricks to show us. In 1951, botulism was recognized as being caused by contamination of a wound with in situ toxin production [1]. Originally the result of injuries and occasionally surgery, wound botulism has increased around the world due to certain illegal drug-use practices. In 1976, infant botulism, currently the leading botulism form in the USA, was recognized as a distinct syndrome as a result of intestinal colonization [2, 3]. The recognition of infant botulism introduced us to an astounding diversity of neurotoxin-producing *Clostridium* sp. New botulinum toxin-producing species, *C. baratii* type F and *C. butyricum* type E, were identified. Additionally, some *C. botulinum* isolates were found with the capacity to produce more than one toxin serotype; usually, one of the two toxin genes produce more toxin than the other (i.e., Af, Ab, Ba, Bf, Ae, where the lower case letter reflects a lower level of toxin produced). In 1988, the first cases of intestinal colonization of adults were reported in the USA; diagnosis of this form of botulism continues to perplex physicians and public health officials. Early work on purification, stabilization and characterization of botulinum toxin by researchers at the US Department of Defense and later at the University of Wisconsin laid the groundwork for the discovery of unique therapeutic properties of this deadly toxin [4]. While new therapeutic uses are continuing to be discovered, botulinum toxin reminds us of its danger through reports of localized and systemic paralytic effects following injections (iatrogenic botulism). This chapter highlights the surprises and diversity of human botulism.

12.2 Surveillance and Control

Countries with a relatively high number of botulism cases, such as the USA, Canada, Argentina, UK, Germany, Italy, France, Poland, China, Thailand and Japan, conduct surveillance and have a response network to investigate and control botulism. However, there is no worldwide reporting mechanism to accurately capture the global incidence of botulism. Limited understanding of global occurrence of botulism is probably due to a number of factors, including availability of inadequate resources to identify botulism cases, differences in internal reporting requirements, language barriers and limited external accessibility to data collected by public health agencies of individual countries. In 1999, a summary of responses from 14 European countries concerning botulism surveillance was published [5]. This publication revealed that 13 of the 14 countries surveyed included botulism as a notifiable disease; most had required reporting of botulism for decades. However, most did not report in-

Table 12.1 2008 European Commission Case Classification for botulism. [7]

	Clinical criteria	Laboratory criteria	Probable	Confirmed
Foodborne	At least one of the following: Bilateral cranial nerve impairment (e.g., diplopia, blurred vision, dysphagia, bulbar weakness) Peripheral symmetric paralysis	Detection of botulinum toxin in a clinical specimen	Any person meeting the clinical criteria and with an epidemiological link	Any person meeting the clinical and the laboratory criteria
Wound	At least one of the following: Bilateral cranial nerve impairment (e.g., diplopia, blurred vision, dysphagia, bulbar weakness) Peripheral symmetric paralysis	Detection of botulinum toxin in a clinical specimen Isolation of *Clostridium botulinum* from wound	Any person meeting the clinical criteria and with an epidemiological link	Any person meeting the clinical and the laboratory criteria
Infant	Any infant (< 12 months of age) with at least one of the following: Constipation Lethargy Poor feeding Ptosis Dysphagia General muscle weakness	Detection of botulinum toxin in a clinical specimen Isolation of *Clostridium botulinum* from stool	Any person meeting the clinical criteria and with an epidemiological link	Any person meeting the clinical and the laboratory criteria

dividual botulism cases, only total number, and many of these were reported as "food poisoning" rather than specifically for botulism. Additionally, there was little uniformity in the actual case definition used; some only used clinical criteria and others required both clinical and laboratory data. Currently, 30 countries within the European Union (EU) or European Economic Area (EEA) participate in voluntary reporting of botulism cases using a set of standard definitions as described in Table 12.1 [6, 7]. Since 2007, the European Centre for Disease Prevention and Control (ECDC) has collected and published an annual report summarizing the incidence of botulism. Participating countries mainly report to the ECDC through passive surveillance. Each country collects data through a compulsory reporting requirement primarily from laboratories, physicians and hospitals. Worldwide, a single suspect case of foodborne botulism is considered to be a public health emergency, because the first case may signal the occurrence of an outbreak. In EU countries, public health authorities quickly withdraw incriminated commercially distributed food products. Product alerts are delivered through the Rapid Alert System for Food and Feed (RASFF) and Early Warning and Response System (EWRS). This system provides a mechanism for rapid notification of potentially risky food exported to both EU and non-EU countries.

In the USA, the Centers for Disease Control and Prevention (CDC) maintains 24/7 epidemiology and laboratory consultation services through the CDC Emergency Operations Center (770 488–7100) to support state/local response to suspect cases [8]. CDC staff work with both state/local epidemiologists and hospital personnel to try and quickly identify clinical specimens and potential food sources which may need to be recovered for laboratory tests. If commercially produced products are suspected, then CDC ensures that the proper food regulatory agency (Food and Drug Administration, FDA or US Department of Agriculture, USDA) is involved to facilitate product recalls and to evaluate production records. CDC provides antitoxins for suspect adult botulism cases either directly or through established distribution systems located in both California and Alaska. Previously, the available therapeutic antitoxin products (a licensed A/B product, and an Investigational New Drug type E product) were limited to the three toxin serotypes (A, B and E) which primarily cause human botulism; however, an equine-derived heptavalent botulism antitoxin (BAT, Cangene Corp.), approved in the USA by FDA in March 2013, is currently used to treat adult cases and covers all known serotypes (A, B, C, D, E, F and G) which could occur naturally or as a result of an intentional act [9]. A human-derived antitoxin product (BabyBIG®) is available from The California Infant Botulism Treatment and Prevention Program (CA IBTPP) and is approved for the treatment of type A and B infant botulism cases [10]. Additionally, CA IBTPP provides 24/7 consultation (510–231–7600) for infant botulism cases throughout the USA and provides laboratory support for infants residing in California. CDC collates morbidity and mortality surveillance data from a variety of sources, including epidemiology and laboratory investigations reports from state/local health departments, FDA or USDA food investigations reports, CDC clinical consultations, antitoxin distribution reports and CA IBTPP antitoxin release summaries [8]. Each year, CDC prepares a summary of laboratory-confirmed botulism cases to help identify trends and potential high-risk areas for education and control. Since 1992, the definition of a botulism outbreak includes events involving two or more persons, whereas previously, a single case met the definition of an outbreak as long as a food source was also identified. Consensus definitions for different botulism types are developed through the Council of State and Territory Epidemiologists (CSTE) to ensure uniform reporting across the USA; the current definitions are provided in Table 12.2 [11]. CDC's annual summary report identifies the form of botulism as defined by CSTE for each case. Laboratory confirmation of botulism is achieved through detection of toxin or identification of a neurotoxin-producing *Clostridium* sp. in clinical specimens or by detection of toxin in foods as described for the different forms of botulism in Table 12.2 [12]. In addition, at CDC and other federal facilities such as FDA and USDA, laboratory testing occurs in state health departments and select county or city public health laboratories. A number of investigators have conducted studies to try and find an alternative to the mouse bioassay [13, 14]. However, laboratory confirmation in the USA continues to depend on the mouse bioassay because there is no alternative method approved or cleared by FDA for clinical specimen testing. Methods for detection and control of botulinum neurotoxins are discussed in Chap. 11.

Table 12.2 2011 US CSTE Position Statement for Botulism. [11]

	Clinical Description	Laboratory Criteria	Probable	Confirmed
Foodborne	Ingestion of botulinum toxin results in an illness of variable severity. Common symptoms are diplopia, blurred vision and bulbar weakness. Symmetric paralysis may progress rapidly	Detection of botulinum toxin in serum, stool or patient's food or Isolation of *Clostridium botulinum* from stool	A clinically compatible case with an epidemiologic link (e.g., ingestion of a home-canned food within the previous 48 h)	A clinically compatible case that is laboratory confirmed or that occurs among persons who ate the same food as persons who have laboratory-confirmed botulism
Infant	An illness of infants, characterized by constipation, poor feeding and "failure to thrive" that may be followed by progressive weakness, impaired respiration and death	Detection of botulinum toxin in stool or serum, or Isolation of *Clostridium botulinum* from stool	None	A clinically compatible case that is laboratory confirmed, occurring in a child aged less than 1 year
Wound	An illness resulting from toxin produced by *Clostridium botulinum* that has infected a wound. Common symptoms are diplopia, blurred vision and bulbar weakness. Symmetric paralysis may progress rapidly	Detection of botulinum toxin in serum or Isolation of *Clostridium botulinum* from wound	A clinically compatible case in a patient who has no suspected exposure to contaminated food and who has either a history of a fresh, contaminated wound during the 2 weeks before onset of symptoms or a history of injection drug use within the 2 weeks before onset of symptoms	A clinically compatible case that is laboratory confirmed in a patient who has no suspected exposure to contaminated food and who has a history of a fresh, contaminated wound during the 2 weeks before onset of symptoms or a history of injection drug use within the 2 weeks before onset of symptoms
Other	See Botulism, Foodborne	Detection of botulinum toxin in clinical specimen or Isolation of *Clostridium botulinum* from clinical specimen	None	A clinically compatible case that is laboratory confirmed in a patient aged greater than or equal to 1 year who has no history of ingestion of suspect food and has no wounds

12.3 Foodborne Botulism

In the USA, 1,184 foodborne botulism events (single cases and outbreaks) involving 2,727 persons were reported between 1899 and 2009 [8, 15, 16, 17]. The mean annual incidence of foodborne botulism in the USA is ~0.1 cases per 1,000,000 persons [8]. However, the annual incidence rate in the Alaska Native communities is 800 times greater than that in the contiguous states [18]. The number of foodborne botulism events in the USA has remained relatively stable (104 ± 33 events per decade) for the past five decades (Table 12.3). One obvious trend is the reduction in the number of foodborne botulism events in which toxin type was not determined. During the 1950–1959 reporting period, toxin type was determined in only 23% of events but by the 1980–1989 reporting period, almost 97% of events were categorized by toxin type. This trend is likely reflective of the improvements in awareness of this disease, accompanied by earlier clinical recognition of botulism and earlier collection of appropriate test samples. It also may be the result of the incorporation of new laboratory testing recommendations to include both detection of toxin and isolation of *C. botulinum* from stool [19]. In the USA, the majority of events (~60%) are caused by botulinum toxin type A; type E was identified in 30–45% of foodborne botulism events, primarily in Alaska, since 1980 (Table 12.3). Only two foodborne botulism events, both in California, have been caused by type F toxin; one caused by *C. botulinum* type F involving three persons and the other a single case caused by *C. baratii* type F toxin [20, 21].

There have been no major trends in the USA over the past 20–30 years in the major food types which cause botulism [8]. Vegetables and fish/aquatic animals continue to be responsible for the majority of foodborne botulism cases (~80%). Common home-preserved foods associated with botulism in the USA, excluding Alaska, include asparagus, green beans, peppers and mushrooms [8, 22]. In Alaska, botulism is associated with the consumption of Alaska Native traditional foods of fish (48%), marine mammals (47%) and beaver (6%) [18]. A Hispanic traditional food (*sierra en escabeche*) caused the only reported botulism outbreak (type A) in Puerto Rico [23]. This unusual food vehicle consists of pan-fried fish marinated with oil, vinegar, onions, peppercorns and bay leaves; the marinated fish is stored in large glass jars at room temperature for several days. Three of ten persons who ate the food over an 18-day period became ill. One surprising feature of this outbreak was that the food was acidic (pH 4.4) which is inhibitory to the growth of *C. botulinum*. While rare, botulism associated with acidic foods does occur, an interesting case of type B botulism due to pickled eggs occurred in 1997 [24]. Freshly hard-boiled eggs were covered with commercially canned beets, hot peppers and vinegar in a large jar and stored at room temperature in occasional sunlight. Just prior to closing the jar, a toothpick from the kitchen counter was used to pierce each egg so that juices could enter the center of the egg. The laboratory investigation demonstrated, perhaps not surprisingly, that the toxin level in the yolk of the egg was 1,000 times higher than in the liquid surrounding the eggs. No individual ingredient used to prepare the product contained *C. botulinum* spores, so spores in

Table 12.3 US foodborne events by toxin type, 1950–2009[a]

	1950–1959[b]	1960–1969[b]	1970–1979[b]	1980–1989[b]	1990–1999[c]	2000–2009[d]
A	14 (13.5%)	12 (15.4%)	68 (53.5%)	48 (60.8%)	72 (47.7%)	59 (60.8%)
B	3 (2.9%)	10 (12.8%)	28 (22.0%)	13 (16.5%)	23 (15.2%)	9 (9.3%)
E	7 (6.7%)	9 (11.5%)	15 (11.8%)	16 (20.3%)	51 (33.8%)	25 (25.8%)
F	0	1 (1.3%)	0	0	0	1 (1.0%)
Unknown	80 (76.9%)	46 (59.0%)	16 (12.6%)	2 (2.5%)	5 (3.3%)	2 (2.1%)
Total	104	88	127	56	151	97

[a] A foodborne event involving one or more cases
[b] Data from ref [12]
[c] Data from ref [22]
[d] Data from ref [8, 15, 16, 17]

the kitchen were likely to have been introduced into the center of the egg through the insertion of the toothpick (CDC unpublished data). Cases of botulism associated with traditional foods from other countries also have occurred in the USA. In 2006, home-prepared fermented tofu, commonly prepared in Asia and a common cause of botulism in China, caused type A botulism in an Asian woman living in California [25]. In 2012, two unrelated botulism cases (1 confirmed type B) in New York were suspected to be associated with a vendor-prepared tofu purchased at different times from a local market. The tofu was independently fermented by each of them. Type B toxin was detected in the tofu fermented by one of the patients [26]. In 2005, five individuals in New Jersey became ill, two were confirmed with type E botulism, after consuming a traditionally prepared Egyptian salted fish; one of the patients previously had botulism from consuming a similar product in 1992 [27]. Other unusual home-processed foods, such as peyote tea (a traditional drink of some American Native populations prepared from cactus) and pruno (a contraband alcoholic beverage prepared by prisoners), have caused botulism [8]. Surprisingly, even foods considered fresh (e.g., consumed soon after preparation, such as baked potato, potato salad and sautéed onions) rather than preserved have caused botulism (8). A common feature of these "fresh food" events is that each of these products usually consumed immediately was held at room temperature for 1 or more days, under at least partial anaerobic conditions (e.g., foil-wrapped potato, under an oily layer), prior to consumption with minimal or no heating.

Most foodborne botulism events in the USA occur as single cases, involve family members, or in defined social gatherings so the food source is relatively easy to identify even if unavailable for testing. However, several large outbreaks have occurred in restaurants which have challenged both epidemiologic and laboratory investigations. The largest outbreak (59 persons) in US history occurred in 1977 over a 1-week period in Michigan; all patients ate a meal at a single restaurant [28]. The investigation of the cause of botulism was complicated because of the number of people who ate at the restaurant and the number of foods served during the probable exposure period. However, type B toxin was detected in a jar of home-canned jalapeno peppers used to prepare various foods. The peppers were canned using an open kettle method (without pressure) approximately 5 months before the

outbreak; interviews with employees indicated that only a few jars were available since many exploded several months earlier. A very complicated type A outbreak occurred in New Mexico in 1978, involving 34 persons who ate a buffet dinner at a country club [29]. A commercially canned three bean salad was epidemiologically linked to the cases but was not available for testing. Both potato salad and coleslaw retrieved from the buffet were positive for botulinum toxin type A; however, all patients did not report eating potato salad and no ill persons reported eating coleslaw. An extensive investigation was unable to establish a clear mechanism for cross-contamination between foods served on the buffet although all three items were stored in the same refrigerator. Seven cases of type A botulism also occurred in 1978 in Colorado [30]. Potato salad, prepared from unwashed aluminum foil-wrapped baked potatoes held at room temperature for several days, was epidemiologically implicated as the cause of the outbreak, but left over food samples were negative for botulinum toxin. In 1994, 30 persons became ill after eating potato- or eggplant-based dip [31]. Botulinum toxin type A was detected in both dips; the epidemiologic investigation suggested that the eggplant-based dip was cross-contaminated by the potato-based dip through a shared spoon. Both dips were prepared using aluminum foil-wrapped baked potatoes that were stored at room temperature for at least 18 h. Studies conducted at the University of Wisconsin demonstrated that botulinum toxin was produced in aluminum foil-wrapped baked potatoes stored at 37 °C for 3–4 days. However, botulinum toxin was not produced in foil-wrapped potatoes held as long as 10 days at either 21 °C or 50 °C nor in foil-wrapped potatoes held up to 21 days at 4 °C [31]. In 1993, botulinum toxin type A surprisingly was detected in a cheese sauce served in a restaurant which caused eight botulism cases in Georgia [32]. Investigators suspected that the cheese sauce, which had expired 1 year prior to the outbreak, was contaminated with type A spores from baked potatoes served in the restaurant; just prior to the outbreak, the restaurant reopened after an extended closure. Although denied by the owners, the cheese sauce served in the outbreak was likely held in the restaurant since the closure. Since more than 50 cases of botulism were reported to be caused or suspected to be caused by baked potatoes, the FDA classified baked or boiled potatoes as “potentially hazardous food” [31]. Another surprising outbreak of 28 cases of type A botulism occurred in Illinois in 1983, as a result of consumption of restaurant-prepared sandwiches served with sautéed onions which were held throughout the day in a warm pan. Leftover food was not available from the restaurant, but a wrapper that previously contained one of the sandwiches was retrieved from the home of one of the patients; type A toxin was identified from washings of the wrapper [33].

Outbreaks in the USA also have occurred through consumption of commercially processed foods. One such type A outbreak (15 cases) in 2001 involved a commercially processed frozen meat-based chili [34]. The risk associated with this frozen meat-based chili was determined to be caused by mishandling of the product by a retail establishment rather than a manufacturing error; the product was frequently removed from the freezer and placed on a discount sale table outside the store. Another outbreak involving a commercially produced meat-based chili occurred in 2007 [35]. The 2007 outbreak had the capacity to be one of the largest foodborne

botulism outbreaks in the USA because it involved a commercially canned product that was widely distributed. Sixteen of 17 cans tested by the FDA were found to be positive for botulinum toxin type A. Significant deficiencies with the manufacturer's retort process resulted in the recall of 111 million cans of food involving 91 different products and the closure of the manufacturing plant. This was the first botulism outbreak associated with commercial canning in the USA in over 30 years, and it was the largest outbreak associated with commercial canning since 1919. From 1950 until this outbreak, only four botulism events (canned tuna, liver paste, vichyssoise and beef stew) in the USA were attributed to deficiencies in a commercial canning process [8, 35]. Remarkably, considering the number of botulinum toxin positive cans of chili identified by the FDA, only eight botulism cases, from three states, occurred as a result of this product failure [35]. Most consumers may have appropriately heated this product which destroyed the toxin and limited the number of persons exposed. The underlying cause of this canned chili outbreak was different from an outbreak earlier involving canned beef stew. The chili outbreak occurred as a result of a catastrophic breakdown of the retort process such that millions of cans were not properly heated. The beef stew outbreak in 1974, also type A, likely occurred as a result of either a two-line process which allowed cans to bypass the retort process or a lack of venting which may have created air pockets causing inconsistent heating [36]. No beef stew cans, other than the one consumed by the two patients, were positive for botulinum toxin, suggesting that this was a single can incident. Additionally, the contents of the can were reported by the consumers to be only warmed before eating. Three cases of type A botulism occurred in the USA in 1989 from the consumption of commercially prepared garlic in oil [37]. This outbreak followed a type B outbreak involving 36 persons in Canada with a similar product [38]. In response to these outbreaks, the FDA required new safety standards for these types of commercially prepared products [37]. A minimally processed commercial carrot juice product was the cause of an international type A outbreak in 2006 involving six total cases in Florida, Georgia and Ontario, Canada [39]. Except for the leftover product retrieved from the homes of the patients, no other bottles were identified with botulinum toxin, including bottles from the same lot consumed by the patients; however, several were found to contain *C. botulinum* type A spores. It was likely that the commercial carrot juice was inadequately refrigerated during transport, storage by the retailer or by the patients; however, at least two of the patients reported meticulous refrigeration control of the product following purchase. In 2011, two unrelated cases of type A botulism in different states were reported to CDC; both cases consumed commercial chilled soup sold by different companies that was left unrefrigerated by the consumers for several days [40]. A recent review of minimally heated, chilled foods suggests that these types of food products may be an emerging issue for botulism outbreaks [41]. Recent botulism events involving chilled foods, such as carrot juice and soup, which are designed to be consumed without heating or which are minimally heated even if required, unfortunately demonstrate that these products bring new challenges for public health, including assuring adequate labeling by the manufacturer and education of the consumer.

Foodborne botulism occurs worldwide, and rates of reporting vary by continent. However, the reasons for continental or country variations in reporting are not known. Foodborne botulism could be rare in some countries because of cultural differences in food preparation and consumption (low risk for botulism) or a result of limited medical and public health capacity in some areas (unrecognized cases). From publically available information, the toxin type shows some geographical distribution with type B predominating in Europe and type A predominating in Asia [42]. Botulism is rare in Canada but it is the second highest country in North America to report cases [43, 44]. From 1985 to 2005, 91 outbreaks (outbreak defined as ≥1 case) involving 205 cases with 11 known deaths were reported in Canada [43]. Most (~85%) foodborne outbreaks in Canada are similar to those that occur in Alaska in that they occur in native communities, primarily Inuit or First Nations, and involve traditional preparations of foods, including fish and marine mammals [43, 44]. All outbreaks in Canada involving native communities were caused by type E toxin. Most (64%) outbreaks in nonnative communities were caused by home-preserved foods; 50% type A, 34% type B and 14% type E. Two outbreaks in restaurants (chopped garlic in oil previously mentioned and bottled chanterelle mushrooms) accounted for 72% of all foodborne botulism cases in nonnative communities during the 1985–2005 reporting period [43]. Eight outbreaks or single cases occurred in Brazil from 1981 to 2001; the majority was caused by type A botulinum toxin in home-preserved foods [45]. In 2011, Brazil reported an outbreak among seven persons who consumed commercially produced sausage [46]. Foodborne botulism is considered a public health threat in Argentina; the first case occurred in Mendoza in 1922 due to home-canned asparagus [47]. Forty two cases were reported between 1992 and 2004; foods involved included home-processed ham, chili peppers, eggplant, cucumbers, heart of palm, tomatoes, peaches, spinach, cheese with onions, pickled octopus, asparagus, canned fish, sweet corn and rodent vizcacha. The only worldwide known outbreak due to an unusual dual toxin-producing strain (*C. botulinum* Af; type A produced at a higher level than type F) occurred in Mendoza and was caused by home-prepared pickled trout [48]. In 1998, type A botulism occurred in nine bus drivers who ate *matambre*, a traditional meat roll prepared with vegetables, spices and egg; this event increased awareness of foodborne botulism in Argentina and helped support a national surveillance system to include an antitoxin stockpile [49].

Reports of foodborne botulism are rare in the continent of Africa. The highest numbers have occurred in South Africa; however, only five events (3 type B, 1 type A, 1 unknown) have been reported [50]. All, in which a food was identified, have been associated with a meat product. A type A outbreak involving two young persons resulted from a damaged commercially canned fish with tomato sauce which was given to their economically distressed family. In 1991, a large outbreak of type E botulism occurred in Egypt which was traced to consumption of a traditional fish dish, *faseikh* (8). Recently, CDC provided assistance to two separate foodborne outbreaks one type B, affecting 32 persons in Rwanda and one type A, affecting three persons in Uganda. Although the food sources were not confirmed, a rice and bean mixture was epidemiologically linked to the Rwanda cases, and a home-prepared

herb-in-oil condiment was the suspected source in the Uganda outbreak (CDC unpublished data).

Botulism occurs frequently in various parts of Europe. As stated in Sect.12.2, 30 countries participate in the ECDC annual collection of botulism surveillance data. In 2011, the ECDC reported that 185 cases of botulism occurred among the 30 countries; however, the report does not delineate the number of cases attributed to foodborne botulism [6]. Four countries (France, Italy, Poland and Romania) accounted for 70 % of all reported cases. Sixteen participating countries reported no botulism cases during at least 1 of the 4 years of the reporting period; eight countries had no reported cases for the entire 4 years. The first reported foodborne outbreak in the UK occurred in 1922, involved eight persons, and was caused by type A botulinum toxin in duck paste [51]. Until 1999, ten additional events occurred involving 50 persons; three were type A, 3 type B, 1 type E and 3 unknown. The largest outbreak (type B), involving 27 persons, was caused by a hazelnut purée which was prepared with a sugar substitute. The UK reported 10–13 confirmed cases of botulism to the ECDC between 2006 and 2009, except for 2008 when only one case was reported [6]. In 2011, three children in a single family were confirmed with type A botulism; the outbreak was caused by a commercially produced product (korma sauce) only available in the UK [52]. The implicated batch of korma sauce was quickly withdrawn from sale. Additionally, alerts were issued throughout the UK and other areas of Europe through EWRS and RASFF. No other cases occurred. A dessert (tiramisu) containing contaminated mascarpone cheese caused a type A outbreak among eight young persons in Italy in 1996 [53]. Italy reported 12–32 botulism cases per year to the ECDC between 2006 and 2009 [6]. Most outbreaks in France are associated with canned ham, but recently nine cases were associated with home-canned asparagus [54]. Although most foodborne botulism cases in France are caused by type B toxin, at least 16 cases of type E botulism have occurred [55]. These type E cases in France differ from type E cases in North America, because they are primarily due to vacuum-packed fish and seafood products rather than “fermented” foods. France reported 4–23 confirmed botulism cases to the ECDC during the period of 2006–2009 [6]. In 2011, two type A outbreaks involving a total of nine cases were determined to be caused by a commercially prepared green olive paste (containing green olives, garlic, capers, and olive oil), produced in a small batch (total of 60 jars) [56]. The jars were primarily distributed to a small geographic area, but were also sold through the Internet. An investigation determined that the product was not sterilized correctly. Coincidently, during the same time period, Finland reported two cases of type B botulism confirmed to be caused by commercially prepared olives stuffed with almonds [57]. These are the only cases of foodborne botulism that have occurred in Finland except for two persons who had botulism from eating vacuum-packed smoked fish. Poland reports some of the highest number of foodborne botulism cases in Europe [58]. From 1988 to 1998, almost 2,000 cases have occurred with increases observed during times of social disruption and food shortages. The most common foods that cause botulism in Poland are home-canned pork, sausage, cheese and bacon; type B toxin was identified in > 80 % of cases. Recently, a case of type B botulism caused by home-preserved marinated mushrooms

was reported indicating that botulism in Poland is not restricted to home preservation of meat and cheese products [59]. Poland reported 15–24 annual cases of botulism during the period of 2006–2009 to the ECDC [6]. Presumably, all cases were foodborne since there have been no reports of other forms of botulism in Poland. Botulism in Romania from 1990 to 2007 was similar to Poland in that 93 % of foods involved were ham, pork, bacon and sausage; only type B outbreaks have been reported [60]. Romania reported 14–31 annual cases of botulism to the ECDC during 2006–2009 [6]. As in Poland, all reported cases are presumed to be food related. Foodborne botulism is rarely reported in Austria; only 15 cases have been reported since 1990 [61]. Botulism is rare in Denmark but usually involves type E toxin from the consumption of fish products [42]. However, a type B outbreak occurred in three Danish tourists on a mini cruise in Turkey; cheese rolls and fish were epidemiologically linked to the cases but no food was confirmed [62]. Notably, type F toxin first was discovered following an outbreak of three persons on the Danish island of Langeland [63]. While not part of the ECDC surveillance report, botulism is also rare in Turkey but three cases of unknown toxin type were epidemiologically linked to home-canned roasted mushrooms [64]. The Republic of Georgia has one of the highest incidences of foodborne botulism in the world (up to 6.7 per 100,000), primarily attributable (80 %) to unsafe home-preserved vegetables (85 % type B), while smoked fish and other food products also cause some illnesses [65].

As with Republic of Georgia, foodborne botulism is relatively common in Asia. From 1958 to 1989, 745 outbreaks involving 2,861 cases have occurred in China [66]. Over 60 % of these events were caused by home-preserved bean products, including bean curd and bean paste; most cases occur between February and May when these products are mainly prepared and consumed. Although toxins A, B and E have been identified, most cases (~ 79 %) were caused by type A. In contrast to type E cases in Alaska and Canada which occur along coastal waters, type E cases in China occur inland [67]. Surprisingly, one type E outbreak was found to be caused by *C. butyricum* type E in home-preserved soybeans [68]. Another outbreak caused by this rare *Clostridium* sp. occurred in India in 1996; the food epidemiologically associated with this outbreak was *sevu*, a crisp made from flour [69]. Japan first reported botulism in 1951 [70]. Although less common now, 86 outbreaks involving 351 cases occurred between 1955 and 1998. Most (88 %) of the outbreaks were due to type E botulinum toxin; only six were due to type A and three due to type B. All of the type E outbreaks were caused by *Izushi* (fermented fish and cooked rice) or *Kirikomi* (fermented fish without rice). The non-type E outbreaks occurred from commercially produced foods some of which were prepared using materials exported from other countries. In 1969, 23 type B cases occurred from food manufactured using bottle caviar imported from Germany. Eighteen type B botulism cases occurred in 1998 from the manufacturer of a food product using green olives imported from Italy. In 1984, type A botulism occurred in 36 individuals after eating vacuum-packaged deep-fried mustard stuffed lotus root. Botulism is rare in Thailand; however, since 1997, three outbreaks (all type A) have occurred from the consumption of community-prepared bamboo shoots [71, 72, 73]. The first two outbreaks affected < 12 persons each; however, the 2006 outbreak affected

209 individuals because the bamboo shoots were consumed during a highly attended religious event. The locally prepared bamboo shoots in 2006 were prepared by placing bamboo shoots in large tin containers over an open fire. Some of the bamboo shoots added last were likely not heated adequately, and the containers were filled to capacity likely creating an anaerobic environment when the cans were sealed. Another botulism outbreak occurred in Thailand in July 2006; 21 persons were affected after consuming a traditional meal that included raw deer meat. CDC provided laboratory assistance and identified type F toxin in the stool from one patient and from the samples of raw deer meat; *C. baratii* type F was identified in both the stool and the deer meat (CDC unpublished data). Commercial products have been responsible for some foodborne botulism in Asia. Type A toxin caused an outbreak in Taiwan involving nine persons who consumed commercially canned peanuts from an unlicensed manufacturer [74]. Recently, a single type E botulism case was reported in Taiwan associated with commercially vacuum-packaged dried bean curd [75].

Other than consumption of home-canned or home-processed food, there are no known specific risk factors for foodborne botulism; men and women appear to be affected equally. In the USA, foodborne botulism occurs primarily in persons aged 30–60 years probably due to the higher consumption of home-processed food products in this age group [8]. However, foodborne botulism affects all age groups. Foodborne botulism occurred in a 6-month-old US infant after eating botulinum toxin-contaminated home-canned baby food [76]. In 2011, Italy reported a case of type A foodborne botulism in an 8-month-old infant; the infant was exposed to improperly preserved turkey [77]. These two cases demonstrate that persons of all ages, even infants, are susceptible to this form of botulism. There has been speculation that younger persons may be less susceptible to the effects of botulinum toxin because lower case–fatality ratios are reported in this population, but the number of cases of foodborne botulism in younger persons is too low to establish a significant difference [8]. The trend observed simply may be due to fewer complications during intensive care respiratory support in younger patients rather than to inherent resistance to the effects of botulinum toxin [28].

Home-processed foods (served in homes, social settings and restaurants) account for 95 % of foodborne botulism outbreaks in the USA [8, 22]. Temperatures obtainable only with a pressure cooker are usually necessary to kill *C. botulinum* spores, particularly spores of proteolytic strains. The toxin itself is heat labile; heating to 80°C for 10 min is sufficient to destroy the toxin. However, some foods are not heated before being eaten, and others may not be heated sufficiently to reduce botulinum toxin below hazardous levels. A recent review of food preparation practices associated with three different home-processed foodborne outbreaks revealed that there was no evidence that a pressure cooker was used to preserve the foods, and in at least one outbreak, food was not adequately heated prior to the meal [78]. In 2010, five of seven persons that consumed a meal consisting of home-preserved food were determined to have type A botulism suggesting that even in Europe, additional education is needed to prevent this potentially deadly illness [79]. Public health investigators identified increased numbers of botulism cases in the USA dur-

ing periods of economic crisis, such as during its participation in World War I and II and the depression [80, 81, 82]. Both Polish and Romanian public health officials observed similar trends in their countries during times of social and political strife including post-communism eras [58, 60]. While home-canning practices, at least in the USA, have reduced in the past few decades, there is a fairly new trend around the world that encourages individuals to accept individual responsibility for preparedness against all types of disasters, such as hurricanes, tornados, earthquakes, even The End of The World as We Know It (TEOTWAWKI) campaigns. These "preppers" have created networks in various countries to serve as resources for those who want to prepare for upcoming disasters. One such group in the USA is the American Prepper Network [83]. A review of this website shows a wide range of advice on disaster planning. Unfortunately, the website includes outdated (circa 1912–1915) resources on home-canning practices. One of the documents provided on the website does mention pressure cooking as a means for home canning, but it only provides this as a suggestion to reduce the time to prepare the food rather than being a requirement for safe food production. Use of these documents by Network member or website visitors may result in an increase in the number of foodborne botulism cases around the world over the coming few years. While accepting individual responsibility for preparedness should be encouraged, perhaps networks such as this should be targeted for educational messages that make sure home-based disaster planners prepare safe food.

12.4 Wound Botulism

Wound botulism results from the growth of *C. botulinum* spores in a contaminated wound with *in situ* toxin production. The first report of a wound botulism case (occurred in 1943) was published in 1951 on a 15-year-old female who sustained a compound fracture of the leg and ankle during a fall [84]. Neurological symptoms began 14 days after the injury, and the patient died. A review of 18 other wound botulism cases in the USA resulting from injury showed that botulism symptoms began within 4–17 days [85]. Only 27 wound botulism cases were reported between the first case in 1943 and 1982 when the first wound botulism case associated with illegal drug abuse was reported in New York [86]. Between 1943 and 2009, 461 cases of wound botulism were reported in the USA [8, 15, 16, 17]. In cases in which toxin type was identified, 408 (88%) were type A, 35 (8%) type B, one (<1%) a mixture of type A and type B organisms, and 17 (4%) had serum quantity insufficient to do toxin-specific neutralization. Forty-seven wound botulism cases were reported between 1951 and 1990 [87]. Only 15% of the cases were associated with injection drug use; the remaining cases were associated with trauma or post-surgery complications. Recently, the fifth case of post-surgery botulism case (type A) was reported in a 54-year-old who had a laparoscopic appendectomy; this patient's risk factor for wound botulism was suspected to be due to broad-spectrum antibiotics used to treat an abdominal abscess in the patient 6 days after surgery [88]. In con-

trast, 389 wound botulism cases were reported to CDC between 1990 and 2009; 97% were due to injection drug use [8, 15, 16, 17]. The change in wound botulism in the USA is largely due to changing trends in California. A review of California botulism cases showed that prior to 1988, wound botulism occurred < 1 per year; 10 years later, 28 cases occurred in 1998 alone [89]. Prior to 1988, the rare cases of wound botulism were primarily male with median age of 28. After 1988, the median age increased to 40, 45% were women, a higher proportion (57%) occurred in the Hispanic population, and almost all were associated with injection drug use, primarily "skin popping" black tar heroin. The risk of wound botulism in injection drug users does not seem to be a behavioral deterrent. Seventeen wound botulism patients previously treated in California returned to the hospital within 1–71 months with additional episodes of wound botulism, presumably from continued exposure to contaminated illegal drugs; one case had three wound botulism episodes [90].

Wound botulism has been reported in Europe, Asia, Australia and South America; rarely, these cases are associated with trauma [91, 92]. Wound botulism was not recognized in the UK and Republic of Ireland prior to 2000 [93, 94]. Between 2000 and 2004, 88 cases were reported (80% occurred in England) all in injecting drug users. Similar to cases in California, most laboratory confirmed cases (88%) were caused by *C. botulinum* type A. In 2004, a geographic clustering (36 of 40 total cases) of wound botulism cases occurred in England [94]. This unusual cluster suggested that the patients were linked; however, a causal association could not be identified. To date, no laboratory has provided a definitive link between wound botulism in injection drug users and any contaminated drug source. Sixteen cases of wound botulism were reported to German public health officials between October 13 and December 5, 2005, when only one wound botulism case was reported for all the previous 3 years [95]. The sudden surge in the numbers of wound botulism cases prompted an extensive epidemiological investigation, but no link could be identified. However, seven *C. botulinum* type B isolates from six patients (two isolates were obtained from two wound sites on a single patient) had indistinguishable pulsed-field gel electrophoresis (PFGE) patterns suggesting a possible link.

In spite of recent reporting increases, the number of wound botulism cases may be underestimated. Wound botulism is laboratory confirmed by detection of toxin in serum and/or isolation of *C. botulinum* from a wound. Investigators in California reviewed medical records and laboratory data for 73 patients with illness compatible with wound botulism. Their review showed that serum was positive by mouse bioassay in only 68% of the cases [96]. Similar difficulties in laboratory confirming wound botulism have been reported elsewhere. Only 58% of suspect cases were laboratory confirmed by either toxin detection in serum or isolation of *C. botulinum* from tissue or pus in the UK between 2000 and 2002 [93]. Similarly, 58% of patients associated with a wound botulism cluster in Germany were laboratory confirmed by toxin detection or positive culture results [97]. Only 33% of 36 suspect wound botulism cases in England in 2004 were laboratory confirmed [94]. In Ireland, only 17% of cases were laboratory confirmed in 2008 [98]. The California study also showed that of the 23 serum negative patients, 26% did not have "botulism" or "suspected botulism" in their discharge summaries, in spite of

having classic botulism symptoms, such as ptosis, dysphagia, dyspnea and bilateral, descending muscle weakness. The lack of documentation of a diagnosis of botulism in the medical records for clinically consistent patients with negative test results suggests that wound botulism may be significantly under reported worldwide. As a result of this study, a change was made in 2011 to the National Notifiable Diseases Surveillance System CSTE Position Statement for Botulism (Table 12.2) to include a definition for a probable case for wound botulism which previously only contained a classification for confirmed cases.

12.5 Intestinal Toxemia Botulism

As early as 1937, investigators in the former Soviet Union recognized a secondary type of botulism sometimes termed "toxinfection" which resulted from growth of organisms and toxin production in the intestinal tract; however, this form of botulism was universally discounted for decades [99]. The Russian investigators demonstrated this in experimental animals and described extended (>14 days) detection of botulinum toxin in several patients and reoccurrence of severe disease over 3 months after initial exposure to a contaminated food product in the mid- to late-1950s. Two forms of intestinal toxemia botulism are now recognized worldwide: infant botulism and intestinal colonization botulism (adult and child) even though there are no standard measures (clinical or laboratory) to definitively define the adult form. Infant botulism is the most frequently recognized form of botulism in the USA, although it is rare in other countries [8].

12.5.1 Infant Botulism

Infant botulism as a unique syndrome first was reported in the USA in 1976 [2, 3]. Within 2 years of the first report, infant botulism became the most common form of botulism reported in the USA; currently, 80–100 cases are reported each year representing a fourfold to fivefold increase over reports of foodborne botulism [8]. The total number of US infant botulism cases reported to CDC between 1976 and 2009 was 2,709 [15, 16, 17, 100]. In 2008, Rhode Island became the last state in the USA to report its first infant botulism case (type B) [16]. Since 1985, 39% of all infant botulism cases have been reported from California.

Recently, a review of worldwide infant botulism from 1976 to 2006 showed that this illness has been reported from almost every continent; no cases were reported in Africa [100]. Only 18% of all cases occurred outside the USA. Continent-specific clustering also occurred. Most cases (99%) on the continent of North America occurred in the USA; only 13% of countries reported any cases with Canada reporting the second highest number of cases. Almost all (99%) cases in the South American continent occurred in Argentina, with only three countries (25%) reporting

even a single case. Thirteen of 47 (28%) countries spread throughout the European continent reported infant botulism cases; however, 40% of these cases occurred in Italy. Interestingly, there were no reports of infant botulism cases in Poland or in the Republic of Georgia both of which represent high incidents of foodborne botulism on this continent [6, 58, 65]. A third infant botulism case in Denmark was recently reported; however, foodborne botulism was also suspected since the commercially produced infant food, not available for testing, provided to the infant was reported by the mother to smell bad [101]. No organisms were recovered from the infant stool, and toxin was only detected in the serum. A product recall was issued although no toxin was detected and no *C. botulinum* contamination was identified in an unopened identical product. Only 6 of 44 (14%) countries on the continent of Asia reported infant botulism cases; 76% of the cases occurred in Japan [100]. Recently, investigators in Iran reported their first case of laboratory-confirmed (type A) infant botulism in a 6-month-old girl with a history of consuming honey 3 weeks prior to illness [102]. While a full food history was not provided, other than exposure to honey, type A toxin was detected in the stool and no other family members were reported to be ill. This report in Iran adds to the growing number of countries reporting cases of infant botulism. Australia, the largest of 14 countries consisting of mostly small islands on Oceania and the only one with reports of infant botulism, had 32 cases; 56% of these occurred in South Australia and Victoria [100].

All known botulinum toxin types, except types D and G, have caused infant botulism, and dual toxin-producing *C. botulinum* strains have been identified [100]. Toxin types A and B are the most commonly reported (98.6% of cases with known toxin type) cause of infant botulism worldwide (Table 12.4). An additional 45 cases were reported to be caused by a single strain which produced both type A and B toxin. The percentage of type A versus type B cases was nearly equivalent in most continents except for Asia, where the number of type A cases was approximately two times the number of type B cases and South America, where 99% of cases were type A. Type E toxin was the next highest toxin type reported in Europe; four cases of infant botulism due to *C. butyricum type E* were reported from Italy [100]. *C. butyricum* type E was also identified in one case in Japan and in one case in the USA. A second type E case was identified in 2007 in the USA and is the first case of infant botulism caused by *C. botulinum* type E [103]. The first type F infant botulism case was described in 1975 and determined to be caused by *C. baratii* type F [104]. To date, nine type F infant botulism cases in the USA were either suspected or proven to be caused by *C. baratii* type F, the most recent was identified in 2008 in Colorado [16, 100]. Another case of infant botulism due to *C. baratii* type F occurred in Hungary and represents the only reported infant botulism type F case outside the USA [100]. A single case (5½-month-old female) of infant botulism due to *C. botulinum* type C was reported in Japan in 1990 [105].

Infant botulism is reported in the USA most commonly in the second month of life and occurs somewhat earlier in cases of type B disease (median 9 weeks) than in cases of type A disease (median 11 weeks); type F cases have been reported in infants as young as 1–2 days old [10]. There is no sex predilection and no apparent pattern of seasonal variation. Little is known about how infants are exposed

Table 12.4 Global distribution of reported infant botulism toxin type

Area	A	B	C	E	F	Ba[b]	Bf	Ae	Unknown	Total
North America	1,213 (44%)[a]	1,489 (54%)		2 (0.0 7%)	9 (0.33%)	16 (0.58%)	5 (0.18%)		2 (0.07%)	2,736
USA[c]	1,191 (44%)	1,484 (55%)		2 (0.07%)	9 (0.33%)	16 (0.59%)	5 (0.18%)		2 (0.07%)	2,709
Canada	22 (81%)	5 (19%)								27
South America	368 (99%)	1 (0.27%)							1(0.27%)	370
Europe	20 (30%)	27 (41%)		4 (6%)	1 (1.5%)	1 (1.5%)	2 (3%)	1 (1.5%)	10 (15%)	66
Oceania	12 (37%)	15 (47%)				1 (3%)			4 (12%)	32
Asia	14 (48%)	8 (28%)	1 (3%)	1 (3%)					5 (17%)	29
Africa	0	0	0	0	0	0	0	0		0
Total	1,627 (50%)	1,539 (48%)	1 (0.03%)	7 (0.2%)	10 (0.3%)	18 (0.6%)	7 (0.2%)	1 (0.03%)	22 (0.7%)	3,233

[a] Percentage calculated from total number of cases for each continent or country

[b] Most identified as predominately B with minor level of type A toxin; in one case, the relative toxin levels were not available [100]

[c] Data for the USA from refs [15, 16, 17, 100]; all data from other countries/continents were from ref [100]

to neurotoxin-producing *Clostridia* sp. Although spores have been found in some foods, only honey has been identified as a specific risk to infants [8]. Several studies from the late 1970s and early 1980s showed that more than 20 % of affected infants in the USA had ingested honey before the onset of botulism. Additionally, *C. botulinum* spores of the same type as those isolated from feces were cultured from honey known to be fed to the infant in a few cases suggesting a causal relationship. However, since most infants with infant botulism, at least in the USA, have had no exposure to honey, the risk factors and vehicles of transmission of *C. botulinum* for the majority of cases remain unclear [8, 100]. A survey of foods revealed *C. botulinum* in samples of corn syrup as well as honey, but in no other tested category of foods. Some infant botulism cases, particularly those < 8 weeks old received no other food then breast milk. Some environmental studies demonstrated that the same serotype of *C. botulinum* that caused disease could be isolated from the soil in an infant's yard and from vacuum cleaner dust; investigators have also frequently noted environmental conditions that might expose infants directly to environmental sources of *C. botulinum* spores, such as a shared crib, dusty or windy locales, nearby building construction or outdoor activities [8]. *C. botulinum* type A was isolated from a left over rice pudding and *C. botulinum* type B was isolated from both opened and unopened powdered infant formula (PIF) fed to a type B infant botulism case in the UK [106]. Ten *C. botulinum* type B isolates obtained from the opened PIF were clustered into four amplified fragment length polymorphism (AFLP) patterns, and nine *C. botulinum* type B stool isolates were clustered into two patterns; two of the AFLP patterns from the opened container of PIF were indistinguishable from the two AFLP patterns from stool isolates. Cultures of 25 unopened cans of PIF (same brand and batch as fed to infant) resulted in only one can with *C. botulinum* type B; the AFLP pattern of the isolates from this unopened container was distinct from both the AFLP patterns from the stool and the four AFLP patterns obtained from the opened food. PFGE studies confirmed that some isolates from the opened PIF were indistinguishable from stool isolates, but the isolates from the unopened PIF were unique [107]. Both these related studies demonstrate that simple identification of the same toxin type (type B in this case) in cultures of stool and implicated food cannot be used to assign a causal relationship between infant botulism and foods known to be consumed by these cases. Isolation of *C. botulinum* type A from the left over rice pudding demonstrates that simply isolating a neurotoxin-producing *Clostridium* sp. from a leftover food also cannot be used as proof of source of exposure in infant botulism cases, since this infant was confirmed to have type B disease. Additionally, infants may be colonized with several different strains of *C. botulinum* and products, such as PIF fed to infants, may contain several unique strains. The studies also showed that *C. botulinum* type B spores with indistinguishable AFLP and PFGE patterns from the stool isolates were present in the opened container of PIF suggesting that spores in this opened food may have caused infant botulism in this case. However, the studies did not prove that contamination of the PIF by the strain which caused illness occurred during manufacturing, since an unopened container of PIF of the same brand and batch number contained spores which were clearly distinct from those which caused this infant botulism case. The source of the

spores found in the opened container is not known, but could have occurred after the product was opened by the consumer. While these studies demonstrated that it may be possible to use AFLP or PFGE to analyze products containing *C. botulinum* spores and to suggest that a particular product may be the source of infant botulism, additional studies are needed to establish the diversity of *C. botulinum* before they can be used to establish a causal relationship between isolates recovered from clinical samples and suspect food products.

12.5.2 Intestinal Colonization Botulism (Child or Adult)

Intestinal colonization botulism in an adult or child is an elusive disease to classify. These are isolated cases of botulism in patients >1 year of age in which extensive investigations failed to implicate a specific food as the cause of the disease and for which an infected wound could not be identified and illegal drug use was not suspected. In the USA, the initial investigation of these is usually recorded by CDC as cases of "undetermined origin" or "other." There is currently no standardized CSTE position statement for these cases and so these are nationally reported as "Other" if laboratory confirmed (Table 12.2). Since 1976, 73 botulism cases could not be definitively classified as either foodborne or wound botulism (CDC unpublished data). Of the 64 for which toxin type was known, 63% were type A, 13% were type B and 25% were type F. This somewhat contrasts with the relative distribution of toxin type associated with foodborne botulism in the USA from 1975 to 1992: type A (57%), type B (18%), type E (21%) and type F (0.3%) [42]. Although there has been speculation on the matter since the 1920s, careful investigation has now demonstrated that some of these cases of "undetermined origin" are caused by colonization of the gastrointestinal tract by neurotoxin-producing *Clostridia* sp. with *in vivo* production of toxin, analogous to the pathogenesis of infant botulism [8, 108, 109, 110].

Remarkably, 25% of the USA cases of "undetermined origin" were associated with type F toxin. The first such case in 1987 was determined to be caused by *C. baratii* type F [111]. A review of botulism cases from 1981 to 2002 identified 13 type F adult cases [112]. *C. baratii* was isolated from stool for 9 of these 13; 8 stool cultures produced *C. baratii* type F. One type F case from California was due to foodborne botulism since type F toxin was detected in an implicated food consumed by the patient; *C. baratii* type F was also isolated from the food [21]. Toxin type F was detected in stool culture of two additional cases but the causative organism was not identified [112]. Five of the nine patients in which botulism was caused by *C. baratii* type F, including the California foodborne botulism case, had a medical history of major gastrointestinal surgery, recent gastrointestinal procedures, and/or recent antimicrobial treatment consistent with other reported intestinal colonization botulism cases. Few cases of intestinal colonization botulism are reported outside the USA. Two unrelated cases, both caused by *C. butyricum* type E, were reported in Italy [113]. Both cases presented to the hospital with acute abdominal pain and

underwent surgery for suspected appendicitis; during surgery, each was found to have inflamed Meckel's diverticulum which was resected. *C. butyricum* type E was isolated from stool 11–16 days from symptom onset. Five cases of suspected adult intestinal colonization were identified between November 2006 and May 2008 in or around Toronto, Ontario, Canada; three of which were described in detail in 2012 [114]. Two of the three patients were confirmed as type A botulism; both had medical histories significant for gastrointestinal complications. The third patient (type B botulism) had no significant medical history to suggest a higher risk for intestinal colonization. In 2003, investigators in Japan reported an intestinal colonization botulism case in a 12-year-old girl following foodborne botulism involving consumption of vacuum-sealed hashed beef [115]. The description of this case in Japan is similar to early reports by Russian investigators in which prolonged botulism followed a shorter typical foodborne botulism episode [1]. *C. botulinum* type Ab was isolated from the patient's stool up to 122 days from hospital admission; serum collected at day 250 was able to neutralize the effects of type A toxin, suggesting that the patient produced protective antibody to the toxin produced in the gut [115]. Support for the diagnosis of botulism from intestinal colonization can be obtained by the demonstration of the prolonged excretion of toxin- and neurotoxin-producing *Clostridium* sp. in the stool [109]. Unfortunately, few laboratories collect and test additional clinical specimens on these cases. Definitive classification of this form of botulism is difficult and in most cases relies on the absence of association with a botulinum toxin-contaminated food or a reliable history of consuming home-canned foods. Intestinal colonization botulism patients may produce detectable antibody due to the long-term exposure to low levels of toxin [109, 115]. However, identification of circulating antibody is rarely, if ever, done. The limited data available on intestinal colonization botulism cases have not identified any trends in specific food risks and except for alterations in the gastrointestinal tract, no other specific risk factors have been identified [8].

12.6 Iatrogenic Botulism

Remarkably, one of the most potent toxins known has brought incredible relief to individuals with a wide range of conditions. As with all pharmaceuticals, there are potential risks in therapeutic administration of the botulinum toxin. Several products are currently approved for use in both the USA and Europe for defined conditions, such as blepharospasm, strabismus, cervical dystonia, severe primary axillary hyperhidrosis, spasticity and for the temporary improvement in the appearance of moderate to severe facial frown lines. However, off-label use of botulinum toxin for the treatment of other conditions occurs. Adverse events associated with botulinum toxin injections that lead to unanticipated local or systemic effects consistent with botulism symptoms are classified as iatrogenic botulism, although there is no formal definition in the CSTE guidelines (Table 12.2).

Almost all approved products have caused some adverse effects. A 3-year-old female with cerebral palsy was off-label treated in multiple limbs with a total of 400U onabotulinumtoxinA (BoTox BT/A) (marketed by Allergan) [116]. Within 3–4 weeks of injection, the child experienced worsening dysphagia, excessive drooling and aspiration of liquids. Respiration would sometimes cease when the child was sleeping and required resuscitation by her mother. Severe generalized weakness lasted for 6 weeks and she was unable to lift her head for 3 months. A 10-year-old boy was treated in multiple limbs with a total of 19,000U rimabotulinumtoxinB (Myobloc BT/B; marketed by Solstice Neurosciences) for spastic quadriplegia [117]. Within 1 week, the patient began experiencing problems breathing when sleeping and progressed to cranial nerve symptoms by week 5; full recovery did not occur until 6 months from the injections. An adult patient treated with 18,500U of type B toxin (product not identified) for cerebral palsy-related leg spasticity developed severe dysphagia that required hospitalization for 12 days; full resolution of symptoms did not occur for 75 days [118]. In 2010, adverse events in three adult patients with spasticity treated with 1,000–1,500U abobotulinumtoxinA (Dysport BT/A; marketed by Ipsen Biopharm Ltd.) were reported [119]. Two of the patients presented within 1–3 weeks from injection with symptoms of botulism (dysphagia and moderate to severe weakness of the upper body; one required intubation); the adverse symptoms resolved within 2 months. The third patient did not show systemic symptoms (dysarthria, dysphagia, shortness of breath, mild weakness of head, neck and upper extremity muscles) until 1 week after the fourth injection (identical injections were spaced 3–4 months apart); adverse symptoms did not resolve for 6 months. An interesting case of iatrogenic botulism was reported in a weight lifter [120]. The patient was initially treated with 1,000U Dysport BT/A in his lower limbs for a gait disorder. On follow-up, the patient reported a tremendous improvement in gait coordination but also some feeling of fatigue and generalized weakness that started about 2 weeks after injection. Three months later, the patient was injected with a lower dose (600U) and asked to keep a diary of his weight lifting capacity. His meticulous documentation showed almost a 50% reduction in both upper and lower limb strength 6 weeks after injection, but strength capacity returned by week 12. While adverse symptoms were fairly mild and the benefit of treatment clearly outweighed the risks, this report demonstrates that even low doses of botulinum toxin migrate beyond the injection site and can affect distant muscles. Some adverse events may be more localized. A review of 211 patients injected into extraocular muscles showed that ptosis occurred in 8.7% of patients; most events were mild and most recovered within 6 weeks [121].

Only a small number of peer-reviewed publications report on adverse events associated with either therapeutic or cosmetic injections. Perhaps there is reluctance on the part of a physician to report in the scientific literature adverse events for off-label use or changes in recommended dosing of pharmaceuticals. Additionally, suspected iatrogenic botulism cases are rarely reported (<eight reports documented) in the USA to CDC's Botulism Surveillance System or other state health departments, and none have been laboratory confirmed. However, in the USA, many of these events are reported through MedWatch, a voluntary system for reporting adverse

events, product quality problems or product use errors. A review of adverse events reported to MedWatch during and following administration of licensed botulinum toxin was conducted in 2005 [122]. Serious adverse events reported (1,437) in patients from 1989 to 2003 treated with licensed toxin for therapeutic reasons (for both labeled and unlabeled indications) covered a wide variety of reactions, including death, ptosis, diplopia, dysphagia, dysarthria, dystonia, fatigue, and generalized muscle weakness, muscle spasm, allergic reactions, flu-like syndromes, gastrointestinal symptoms, cardiovascular system-related symptoms, respiratory system-related symptoms and seizure.

In January 2008, the Public Citizen's Health Research Group, a US consumer advocacy group, petitioned the US FDA to follow and expand on the EU 2007 safety recommendations and require manufacturers of therapeutic botulinum toxin products to add warning labels on product vials and to provide health-care providers and consumers information on potential risks to toxin injections [123]. This group's petition included an analysis of adverse events reported between 1997 and 2006. Their analyses identified 658 adverse events with 27% ($N=180$) reported as aspiration, dysphagia or pneumonia. Almost 50% of these more serious events required hospitalization and 16 deaths were reported; four deaths were in children < 18 years of age. As a result of the petition, FDA reviewed available data from clinical trial studies, published reports and MedWatch adverse events reports. In April 2009, the FDA determined that these adverse events warranted the requirement of a "Black-Box" warning label to be applied to all botulinum toxin products approved for use in the USA [124, 125]. In addition, the FDA required manufacturers to develop a risk mitigation strategy (RMS) to include information documents for both practitioners and patients. FDA determined that the RMS documents must have plain language which informs patients of possible adverse events and also language that makes it clear to practitioners the importance of following manufacturers' suggestions for dosing. FDA's review did not identify any deaths in patients treated with toxin type A for cosmetic purposes, but commonly reported reactions included headaches, focal facial paralysis, muscle weakness, dysphagia, flu-like syndromes and allergic reactions [122, 124, 125]. However, FDA's requirement included "Black-Box" warning labels and an RMS for products intended for cosmetic use only. This action by FDA may reduce the number of adverse events reported in the USA, since patients will be provided clear information on the risks of treatment, and practitioners will be provided with guidance on approved uses and information on the potential inequitable results when using identical dosing using different products.

It is not clear whether all adverse events are directly related to botulinum toxin. The summary report by FDA investigators in 2005 is clear in that not all adverse events reported to the FDA can be clearly identified as caused by the product [122]. A recent study [126] summarizes a prospective evaluation of adverse events, following botulinum toxin injection of 334 pediatric cerebral palsy patients. This study suggests that when used properly, even in severely affected cerebral palsy patients, the improvements in quality of life, following botulinum toxin treatment, outweigh the risk of primarily temporary adverse events. However, it is the physician's responsibility to know and understand the risks, including any maximum dose recom-

mendations [127]. Additionally, they need to understand that there are no dosing recommendations for pediatric patients since botulinum toxin is not approved in the USA for this population. Instituting strong requirements for "Black-Box" warning labels and development of a product RMS in all countries should provide physicians with the information they need to clearly explain the risks and benefits so that the patient's caregivers can make informed decisions on treatment options involving botulinum toxin.

An additional risk factor for iatrogenic botulism is in counterfeit or other non-approved materials marketed for human use and the availability of bulk research toxins not intended for human use. A list of several counterfeits marketed as alternatives to legitimate therapeutic toxin products was recently reported [128]. Independent laboratory analyses of these products indicated that most did not meet toxin potency as indicated on the vial; some were much lower, but even more problematic was that some were found to be more potent than the label indicated. Use of these bogus products could result in overtreatment of patients with subsequent severe consequences. A recent report by security analysts suggests that the botulinum toxin counterfeit market is growing around the world [129]. The groups marketing these products primarily through the Internet, in many cases, use deceptive practices to make their material resemble licensed products. Unfortunately, commercial industry and the health-care field contain unethical individuals who potentially profit from the production and administration of these bogus materials.

Perhaps the most incredible outbreak of iatrogenic botulism reported to date occurred in 2004, when a physician, on a suspended license, injected himself and three others with a research grade botulinum toxin type A product to treat moderate to severe facial frown lines; each patient was exposed to as much as 20 μg of type A complex through 4–6 injections in the facial area [130]. Onset of symptoms of dysphagia, diplopia and generalized weakness began within 1–2 days; all required mechanical ventilation within 3 days following injections. All patients, except for the physician who administered the injections (permission to collect clinical specimens was not provided by this patient), were laboratory confirmed by detection of botulinum toxin type A in serum; stools when available did not contain toxin, and *C. botulinum* was not isolated. All four patients survived; one patient, who was followed up to 10 months after hospitalization, reported persistent mylagias, muscle weakness, shortness of breath, and persistent pain that required treating with an analgesic medication [131]. The toxin used in these cases was labeled as a laboratory research product, not licensed or intended for human use, and was ordered as a single 100-μg vial and shipped from a legitimate manufacturer. The physician was sentenced to 3 years in prison for misbranding a drug [130]. Other vials of non-approved botulinum toxin were recovered from the physician's office but records were not available on whether this product was administered to any individuals visiting this doctor's office. The owners of a business, Toxin Research International, Inc (Tucson, AZ, USA) pled guilty in 2006 to charges of mail/wire fraud, misbranding a drug and defrauding the US government related to the packaging and distribution of vials of bogus Botox [129]. The greed of some individuals, unscrupulous doctors or other health-care providers, the availability of "bogus" improperly tested/labeled therapeutic products and the continued practice of "off-label" use of even approved

products by inexperienced practitioners may only increase the number of iatrogenic botulism cases reported to authorities.

12.7 Summary

Botulism is a rare and often surprising illness. While once thought to only occur as a foodborne intoxication, human botulism now occurs worldwide as one of the several unique forms (foodborne, wound, infant, adult colonization, iatrogenic and, rarely, inhalational) [8]. All provide unique challenges for diagnosis and control. Originally named because the illness was thought to be solely a problem of sausage, surveillance worldwide over the past 200 years has demonstrated the wide variety of foods, including sausage, fish, marine mammals, vegetables of all types, which may cause botulism. The identification of recent outbreaks due to commercial products suggests that this illness may not be on the decline around the world. Minimally processed, chilled foods may present a new era in foodborne botulism and provide opportunities for enhanced control measures. Additionally, wound botulism, infant botulism and adult colonization cases are beginning to be recognized in many countries, suggesting that reports of these botulism forms may become more frequent. Finally, a disturbing number of adverse events are being reported related to therapeutic toxin injections. These events are particularly challenging for public health because: (1) some of these events may be unrelated to toxin injections, (2) there is no current mechanism to capture this botulism form through public health surveillance, (3) no known laboratory tests can differentiate between true iatrogenic botulism and non-related causes of symptoms and (4) counterfeit products may be used by unscrupulous health-care providers which may be difficult to trace to their source.

Botulism has provided surprises to investigators through the identification of unusual *Clostridium* sp. that produce botulinum toxin. Other neurotoxin-producing species may be lurking in the environment waiting to be discovered. Seven serotypes of botulinum toxin so far have been identified but others may exist. Recent studies have demonstrated a diversity within the known toxin serotypes which is discussed in Chap. 10 but, subtype-specific risks to humans are currently unknown. Future surprises in botulism outbreaks, discovery and further study of toxin serotypes/subtypes, and characterization of the neurotoxin-producing Clostridia involved will help us understand and perhaps eventually control human botulism.

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Chapter 13
Prevention and Treatment of Botulism

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Abstract Concerns regarding botulinum neurotoxins (BoNTs) as biowarfare and bioterrorist agents have generated interest in developing medical countermeasures for protection against the neurotoxins. Efforts have focused on improvements in the available vaccines and antisera and de novo discovery of pharmacological inhibitors of toxin action. This chapter reviews the various approaches taken to develop next-generation vaccines, antitoxins and pharmacological treatments against intoxication by the BoNTs. The historical progression, current status and future trends are described.

Keywords Antitoxin · Bioterrorism · Botulinum neurotoxin · Botulism · Metalloprotease inhibitors · Vaccine

13.1 Introduction

13.1.1 Background

The botulinum neurotoxins (BoNTs) are the most potent substances in nature, and exposure to as little as 1–3 ng/kg may be sufficient to cause human lethality [26, 73, 104, 113, 146, 154, 162, 196]. The toxicity of the BoNTs stems from their potent and selective inhibition of acetylcholine (ACh) release at the neuromuscular junction, autonomic ganglia and structures innervated by the parasympathetic branch

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of the autonomic nervous system [19, 44, 46, 83, 86, 126, 159]. Paradoxically, this selective inhibition of ACh release has also enabled BoNT/A to become a highly useful therapeutic agent [22, 41, 43, 59, 140].

Since its approval in 1989 as an orphan drug for the treatment of strabismus, hemifacial spasm and blepharospasm, BoNT/A has come to be regarded as the treatment of choice for an increasing number of neurologic, autonomic and cosmetic conditions [43, 55, 82, 85, 222, 264]. The ability of BoNT to serve in this role is based on its exquisite selectivity for cholinergic nerve terminals, its long duration of action and its ability to remain localized near the intended target when injected at low concentrations and volumes [139, 153].

Although we have learned to harness the therapeutic benefits of BoNT in ways that were not even imagined when the neurotoxin was first approved as an orphan drug, we should not lose sight of the fact that BoNT is inherently a highly lethal toxin, that outbreaks of botulism with devastating consequences continue to occur [73, 76, 198, 277] (Chap. 12 of this volume) and that recovery from botulism can require months of intensive care and rehabilitation, often leaving patients with long-lasting physical and psychological trauma [68, 69, 132, 150, 151, 266]. After severe intoxication by BoNT, restoration of normal muscle function, exercise tolerance and cardiovascular fitness have been reported to take nearly a year for BoNT/F [239], more than 2 years for BoNT/B [266], and greater than 5 years for BoNT/A [151].

13.1.2 BoNT as a Bioterrorist Weapon

In addition to natural outbreaks, the potential use of BoNT by hostile nations or terrorist groups has been a growing concern [26, 29, 101, 116, 162, 191, 196, 198, 219, 263]. The ability of BoNT to cause mass casualties has led to its designation as a Tier 1 select toxin by the US Department of Health and Human Services (HHS), the only noninfectious agent to receive this designation (http://www.selectagents.gov/Select%20Agents%20and%20Toxins%20List.html). The threat of battlefield deployment of BoNT has diminished somewhat following the dissolution of the Soviet Union in 1991 and the regime change in Iraq in 2003. However, use by terrorists has become an increasing concern due to widespread availability of BoNT from both legitimate and illegitimate sources, coupled with ease of concealment and inherent vulnerabilities of our modern food and beverage distribution systems [265]. These factors, in conjunction with a rise of religious fundamentalism and proliferation of failed nations and those that support or sponsor terrorism, make an attack by BoNT more probable than in previous generations [21, 191, 196].

A bioterrorist attack with BoNT is likely to involve aerosol delivery or deliberate contamination of food, beverage or animal feed [1, 21, 26, 162, 263]. The pattern of botulism following a terrorist attack would be expected to resemble that observed after a natural outbreak, except the former may involve a larger number of casualties, originate at multiple locations and, in the case of aerosol exposure, lack an

easily identifiable source [1, 26, 101]. As with naturally occurring outbreaks, signs and symptoms following a terrorist strike by BoNT would consist of cranial nerve palsies, followed by symmetrical descending muscle weakness and respiratory collapse [73, 191, 196, 238]. BoNTs are less potent by inhalation than by injection and least potent by ingestion [26, 162]. The lower potency by the latter routes may be related to the need for BoNT to undergo transcytosis across airway or intestinal epithelial cells prior to entering the general circulation, whereas injection provides direct access to the bloodstream [18]. Moreover, ingested toxin must also overcome the hostile environment of the gastrointestinal (GI) tract (low pH and proteolytic enzymes), a process which is aided by association with a specific nontoxic non-hemagglutinin accessory protein. This protein is co-secreted by *Clostridium botulinum* and is able to shield the neurotoxin by providing it with complementary binding surfaces [107].

13.1.3 Medical Management of BoNT Intoxication

Treatment options for BoNT intoxication have changed little in principle over the past 40 years, although the therapies, guidelines and doctrines have undergone periodic refinement. Medical countermeasures consist of timely administration of antitoxin for those individuals exhibiting clear signs of exposure and treatment in an intensive care facility until patients can be discharged to lower levels of care such as to a rehabilitation unit [196]. Patients exhibiting respiratory collapse would also require mechanical ventilation in addition to the above measures, sometimes for extensive periods [34, 65, 238, 249].

In addition to these measures, vaccination with pentavalent botulinum toxoid (PBT) vaccine was recommended, until recently, for individuals in high-risk groups. Since development of active immunity to BoNT is relatively slow, vaccination would have to be initiated well before an outbreak and would be of no benefit after an exposure to BoNT [94, 202, 221]. On 30 November 2011, PBT was withdrawn by the US Centers for Disease Control and Prevention (CDC) due to problems with reactogenicity and declining immunogenicity; new recombinant vaccines are under development but not yet licensed by the US Food and Drug Administration (FDA) (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6042a3.htm?s_cid=;mm6042a3_x). BoNT vaccines are discussed in detail in Sect. 13.2.1.

It is generally agreed that a large-scale bioterrorist attack would overwhelm our intensive care facilities, and vaccination is not considered to be practical for civilians since vaccinated individuals would be precluded from benefiting from the medical uses of BoNT [191, 202]. The indications for the latter have expanded markedly from rare focal dystonias to more common medical conditions such as genitourinary disorders, hyperhidrosis, pain, headache and neuropathy [222, 264]. Candidates for BoNT therapy now encompass a sizable fraction of the population [41, 43, 82]; consequently, when improved vaccines become available, vaccination should only be considered after a careful risk–benefit analysis [230]. In addition,

reengineering of toxins has opened up additional therapeutic opportunities for treatment of conditions such as chronic pain [98, 157] and asthma [99]. These reengineered toxins are likely to be ineffective in BoNT-immunized individuals [84].

Since it is expected that most victims of a bioterrorist attack with BoNT would not be vaccinated, symptomatic treatment would need to be supplemented by infusion of antitoxin to prevent continued internalization of BoNT into target tissues. The current product is a despeciated heptavalent botulism antitoxin (HBAT) developed originally by the US Army Medical Research Institute of Infectious Diseases (USAMRIID) in conjunction with the University of Minnesota [119]. As with earlier antitoxins, HBAT is effective in reducing the severity of BoNT intoxication, as long as it is administered while active toxin is still circulating in the bloodstream [154].

To overcome this time constraint for therapy and to accelerate recovery, specific pharmacological agents to counteract BoNT intoxication would be desirable, either as stand-alone treatments or as adjuncts to antitoxin [7, 244]. The purpose of this chapter is to use the insights gained in our understanding of the mechanism of BoNT action, especially during the past two decades, to establish a conceptual framework within which to develop effective treatment strategies for intoxication, and to evaluate current and emerging treatment options. Important advances have been made in a number of critical areas. These include identification of antitoxin-binding epitopes [28, 32], determination of BoNT pharmacokinetics in animal models [61, 193], identification of the protein receptors for cell surface binding [79, 80, 123, 173], a more precise characterization of the translocation channels [94] and resolution of the crystal structure of BoNT and its functional domains [14, 42, 138, 247, 248]. Many of these discoveries were set in motion following recognition of the zinc metalloprotease action of the BoNT light chain (LC) [169], and subsequent identification of their SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein targets [168, 204, 205, 206]. The SNARE proteins targeted by BoNT, namely SNAP-25, synaptobrevin and syntaxin, are intimately involved in synaptic vesicle exocytosis, and their cleavage by BoNT is the key event that precedes the onset of toxin-mediated paralysis [182]. Continued advances in our understanding of the mechanism of BoNT-mediated intoxication are expected to lead to sustained improvements in our treatment options in the years ahead.

13.2 Vaccines

13.2.1 Botulinum Pentavalent Toxoid Vaccine

Other than physical protection, vaccination is the sole means to prevent botulism. From the earliest stages of vaccine development, it was recognized that a separate toxoid would be required to generate protective antibodies for each serotype, since neutralizing BoNT antibodies exhibited little cross-reactivity with non-homologous

BoNT serotypes [221]. In fact, the lack of cross-reactivity was the basis for designation of the eight distinct serotypes of BoNT [32, 114]. For the past half century, prophylaxis against botulism had been carried out by use of PBT vaccine developed by the US Army at Fort Detrick, Maryland [94]. The pentavalent formulation encompassed all of the serotypes (A, B, C1, D, E) that were known at the time of original production in 1958 and included the three serotypes that were responsible for most human outbreaks (A, B, E) [94, 114, 221]. PBT is generally administered to personnel at high risk of exposure such as those employed in botulinum research laboratories, BoNT production facilities and public health laboratories that investigate botulism outbreaks or military personnel deployed to regions with high potential for bioterrorism or biological warfare [73, 202, 221]. The individual monovalent toxoid components of PBT were produced separately in bulk culture, partially purified, inactivated with formalin (0.022%) and adsorbed on aluminum phosphate (adjuvant) to increase immunogenicity [94]. PBT vaccine was generated by blending the five monovalent toxoids, which were preserved with 0.01% thimerosal and bottled in multiuse vials [23, 221]. Vaccination of human volunteers with a series of three deep subcutaneous injections of PBT over a 10- to 12-week period followed by a booster at 52 weeks led to measurable titers in virtually all vaccinees.

There is compelling evidence for the efficacy of PBT from animal studies, from which human efficacy can be inferred. Thus, guinea pigs vaccinated with PBT were able to survive a challenge by up to 10^6 mouse median lethal dose (MLD_{50}) of BoNT [94], and passive transfer of antibodies from individuals vaccinated with PBT protected guinea pigs [103] and nonhuman primates (NHPs) from aerosol challenge by lethal doses of BoNT [100]. In addition, purified human immunoglobulins from volunteers vaccinated with PBT have been found to be highly effective in the treatment of infant botulism ([27], cf. Sect. 13.3.4).

PBT vaccine has been available from the CDC as an investigational new drug (IND) since 1965 for civilians at risk (IND 161) and subsequently for deploying military personnel (IND 3723, US Army Office of the Surgeon General). From 1965 to the present time, more than 20,000 injections have been given to those in the high-risk category, and more than 8,000 injections were administered to military personnel. In spite of its long history, PBT has never been licensed by the FDA. This is largely because the FDA's Animal Rule, which allows for licensure of vaccines (and approval of drugs) in the absence of human efficacy data, only came into effect in July 2002, well after PBT was developed [113].

Since 1979, five separate lots of PBT were produced, all using the same monovalent toxoids. New lots were necessitated by shortages in supply or reductions in potency of some of the individual serotypes. The most recent lot (PBP-003) has been available since 1994 [202]. Due largely to the high levels of formalin (to prevent reactivation of toxoid to toxin) and thimerosal in the final product, PBT is reactogenic, although most adverse events have been reported to be local rather than systemic: pain and erythema/induration at the injection site. For lot PBP-003, moderate local reactions (erythema/induration between 30 mm and 120 mm) were associated with 12% of vaccinations, and severe local reactions (erythema/induration more than 120 mm, or axillary lymph node enlargement and/or tenderness) with 2%

of vaccinations. Systemic adverse events (fever, malaise, headache, myalgia) were mild and occurred in 7% of vaccinees. Reactions were more frequent after boosters than after the primary series [202].

13.2.2 Limitations of PBT Vaccine

In spite of its long use, the PBT vaccine was found to have a number of drawbacks, which has led to the search for new-generation vaccines for BoNT intoxication. Among these are high reactogenicity, poor immunogenicity and absence of toxoids for BoNT/F or BoNT/G, both of which were discovered considerably later than the first five serotypes [105, 166]. Although BoNT/F and BoNT/G have been implicated in only a small number of human intoxications [147, 241], both are considered to be potential agents of bioterrorism. With regard to immunogenicity, achieving optimal protective titers requires injections at 0, 2 and 12 weeks (primary series) plus a boost at 52 weeks; annual boosters were recommended to maintain titers [236]. Antibody titers were observed to fall significantly between the end of the primary series and the first annual booster, creating a long window of vulnerability [221]. This problem was addressed in 2004 by adding a 6-month booster.

There have also been periodic issues with reductions in potency, especially for serotypes B and E [202]. The low immunogenicity and loss of potency were presumably the result of the age and relative impurity of the individual toxoids. The monovalent components were manufactured between 1969 and 1971 and contained only 10–15% protein [202]. These problems could be alleviated, in principle, by formulation of toxoids with higher purity [127]. However, the current safety and surety concerns over the large-scale toxin production that would be needed to support the vaccine effort and the increased regulatory requirement by the FDA for product safety make this approach impractical.

13.2.3 Discontinuation of PBT

As of November 30, 2011, the CDC terminated release of PBT for individuals at high risk of occupational exposure to BoNT [57]. This decision was based on the reduction in immunogenicity coupled with a progressive rise in reactogenicity of PBT, as described earlier. To allow current vaccinees to complete the primary series, PBT remained available until May 31, 2012. However, no new personnel were permitted to be vaccinated with PBT, and no licensed or IND vaccine for botulism is currently available in the USA to replace PBT [57].

It has been recognized for more than 20 years that new vaccine candidates could overcome many of the problems associated with the traditional vaccine, and alternative approaches to develop vaccines against the BoNTs were initiated in the early 1990s for eventual replacement of PBT [67]. Many of these efforts are still being pursued, including recombinant subunit vaccines based on the toxin-binding

domain, the combined catalytic and translocation domains, or holotoxin rendered inactive by mutations in the catalytic domain. These will be discussed in the following sections

13.2.4 Recombinant Botulinum Vaccines

Improvements in recombinant DNA techniques allowed for elucidation of the nucleotide and deduced amino acid sequence of clostridial neurotoxins nearly three decades ago [87, 130, 252, 265]. These advances enabled expression of nontoxic fragments of BoNT for use as vaccine candidates. Unlike toxoids, recombinant antigens could be produced in quantities sufficient for vaccine development without the need for large-scale culture of *C. botulinum* and its associated surety and biosafety risks, high cost, and need for chemical detoxification.

13.2.4.1 Recombinant Subunit Vaccines

The first demonstration that a nontoxic component of BoNT/A was able to generate protective antibodies was provided by investigators at USAMRIID [67]. These authors constructed a synthetic gene coding for the ~ 50-kDa binding domain of BoNT/A (Hc) which, unlike the native clostridial gene, could be readily expressed in *Escherichia coli*. The recombinant Hc, corresponding to the C-terminal half of the heavy chain (HC), was selected since it is the most antigenic component of BoNT and the one most likely to produce neutralizing antibodies [28, 33, 254]. An additional advantage of Hc is that it is nontoxic since it lacks both the translocation domain (H_N) and the catalytically active LC [229, 233]. After purification and adsorption on aluminum hydroxide adjuvant, mice were immunized with Hc. A series of three vaccinations at 0, 2 and 4 weeks protected mice against a 10^6-MLD_{50} challenge dose of BoNT/A at week 5. This pioneering study was inspired by earlier efforts with the related tetanus neurotoxin (TeNT) [92], and it clearly demonstrated the feasibility of using a nontoxic fragment to protect against botulism.

Since BoNT/A Hc appeared to be a promising vaccine candidate capable of replacing PBT, Smith and coworkers modified the synthetic Hc gene for expression in the yeast *Pichia pastoris* to take advantage of the superior attributes of this system (high yields, lack of endotoxin and ease of purification), and subsequently extended the recombinant vaccine effort to seven BoNT serotypes [52, 53, 124, 189, 235, 260, 261]. In preclinical studies, recombinant BoNT/A Hc was found to be protective in mice, and Hc of serotypes A and B were also shown to be protective in NHPs challenged by aerosol exposure [40, 113]. Moreover, neutralizing antibody titers were detected for up to 2 years in NHPs following vaccination with Hc domains [40].

To prepare for replacement of PBT by a recombinant vaccine, the US Chemical Biological Medical Systems-Joint Vaccine Acquisition Program (CBMS-JVAP) has been working with DynPort Vaccine Company LLC (DVC) to transition a bivalent

serotype A and B Hc recombinant vaccine (rBV A/B) for licensure under the FDA's Animal Rule. The vaccine was developed against BoNT/A subtype A1 and BoNT/B subtype B1 [113].

In 2004, DVC submitted an IND application to the FDA to carry out phase 1 clinical trials on this vaccine in a group of healthy adult volunteers [236]. The bivalent vaccine was well tolerated in the study population and stimulated serotype-specific neutralizing antibodies at all dosage levels tested. Moreover, passive transfer of antibodies from human subjects to guinea pigs was protective when animals were challenged with a 10-MLD_{50} dose of BoNT/A or BoNT/B [202].

The rBV A/B program was granted Fast Track designation by the FDA, and phase 2 trials were begun in 2008. The trials involved a study population of 440 healthy adult volunteers and assessed the safety of rBV A/B over an 18-month period. Vaccine or placebo was administered at two dosing schedules: 0, 28 and 182 days or 0, 56 and 182 days. Antibody levels were then measured at prescribed time intervals. This study was successfully completed in February 2011. Licensure must now await completion of phase 3 studies. If successful in phase 3, rBV A/B is likely to be the first vaccine approved under the FDA's Animal Rule (http://assets1.csc.com/dvc/downloads/DVC_Botulinum_Vaccine_Case_Study_May_2010.pdf).

Even if rBV A/B achieves licensure as planned, it still leaves the remaining serotypes of BoNT without an approved vaccine. In limited preclinical studies, recombinant Hc fragments of serotypes C and D were also found to be effective vaccine candidates [261], both alone and in combination. Further, a heptavalent Hc-derived vaccine was found to protect mice challenged by 10,000 intraperitoneal (i.p.) MLD_{50} units of each BoNT serotype [31]. At this time, however, there are no plans to conduct advanced studies on these serotypes, largely due to resource limitations.

13.2.4.2 Other Recombinant Vaccine Candidates

In addition to Hc-derived vaccines, a number of other recombinant products have been studied at the preclinical level that may be candidates for future vaccine development. Among these are subunit vaccines consisting of the combined catalytic and translocation domains (LCH_N) [220], subunit vaccines coupled to viral vectors for enhanced immunogenicity [141, 171, 275] and catalytically inactive holotoxin that contains mutations in the LC, rendering the molecule nontoxic (holotoxoid) [186, 262]. This holotoxoid would appear to be the ideal immunogen, since it could elicit neutralizing antibodies to the binding, translocation and catalytic domains of BoNT. Moreover, the holotoxoid could also offer protection following challenge with a reengineered toxin in which one or more domains were altered to evade subunit vaccines targeting a single domain.

Both a double LC mutant of BoNT/A expressed in *E. coli* (R362A, Y365F) [186] and a triple LC mutant expressed in *P. pastoris* (H223A, E224A, H227A) [262] were found to be highly protective in mice. The triple mutant was in fact shown to protect against three of the five subtypes of BoNT/A (A1, A2, A3) after a single

injection, whereas the Hc-derived vaccine was only effective against challenge by A1 under this condition [262]. Although these differences were less pronounced following multiple vaccinations, the atoxic mutant holotoxoid still has the advantage of eliciting protection with less delay, which is significant for enabling rapid military deployment to regions with potential BoNT exposure.

Subtypes within serotypes arise from variations in the primary structure of the toxins, and have significant impact on protective strategies since vaccines and antitoxins are generally less effective in protecting against dissimilar BoNT subtypes [192, 220, 237, 262].

13.2.5 Mucosal Vaccine Delivery

Vaccines delivered parenterally produce antibodies that can neutralize BoNT only after it has gained access to the bloodstream. Vaccines delivered mucosally, on the other hand, are capable of inducing both systemic and mucosal immunity [66, 102, 183]. The addition of mucosal immunity is of potential advantage since botulism, other than wound or iatrogenic, involves initial binding to the airway or intestinal mucosa [133, 149, 194]. Xu et al. [270] demonstrated that intranasal (i.n.) delivery of a vaccine constructed from a replication-incompetent adenoviral vector encoding the Hc component of BoNT/C was able to elicit high levels of immunoglobulin A (IgA) in mucosal secretions and immunoglobulin G (IgG) in sera of mice 2 weeks after a single vaccination with 2×10^7 plaque-forming units. In addition, this dose protected 100 % of mice against a 100-MLD_{50} challenge of BoNT/C at 7–27 weeks after vaccination. The rapid onset, persistence and ability to achieve protection after a single vaccination are remarkable and exceed the performance of traditional and other new-generation vaccine candidates. It is not clear, however, what role mucosal IgA played in the observed protection, since similar results were obtained when the adenoviral vaccine was injected intramuscularly [275]. Even if the mucosal protection was not the major factor in the findings of Xu et al. [270], i.n. delivery still has the advantage of increased efficiency and lower cost [194].

Based on the preceding, we have transitioned from having a single pentavalent toxoid vaccine for botulism (PBT), which was limited by low immunogenicity and high reactogenicity, and are in the process of developing a safer and more effective bivalent subunit vaccine (rBV A/B) that, in principle, can be extended to all serotypes [31]. There are other, perhaps even more promising candidates, such as atoxic holotoxins, that may provide protection against some deliberately modified forms of BoNT whose binding domain might not be neutralized by rBV A/B [186, 262]. Which, if any, additional vaccine candidates will be selected for development is yet unclear. In addition, the benefits of vaccination must be weighed against the considerable cost of developing new vaccines, the likelihood that BoNT intoxication can be successfully treated by a combination of antitoxin and a yet to be developed therapeutic and the probable loss of clinical benefit to the vaccinees.

13.3 Antitoxins

13.3.1 Rationale for Antitoxin Treatment

Although in use for more than four decades, equine antitoxins are still the only postexposure products available for limiting the severity of BoNT intoxication [73, 154]. The efficacy of antitoxins stems from their ability to neutralize BoNT in circulation and thus to prevent further internalization of toxin. Achieving this requires antitoxins to be administered early during the course of illness [115, 215, 249]. The temporal limitation of antitoxin treatment has long been appreciated [117] and is related to the fact that clostridial neurotoxins exert their actions inside the nerve terminal, where they are not susceptible to antibody neutralization [229]. Accordingly, at the time when signs and symptoms of botulism become apparent, a substantial quantity of toxin has already become internalized, and only the fraction that is still in the circulation is available to be neutralized.

In light of this limited therapeutic window, the question arises as to why are antitoxins not administered prophylactically, especially if the threat of BoNT exposure is imminent. There are two compelling reasons why antitoxins are not used without evidence of exposure; both are related to the equine origin of the product. First, equine-derived antitoxins have a high risk of hypersensitivity. The previously licensed trivalent ABE antitoxin (Table 13.1) was associated with a 9% incidence of hypersensitivity [35]. Of 268 patients studied retrospectively over an 11-year period, anaphylaxis was observed in nearly 2% of the study population within 10 min of antitoxin treatment, and nearly 4% in this group developed serum sickness 6–20 days after receiving antitoxin [35]. Second, equine antitoxins are expensive and difficult to produce. Until a substantial quantity of HBAT was delivered to the Strategic National Stockpile (SNS), BoNT antitoxins were only available in extremely limited supply, reflecting production difficulties, high cost, limitations of shelf life and the fact that naturally occurring botulism is a rare disease [215].

13.3.2 Efficacy of Antitoxin

Although equine-derived antitoxins have been demonstrated to be highly protective in animal studies [100], their efficacy in humans has never been established directly in double-blind placebo-controlled clinical trials. Instead, efficacy was inferred from retrospective studies on BoNT-intoxicated patients. In one such study involving 134 patients, those who received antitoxin within 24 h of onset of signs and symptoms had a lower fatality rate (10%) than those who received antitoxin after 24 h of onset (15%) or those who did not receive antitoxin (46%). In addition, patients who received antitoxin within 24 h had shorter hospital stays and spent fewer days on a ventilator than those who received antitoxin after 24 h [249]. To determine the time window for antitoxin administration more rigorously, it would be desirable to have data on plasma levels of BoNT in humans as a function of time

Table 13.1 Botulinum antitoxins

Product	Source	Years used	Availability	Status: year
Heptavalent botulism antitoxin (A–G) (HBAT)	Equine Fab, $F(ab')_2$	2008–current[a]	CDC	IND: 2010 Licensed: 2013
Bivalent A, B	Equine IgG	1999–2010	Withdrawn 2010	IND: 1999–2005; licensed: 2005–2010
Monovalent E	Equine IgG	2000–2010	Withdrawn 2010	IND: 1999
Trivalent A, B, E	Equine IgG	1960–1999	Withdrawn 1997[b]	Licensed: 1960
BIG-IV	Human IgG	2003–current	California Department of Health Services	Licensed in 2003 for infant botulism (BabyBIG®)

CDC US centers for disease control and prevention, *IND* investigational new drug, *IgG* immunoglobulin G, *BIG-IV* botulism immune globulin intravenous (Human)

[a] Until 2008, heptavalent botulism antitoxin (HBAT) was provided from US Army Medical Research Institute of Infectious Diseases (USAMRIID) to the US Centers for Disease Control and Prevention (CDC) on a compassionate basis for type F outbreaks since only types A, B, and E antitoxins were available from the CDC

[b] From 1997 to 1999, trivalent A, B, E was used only for type E outbreaks. Information on some of the dates for usage was provided by Dr. Susan Maslanka, Division of Foodborne, Bacterial, and Mycotic Diseases, National Center for Zoonotic, Vector-borne, and Enteric Diseases, CDC, Atlanta, GA, USA

following potential routes of exposure (ingestion, inhalation, wound, injection). Unfortunately, human pharmacokinetic data for BoNT are lacking since patients are often not seen by medical personnel until well after exposure, and, in addition, BoNT is so potent that the toxin is often difficult to detect by conventional methods, such as the mouse bioassay [267].

In a study conducted on patients with foodborne botulism, toxin was detected by the mouse bioassay in less than half of serum and stool samples examined within 3 days of ingestion (40–44 %) and in only 15–23 % of samples obtained outside of this time frame [269]. Ravichandran et al. [193] investigated the pharmacokinetics of BoNT/A in the mouse and rat by use of relatively high doses of ^{125}I-BoNT/A. The half-life of ^{125}I-BoNT/A in serum was found to be approximately 4 h in both species. As expected, when antitoxin was mixed with toxin and injected in animals, no deaths or signs of botulism were observed. However, when antitoxin was administered 10 min after BoNT, the former only prolonged survival but could not prevent death; if antitoxin was administered 20 min after BoNT, it was unable to even delay death. Similar results were obtained by Cheng et al. [61], using an electrochemiluminescence enzyme-linked immunosorbent assay for detection of BoNT. For lower doses of BoNT, such as those encountered during natural outbreaks, the treatment window is much longer, and antitoxin would confer some benefit as long as toxin remains in circulation [249].

Although it is commonly believed that circulating BoNT is cleared from the bloodstream within 1 or 2 days of exposure, clearance from blood may in fact take considerably longer after severe intoxication. The presence of BoNT in serum 3 days after clear manifestation of symptoms has been documented following an

outbreak of foodborne botulism [115]. Moreover, in the November 2004 Oakland Park, Florida outbreak, BoNT/A in excess of 40 times the estimated human median lethal dose was observed in the serum of one patient 4 days after receiving a massive overdose of toxin. The intoxication occurred during a cosmetic procedure in which the individual was injected for glabellar line reduction with research-grade BoNT/A that was incorrectly diluted [65, 243]. For such severely intoxicated patients, antitoxin administration may still be effective in limiting the severity and duration of illness, since it would prevent continued internalization of circulating toxin. In this context, it is of interest that blood components do not appear to bind, degrade, or alter BoNT, so toxin detected in blood can be considered to be active even days after intoxication [193]. From patient records examined between 1973 and 1980, Tacket et al. [249] concluded that the upper end for antitoxin efficacy was unknown. Unfortunately, we still do not have reliable data to establish this limit.

13.3.3 Equine Heptavalent Botulinum Antitoxin

Presently, the only antitoxin available in the USA for noninfant botulism is an equine HBAT manufactured by the Cangene Corporation of Canada for the CDC's Quarantine stations and for HHS's SNS. HBAT has replaced the licensed equine bivalent AB antitoxin (BAT-AB) originally made by Connaught Corporation of Canada and acquired by Aventis/Sanofi-Pasteur, and the investigational monovalent type E antitoxin (BAT-E) (Sanofi Pasteur). BAT-AB and BAT-E expired on March 12, 2010 [56].

HBAT is prepared from plasma of horses immunized with one of seven serotypes of BoNT toxoid and toxin. For each antitoxin serotype (A–G), purified $F(ab')_2$/Fab immunoglobulin fragments are produced by pepsin digestion of the IgG monomer. The Fc fragments are removed to minimize the risk of hypersensitivity reactions (despeciation). The final product contains $<2\%$ intact IgG and $\geq 90\%$ Fab or $F(ab')_2$ fragments, and therefore adverse reactions to HBAT are expected to be relatively infrequent (http://www.epi.hss.state.ak.us/bulletins/docs/b2010_05.pdf).

Following formulation of the individual components, the seven antitoxin serotypes are blended into a heptavalent product and filled into single-use vials for intravenous (i.v.) infusion. The nominal potency values for HBAT are: 7,500 international units (IU) anti-A, 5,500 IU anti-B, 5,000 IU anti-C, 1,000 IU anti-D, 8,500 IU anti-E, 5,000 IU anti-F, and 1,000 IU anti-G [56, 91]. These units are more than sufficient to neutralize the highest serum levels of BoNT encountered in natural outbreaks [154]. Because HBAT is despeciated, it is less likely to elicit hypersensitivity reactions [119]. Although it may not be as safe as the human-derived antitoxin approved for infant botulism ([27], Sect. 13.3.4), despeciation should in principle increase the safety of HBAT by removing the Fc region of the IgG which binds complement and triggers inflammatory side effects [154]. Accordingly, skin sensitivity tests that were recommended for the previous formulations of antitoxin are not required for HBAT. Data gathered from 148 BoNT-intoxicated patients treated with HBAT under a CDC expanded access program between 2008 and 2011 suggest

that the new product has similar efficacy as BAT-AB and BAT-E (http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/BloodProductsAdvisoryCommittee/UCM338853.pdf). This is based on observations of comparable improvements in the length of hospital stays, time spent in intensive care, and duration of ventilator support with the former and current antitoxins. However, HBAT caused fewer adverse events than had been reported for the former antitoxins, with only one case of serum sickness and no anaphylaxis [120]. This is similar to the safety profile observed with an earlier formulation of HBAT provided by the US Army for a large BoNT/E outbreak in Egypt in 1991 [119].

While despeciation reduced the reactogenicity of the antitoxin, it also shortened its plasma half-life [56]. Perhaps not fully appreciated earlier, the shorter plasma half-life of HBAT can be problematic in cases of intestinal colonization or in wound botulism. A patient with type F intestinal colonization botulism showed initial improvement after HBAT infusion, only to be followed by a relapse 10 days later [91]. The short half-life of type F antibodies in HBAT (14.1 h) was a likely contributing factor since BoNT/F would continue to be elaborated from the gut long after HBAT is cleared from the body. Cases of intestinal colonization and wound botulism will require greater vigilance, with possible repeated infusion of HBAT to prevent recurrence of intoxication [91].

In March 2013, the FDA approved HBAT (BAT™) as an orphan drug for treatment of non-infant botulism in adults and pediatric patients. Unlike previous BoNT antitoxins, which were available in restricted quantities, sufficient doses of HBAT will be stockpiled to meet all expected contingencies. Thus, a total of 200,000 doses will be available in the SNS by 2018. (http://www.fda.gov/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/LicensedProductsBLAs/FractionatedPlasmaProducts/ucm345137.htm). HBAT is the first product to receive licensure by the FDA Center for Biologics Evaluation and Research (CBER) under the Animal Efficacy Rule (21 CFR § 601 Subpart H, Approval of Biologic Products when Human Efficacy Studies are Not Ethical or Feasible).

Data in support of licensure of HBAT included safety studies in humans, pharmacokinetic studies in guinea pigs, rhesus macaques, and humans and efficacy studies in BoNT-intoxicated guinea pigs and rhesus macaques. Human efficacy of HBAT was based on the CDC data described earlier under an IND and was also demonstrated in a small-scale study that took advantage of the phenomenon that injection of low doses of BoNT in a restricted volume can produce complete paralysis of the target muscle while avoiding systemic toxicity. This allows for human efficacy studies to be carried out on a Tier 1 agent in a manner that is both humane and ethical (http://ichgcp.net/clinical-trials-registry/research/index/NCT00636519). Extensor digitorum brevis (EDB) muscles of human volunteers were injected locally with 5 MLD_{50} of Botox® (onabotulinumtoxinA) or 500 MLD_{50} of Myobloc® (rimabotulinumtoxinB) by i.m. administration. The experimental group received an i.v. infusion of HBAT 1 day before toxin and exhibited normal EDB muscle function. The "control" group received placebo in place of HBAT and exhibited paralysis of the EDB muscle over the entire 28-day period of observation.

13.3.4 Antitoxin Treatment for Infant Botulism

Infant botulism is currently the most common form of BoNT intoxication in the USA. Unlike classic foodborne botulism, which involves intoxication by preformed toxin, infant botulism occurs when spores of *C. botulinum* (usually A or B) are ingested, and vegetative cells temporarily colonize the large intestine. Under appropriate growth conditions, *C. botulinum* will proliferate and produce toxin that reaches the target tissues via the general circulation [27]. Infant botulism is most frequently observed at 3–4 months of age, and like adult botulism, severe cases of infant botulism require intensive care, artificial ventilation and antitoxin treatment [25].

Equine antitoxin products are not generally used in infant botulism due to their potential for eliciting hypersensitivity reactions, including a lifelong sensitization to equine proteins. Additionally, equine antitoxins have a brief half-life in humans, which is incompatible with the prolonged duration of toxin production in the colonized intestine. To overcome these constraints, a human antitoxin was developed from plasma of laboratory workers who were hyperimmunized with PBT. This product was designated Botulism Immune Globulin Intravenous (Human) (BIG-IV) and contained ≥15 IU of antibodies against BoNT/A and ≥4 IU of antibodies against BoNT/B. BIG-IV was tested in a 5-year randomized double-blind placebo-controlled study in California as well as a 6-year nationwide open-label study. In both studies, treatment with BIG-IV led to significant reductions in mean hospitalization time, including fewer days of intensive care and mechanical ventilation, with no serious adverse effects. The findings were sufficiently compelling that licensure of BIG-IV was granted by the FDA in October 2003 as BabyBIG® for the treatment of infant botulism. Since BIG-IV is derived from human plasma, it also has a long circulation time (mean serum half-life = 28 days) and generally remains protective for the duration of the intestinal colonization [27].

13.3.5 Recombinant Monoclonal Antibody-Based Antitoxins

From the above, it is clear that a human product like BIG-IV would be desirable for treatment of adult botulism since it is safe and efficacious, and its long plasma residence time would make it possible to use this antitoxin for pretreatment or prophylaxis. Pretreatment with antitoxin prior to BoNT exposure has been shown to prevent botulism in animals, whereas delayed addition led to a relatively poor prognosis [61, 100, 193]. However, since BIG-IV is derived from hyperimmunized human donors, it would not be feasible to obtain the vast quantities of antitoxin needed for the SNS from such a limited source. Another concern is the problem of screening for infectious diseases, which could become an issue if production of BIG-IV were to be expanded to provide coverage for the general population.

As an alternative to antitoxins derived from either equine or human donors, recombinant monoclonal antibodies could, in principle, provide a sustainable source of antitoxin in unlimited quantity without risk of transmitting infectious diseases.

Marks and coworkers pioneered the expression of "recombinant human BoNT antibodies by phage and yeast display technologies" [20, 174, 195]. These investigators demonstrated that a combination of three monoclonal antibodies produced effective neutralization for a single BoNT serotype [174].

These concepts were recently commercialized by XOMA LLC, who expressed anti-BoNT monoclonal antibodies (mAbs) in Chinese Hamster Ovary cells (CHO) and developed processes to scale up the mAbs for eventual placement in the SNS as next-generation therapeutics, if current licensing efforts prove successful. The product furthest in development is designated as XOMA 3AB and has successfully completed phase 1 clinical trials [37, 161]. XOMA 3AB consists of an equimolar mixture of three IgG mAbs designated NX01, NX02, and NX11, which target different nonoverlapping regions on the HC of BoNT/A; NX01 and NX02 bind to BoNT/A Hc, while NX11 binds to the interface between Hc and translocation domains. These binding sites are highly conserved in BoNT/A subtypes A1, A2, A3, and A4, allowing for one triad of antibodies to effectively neutralize four subtypes of BoNT/A. With respect to other serotypes, a botulism serotype B and E antitoxin combination is in advanced preclinical studies, and antitoxins for serotypes C and D have recently entered initial preclinical testing; antitoxin candidates for serotypes F and G are still under evaluation (http://www.phe.gov/Preparedness/mcm/phemce/Documents/2012-PHEMCE-Implementation-Plan.pdf).

The mAbs are considerably more potent than equine-based antitoxins and have a relatively long half-life of approximately 1 month. In addition, these mABs are expected to produce fewer adverse reactions, due to the absence of heterologous antigens. The latter two attributes may allow XOMA 3 AB to be used prophylactically, if desired, rather than only after signs of exposure are observed. Formulations of recombinantly expressed mAb appear to be highly promising for the production of the next generation of antitoxins for BoNT intoxication; they can provide a more reliable, stable, and sustainable source of antitoxin than is possible with current-generation products.

13.4 Pharmacological Intervention

From the time that inhibition of ACh release was established as the mechanism of BoNT action, attempts have been made to antagonize the neurotoxin by measures that enhance ACh release [165] and, subsequently, with specific inhibitors that target its binding [30], translocation [75] and catalytic activity [78]. The search for inhibitors has intensified during the past two decades, driven largely by the need to provide postexposure protection to both military and civilian populations following the rise in the threat of international terrorism. This pursuit has also been aided by recent advances in our understanding of the mechanism of BoNT action following identification of the specific events between exposure and intoxication [182, 229] and elucidation of the SNARE protein targets of the BoNT LCs [169, 207, 233].

An additional factor in support of a pharmacological approach was alluded to in Sect. 13.1.3 in relation to vaccines. Since the indications for the clinical use of BoNT continue to increase [222, 264], the presence of anti-BoNT antibodies in vaccinated personnel would reduce or abolish the therapeutic benefits of BoNT in these individuals. Although antibody levels decline with time after vaccination to the point of failing to protect against a toxic exposure within 2 years [236], residual BoNT antibodies can still interfere with the therapeutic use of BoNT, perhaps for an entire lifetime. Moreover, this resistance to therapy cannot be overcome by increases in the dose of BoNT [84]. Vaccination with selective serotypes is not practical, since those most likely to be used in a bioterrorist attack (A and B) [26] are also the ones that provide the greatest therapeutic benefit.

The question of whether to vaccinate or not is considered by Simpson [230], who notes that a possible answer may be to develop a vaccine that could produce a rapid and robust immunity to BoNT, but only for a limited duration, corresponding to the period of the heightened threat. Antitoxins, especially the XOMA mAbs, can accomplish this to some extent. However, prophylactic use of antitoxins is precluded since the most likely candidates (XOMA mABs) are still in development, and multiple infusion with equine-derived antitoxins would likely create problems with hypersensitivity [249]. Finally, the experience gained in preparation for a potential BoNT threat during the Persian Gulf War made it clear that delays in generating adequate protection by the BoNT vaccine were not consistent with the requirement for rapid deployment of military personnel [29]. Even the new recombinant Hc vaccine requires multiple vaccinations to achieve protective titers [202], and virally vectored vaccine candidates that are effective after a single injection have yet to transition from basic research to product development [270, 275].

13.4.1 Early Treatment Concepts

Some of the earliest putative BoNT antagonists were cholinesterase inhibitors, based on their ability to prolong the actions of ACh. Carbamate anticholinesterase agents such as neostigmine and physostigmine were investigated in animals [86] and in nerve muscle preparations [112], but they were unable to antagonize the effect of BoNT. More recent studies have tended to confirm earlier findings [4], although there have been reports of patients, especially those with less severe signs of botulism, responding to the short-acting cholinesterase inhibitor edrophonium [62]. Other potential antagonists of BoNT action, such as Ca^{2+} ionophores, La^{3+}, black widow spider venom (BWSV), 2,4-dinitrophenol and agents that raise cyclic adenosine monophosphate (AMP) levels, were also examined for their ability to reverse BoNT toxicity. Evaluation of the above compounds in BoNT-intoxicated nerve-muscle preparations revealed increases in the frequency of spontaneous miniature endplate potentials (MEPPs) but little or no enhancement of evoked endplate potentials (EPPs) or of muscle tensions [70, 228, 251]. Accordingly, they were not considered to be of practical value for treatment of BoNT intoxication.

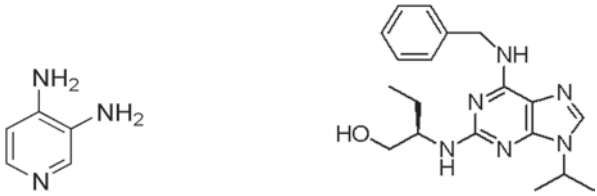

Fig. 13.1 Structure of 3,4-diaminopyrdine (*3,4-DAP; left*) and R-roscovitine (*ROS; right*)

Interestingly, although unable to rescue BoNT/A-intoxicated neuromuscular junctions acutely, BWSV did produce a marked acceleration of recovery of neuromuscular transmission in BoNT/A paralyzed muscles [107]. The acceleration was attributed to rapid destruction of the BoNT/A-poisoned terminals by BWSV, which allowed for reinnervation by a newly formed non-poisoned nerve terminal at the original endplate. In the absence of BWSV, recovery was found to take weeks to months, since the BoNT/A-poisoned terminal does not degenerate but instead prevents nerve sprouts from reinnervating the original endplate. Unfortunately, it has not yet been possible to exploit this phenomenon for accelerating recovery from botulism [160].

13.4.2 K^+ Channel Blockers

K^+ channel blockers were found to be more effective in antagonizing the paralytic action of BoNT than were the former group of compounds. Their higher efficacy comes from their ability to prolong the duration of the nerve terminal action potential [185], leading to a greater influx of Ca^{2+} during nerve stimulation. The increased Ca^{2+} influx enables the K^+ channel blockers to produce striking increases in the amplitude of EPPs and of nerve-evoked twitch tensions [3, 148].

A number of K^+ channel blockers have been evaluated for their ability to antagonize the actions of BoNT, including guanidine, 4-aminopyridine (4-AP), 3,4-diaminopyridine (3,4-DAP), and tetraethylammonium [63, 64, 70, 148, 164, 165, 227], (see Fig. 13.1). Aminopyridines and tetraethylammonium inhibit different K^+ channel subtypes at the mammalian motor nerve terminal [145, 172, 185], and both compounds are potentially useful for counteracting the inhibitory action of BoNT on transmitter release. Of the K^+ channel blockers thus far examined, the most promising candidate was 3,4-DAP; 4-AP exhibited a higher incidence of central nervous system (CNS) hyperactivity, and tetraethylammonium caused a marked postsynaptic depression of EPPs and nerve-elicited muscle contractions that actually exacerbated BoNT-mediated inhibition after an initial potentiation [3, 4, 228, 251].

When added to nerve-muscle preparations prior to BoNT, 3,4-DAP produced a marked delay in the time-to-block of nerve-evoked muscle contractions [155, 227]. When applied after BoNT-mediated paralysis, 3,4-DAP was able to augment tensions to or above control values [4, 148, 164, 227]. Unlike most BoNT antagonists, 3,4-DAP could restore tension even in totally paralyzed muscle [5].

In spite of these successes with 3,4-DAP, two fundamental limitations were noted: the efficacy of 3,4-DAP was largely limited to serotype A [227] and the drug had a brief in vivo half-life [5]. Of the two, the latter is less critical since the short duration of action can be offset by the use of an infusion delivery as demonstrated by Adler et al. [9] with subcutaneously implanted osmotic minipumps. In addition, sustained release formulations of the aminopyridine class of K^+ channel blockers have become readily available following introduction of aminopyridines for treatment of diseases such as multiple sclerosis [259].

The basis for the lack of response to 3,4-DAP by the other serotypes is not well understood. At a functional level, BoNT/A-intoxicated neuromuscular junctions undergo an attenuated but synchronous release of ACh following stimulation; preparations intoxicated by serotypes B, D, and F produce asynchronous release where the ACh quanta are dispersed and cannot summate to produce suprathreshold EPPs [148, 164, 165, 251]. It is readily apparent that the lack of synchrony would prevent 3,4-DAP from restoring transmitter release; however, the factors that lead to asynchronous release are still not well understood in spite of dramatic advances in our understanding of the mechanism of transmitter release during the past two decades [72, 197, 203, 242].

13.4.2.1 K^+ Channel Blockers in Human Botulism

The K^+ channel blockers guanidine, 4-AP, and 3,4-DAP have been evaluated in-human botulism cases, beginning with an assessment of guanidine in 1967 as an adjunct therapeutic for BoNT/A intoxication. The general findings were that K^+ channel blockers caused a modest increase in the strength of limb, extraocular and postural muscles but these drugs were unable to restore spontaneous ventilation [63, 64, 71].

During the last decade, only a single report was published on the use of K^+ channel blockers in human botulism. In this report, dalfampridine (clinically approved formulation of 4-AP) was examined in a recent case of botulism [120]. A patient admitted originally for cellulitis and treated initially for wound botulism with HBAT and antibiotics was ultimately diagnosed with foodborne botulism. On hospital day 19, the patient was treated with 10 mg dalfampridine administered orally twice a day to reduce muscle weakness and paralysis. Although the patient showed an increase in strength over the following 2 days, the improvement was not attributed to dalfampridine, but rather to spontaneous recovery; the drug was considered to have no effect on this patient.

The basis for the failure of K^+ channel blockers to restore function in respiratory (diaphragm and intercostal) muscles is not known. It is possible that these muscles are inherently less responsive to K^+ channel blockers than limb, extraocular or postural muscles, or respiratory muscles may be more sensitive to BoNT and therefore undergo greater paralysis that is more difficult to reverse. Since systematic dose-ranging studies with K^+ channel blockers were not performed in any of the clinical cases, and high doses were not attempted to avoid the risk of seizures and other

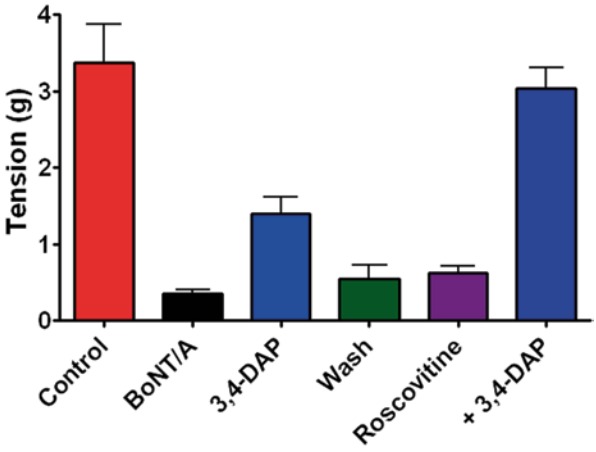

Fig. 13.2 Effect of 3,4-diaminopyrdine (*3,4-DAP*) and R-roscovitine (*ROS*) in reversing botulinum neurotoxin serotype A (*BoNT/A*)-mediated paralysis in isolated mouse hemidiaphragm muscle. Addition of 5 pM BoNT/A depressed the amplitude of indirectly elicited muscle tensions from 3.4 ± 0.5 g to 0.35 ± 0.03 g in 2 h (89.7% reduction). Addition of 10 μM 3,4-DAP restored tensions to 1.4 ± 0.2 g within 15 min; this effect was reversed by a 30-min washout. Addition of 30 μM ROS produced little detectable increase in tension; however, co-application of 10 μM 3,4-DAP to the 30-μM ROS-containing solution restored tension to near-control values. Symbols represent mean ± SEM; $n = 4$

potential side effects, it is likely that the doses of K^+ channel blockers were not adequate for restoring function in these patients [62, 64, 71, 120]. This certainly appears to be the case for 3,4-DAP, since concentrations required to increase tension in BoNT/A-intoxicated diaphragm muscle were found to be ≥10 μM (Fig. 13.2, [13]), and the plasma levels in patients receiving the maximum tolerated dose of 3,4-DAP for conditions such as amyotrophic lateral sclerosis were reported to be almost tenfold lower (1.2 ± 0.5 μM) [16]. Similarly, the dose of dalfampridine in the report of Hill et al. [120] was based on that used for multiple sclerosis and is likely to have been well below the dose required for reversal of BoNT-mediated paralysis [13, 164].

At the present time, the K^+ channel blockers hold promise as potential therapeutic agents, but additional strategies such as development of more selective compounds, targeting of the inhibitors to neuromuscular and neuroeffector synapses or combining these drugs with Ca^{2+} channel activators will be required to exploit their full potential. With regard to more selective inhibitors, Mayorov et al. [155] synthesized new analogs of 3,4-DAP with the goal of finding compounds that displayed both an enhanced affinity for nerve terminal K^+ channels and a reduced propensity to cross the blood–brain barrier. Although none of the analogs was more potent than 3,4-DAP, one was found to have a more favorable peripheral to CNS distribution [155]. Complicating the search for aminopyridines with low CNS toxicity is that their binding site on the K^+ channel is accessible only from the cytoplasmic mem-

brane surface [122, 170]. This makes the goal of finding compounds with reduced CNS penetration challenging, since such compounds would also have an impaired ability to gain access to the cytoplasmic surface of the nerve terminal membrane. For this reason, it may be profitable to focus on K^+ channel blockers that act on the outer surface of the membrane in future studies.

13.4.3 Combination of K^+ Channel Blockers with Ca^{2+} Channel Activators

To address the issue that only high and potentially toxic doses of 3,4-DAP can antagonize the actions of BoNT, we examined the effect of combining 3,4-DAP with the Ca^{2+} channel regulator roscovitine (ROS; Fig. 13.1). ROS, best known as an inhibitor of cyclin-dependent kinases [131], has been evaluated for treatment of human immunodeficiency virus type-1 [110], advanced malignancies [142] and is in phase II trials for non-small cell lung cancer and nasopharyngeal carcinoma [17]. Distinct from its action on cell cycle regulation, ROS has also been found to prolong the open state of nerve terminal N-, P/Q- and R-type Ca^{2+} channels, leading to enhancement of neurotransmitter release [45, 76, 272].

To determine whether ROS could be of benefit in the restoration of tension in BoNT-intoxicated muscles, isolated hemidiaphragms were paralyzed by addition of 5 pM BoNT for 2 h. ROS and 3,4-DAP were evaluated for their ability to reverse paralysis, when added either individually or in combination [11, 13] (Fig. 13.2). Addition of 10 μM 3,4-DAP led to a partial restoration of tension within 15 min of application. Although complete recovery of tension could be elicited with 3,4-DAP alone, higher concentrations of the K^+ channel blocker were required (30–100 μm), which often led to spontaneous muscle fasciculation and multiple twitches following each stimuli [13].

Unlike 3,4-DAP, ROS (30 μM) was not able to reverse BoNT-mediated muscle paralysis on its own. However, when 30 μM ROS and 10 μM 3,4-DAP were co-applied, muscle tensions were restored to near-control levels (Fig. 13.2). As with 3,4-DAP alone, restoration of tension with the combination of 3,4-DAP and ROS was accomplished in ~ 15 min. These results are encouraging since they demonstrate the possibility of achieving a rapid recovery from paralysis by using drugs with synergistic mechanisms of action: increased Ca^{2+} influx via K^+ channel blockade [164] and enhanced Ca^{2+} entry via prolongation of the channel open time [45, 272]. Although the 10-μM concentrations of 3,4-DAP in the combination is still toxic, the concentration of ROS is within the range of plasma levels measured in patients receiving ROS for chemotherapy [156]. By making incremental gains in the margin of safety of the K^+ channel blocker in the combination therapy, it should be possible to achieve efficacy against BoNT in the absence of toxicity. A summary of the role of K^+ channel blockers and Ca^{2+} channel agonists in the treatment of botulism is provided in Table 13.2.

Table 13.2 Candidate pharmacological treatments: Physiological antagonists[a]

Drug candidate	Mechanism	Advantages	Limitations	References
3,4-DAP, 4-AP	K^+ channel blockade	Used clinically	Seizures	[4, 5, 9, 13, 164, 227]
3,4-DAP analogs	Presumed K^+ channel blockade	Reduced CNS penetration	No gain in potency	[155]
Guanidine	K^+ channel blockade	Used clinically	High toxicity	[62, 64]
3,4-DAP + ROS	K^+ channel blockade + increase in Ca^{2+} channel open time	Additive enhancement of agents on Ca^{2+} influx	Toxicity of 3,4-DAP; ROS inhibits cyclin-dependent kinase	[11, 13, 45, 272]

3,4-DAP 3,4-diaminopyridine, *4-AP* 4-aminopyridine, *ROS* R-roscovitine

[a] Advantage of group is rapidity of action and efficacy after intoxication including restoration of muscle tension after total paralysis. Disadvantages of group are that their efficacy is limited to serotype A, and effective concentrations of K^+ channel blockers are toxic in vivo [16]

13.4.4 Inhibitors for Specific Stages of Intoxication

Following the recognition that BoNT enters motor nerve terminal through a series of discrete steps, but prior to establishment of SNARE protein cleavage as the mechanism of BoNT toxicity, attempts were made to develop inhibitors for the binding and internalization of toxin as potential therapeutic candidates. Some of the efforts were intended more to shed light on the mechanisms of action rather than to discover actual treatments, but the search for therapeutics was at least an implicit goal [228]. The current emphasis for therapy is on development of drugs for inhibiting the catalytic activity of the LC, which will be discussed in Sect. 13.4.5, and strategies to accelerate removal or degradation of the LC from intoxicated nerve terminals. The latter is dealt with in Chap. 9 of this volume.

13.4.4.1 Inhibitors of Binding

A reasonable approach to prevent BoNT intoxication is to use receptor antagonists to inhibit the binding of toxin to the nerve terminal. Complications with this approach are that many BoNT serotypes bind to dual polysialoganglioside and protein receptors on the cell surface, and that different BoNT serotypes recognize different protein–ganglioside combinations [134, 167, 274]. This implies that multiple receptor antagonists would need to be developed to protect against all the BoNT serotypes responsible for human intoxications. In addition, although the role of gangliosides in the binding of clostridial neurotoxins was firmly established by the early 1960s [256], the protein receptors for BoNT were not elucidated until 40 years later [79, 80, 134]; by this time, emphasis had shifted to inhibitors of the catalytic activity for potential treatment of BoNT intoxication, as will be discussed in Sect. 13.4.5. The inhibitors of toxin binding that showed the greatest promise were lectins from

Triticum vulgaris and *Limax flavus*, both of which delayed the time-to-block of nerve-elicited muscle contractions with all BoNT serotypes examined [30].

13.4.4.2 Inhibitors of Internalization/Translocation

Following binding of BoNT to receptors on cholinergic nerve terminals, the neurotoxins undergo internalization prior to reaching their ultimate intracellular targets [207, 229, 233]. Internalization is thought to involve endocytosis of the BoNT–receptor complex, acidification of the resulting endocytotic vesicle, dissociation of the LC and HCs, and translocation of the LC into the cytosol [95, 96, 135]. Although it is not known whether LC exists as a discrete entity inside cells or retains some association with other components of the neurotoxin or with cellular components, the isolated LC is known to be the most catalytically active form of BoNT [111].

Translocation affords the next opportunity to ameliorate the toxic actions of BoNT. A number of pharmacological agents have been examined for inhibition of this process with various degrees of success. Simpson [226] demonstrated that pretreatment of phrenic nerve-hemidiaphragm preparations with the lysosomotropic agents ammonium chloride or methylamine hydrochloride delayed the time-to-block of nerve-evoked muscle contractions after exposure to TeNT or BoNT serotypes A, B or C1. Incubation of nerve-muscle preparations with ammonium chloride and methylamine hydrochloride was effective if applied before, concurrently or 10–20 min after toxin exposure. The efficacy of the lysosomotropic agents was reduced rapidly with further delays such that no effect was observed if they were administered ≥30 min after toxin exposure. At optimal concentrations, these compounds produced an ~twofold delay in the time-to-block, but were unable to reduce the degree of paralysis [226].

Other candidates examined for inhibiting BoNT-mediated translocation were the 4-aminoquinoline antimalarial agents, chloroquine and hydroxychloroquine [225]. These drugs were selected on the basis of their ability to accumulate in acidic intracellular compartments and interfere with receptor-mediated endocytosis [268]. The maximal efficacies of the above 4-aminoquinolines were similar to those of ammonium chloride and methylamine hydrochloride, and both groups exhibited a comparable limited therapeutic window. They differed in that effective concentrations of the 4-aminoquinolines also produced a reversible depression of neuromuscular transmission by an unknown mechanism.

Studies on antimalarial agents were extended by Deshpande et al. [75] to identify candidates that did not block neuromuscular transmission, had a longer therapeutic window and could delay the BoNT-mediated time-to-paralysis to a greater extent than the former drugs. These investigators examined a large group of 4- and 8-aminoquinoline compounds as well as analogous acridines for efficacy against BoNT in mouse hemidiaphragm preparations. The most effective compounds were quinacrine and amodiaquine, while 8-aminoquinolines such as primaquine were ineffective. Amodiaquine (20 μM) gave the highest protective index (3.9), defined as the ratio of BoNT-mediated time-to-paralysis in the presence and absence of drug.

Moreover, 20 μM amodiaquine did not impair neuromuscular transmission. The therapeutic window could not be extended, however, and protection was lost if the antimalarial agents were added ≥30 min after exposure to BoNT/A or BoNT/B.

As is clear from the above, strategies targeting BoNT binding or internalization are constrained by a narrow therapeutic window, similar to that which limits the duration of antitoxin efficacy. However, none of the inhibitors of binding and translocation examined to date possess the exquisite potency or selectivity of antitoxins, especially that of the recombinant human mAbs [61, 152]. For these reasons, it is unlikely that pharmacological inhibitors of toxin internalization/translocation will play a prominent role in future drug development efforts.

13.4.5 Inhibitors of Catalytic Activity

The third area for therapeutic intervention is inhibition of the metalloprotease activity of the BoNT LCs. This target is potentially the most promising, since it is not limited by a narrow treatment window as are antitoxins or inhibitors of binding and translocation. In addition, the crystal structures of the LCs for all known serotypes have been solved [14, 24], facilitating the design of antagonists to the active site of the LCs. The presence of a Zn^{2+} binding motif in the LC of clostridial neurotoxins and the finding that Zn^{2+} is required for neurotoxin-mediated proteolysis of SNARE proteins [204] suggest that three classes of potential inhibitors may be effective in antagonizing the toxic actions of BoNT LCs: metal chelators, metalloprotease inhibitors and exosite inhibitors [81, 223].

13.4.5.1 Zn^{2+} Chelators

Simpson et al. [231] demonstrated that the Zn^{2+} chelator *N, N, N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) caused a marked slowing in the time-to-block of nerve-evoked muscle contractions when administered prior to BoNT in phrenic nerve-hemidiaphragm preparations. The maximum efficacy was equivalent to that achieved with *T. vulgaris* lectin, ammonium chloride, methylamine hydrochloride, or the more potent antimalarial drugs.

In common with the above inhibitors, TPEN was effective against all BoNT serotypes examined. In addition, when co-applied with *T. vulgaris* lectin or the lysosomotropic agents, the protection observed with TPEN was approximately additive with that of the former compounds. These results are encouraging since they demonstrate that, in principle, concerted inhibition of the different stages in the production of toxicity is a viable strategy for managing BoNT intoxication. Sheridan and Deshpande [216] examined a number of additional chelators on nerve-evoked twitch tensions and concluded that both a high affinity for Zn^{2+} and membrane permeability were required for antagonism of BoNT.

The efficacy of TPEN in isolated nerve-muscle preparations suggested that it may be able protect mice against BoNT-mediated toxicity in vivo. Thus, TPEN was injected prior to and at 0, 2, 4 and 6 h following a 20-MLD_{50} challenge of BoNT/A or BoNT/B. TPEN prolonged the time to death by 2.1 and 3.3 h for serotypes A and B, respectively. Although TPEN was not able to increase survival, the study clearly demonstrated for the first time that an approach targeting the catalytic activity of BoNT via the active site Zn^{2+} could produce a statistically significant prolongation in the time-to-death [6]. The limitation of TPEN was considered to be its high toxicity; the chelator produced rapid lethality at doses above 30 mg/kg in mice. TPEN was also found to be toxic in primary and clonal cells. TPEN concentrations ≥10 μM produced morphological alteration with characteristics of apoptosis and necrosis [8]. Studies with ion replacement indicated that chelation of Zn^{2+} was the proximal cause of cytotoxicity, and examination of a variety of chelators suggested that those with high membrane permeability were especially likely to produce cell death [217].

Based on these findings, metal chelators have little promise for treatment of BoNT intoxication, since the requirements for efficacy against BoNT are the same ones that promote cellular toxicity [6, 7, 217]. These studies did, however, demonstrate that death could be delayed by removal of the active site Zn^{2+} and suggested that inhibition of the catalytic activity in a more targeted fashion may lead to a practical therapeutic.

13.4.5.2 Rationale for Development of Inhibitors Targeting BoNT LC

Since the toxicity of Zn^{2+} chelators discouraged their further consideration, the major emphasis for therapeutic intervention has been focused on metalloprotease inhibitors. This target was selected because of the general consensus that the toxicity of BoNTs stems from the protease activity of the LC [168, 199], and that both peptide and small-molecule inhibitors (SMIs) can, in principle, impede this activity [111, 144].

The basis for targeting BoNT LC with protease inhibitors is to halt SNARE protein cleavage and allow the normal cellular processes to replace cleaved fragments with intact, newly synthesized proteins to promote recovery of function [97]. Moreover, since the time course of BoNT intoxication is relatively long, and the LC persists in a catalytically active state for much of this time [10, 12, 129] ([128], Chap. 9), the LC remains an appropriate target for inhibitors, whereas the binding and translocation domains lose their relevance as therapeutic targets soon after the onset of intoxication [229].

13.4.5.3 Rationale for Focusing on High-Affinity Inhibitors

BoNT/A represents a significant biowarfare and bioterrorism threat [26] owing to two fundamental traits: its unusually high potency, with less than 0.1 μg capable of causing paralysis and death in humans [113], and its long duration of action

following severe intoxication [65, 238, 243]. After exposure to a supralethal dose of BoNT, treatment with 1 μM of a relatively potent LC inhibitor drug of $K_I = 1$ μM would lead to inhibition of only 50% of LC active sites, even if the compound exhibited ideal solubility and membrane permeability characteristics. If a tenfold increase in drug concentration to 10 μM increases inhibition to 90%, the remaining 10% active LC may still be sufficient to maintain paralysis. This is inferred from findings that paralysis in skeletal muscle requires cleavage of only a small fraction of SNAP-25 [125], and it is generally assumed that this can be achieved with relatively few LC molecules per nerve terminal (see however Simpson et al. [232], who suggests that the process of high-affinity binding of BoNT and internalization can produce a higher intraneural LC concentration than generally assumed). Extending our argument further, if raising the drug concentration to 100 μM leads to inhibition of 99% of protease activity, this may perhaps be sufficient to reverse paralysis, if the rates of de novo synthesis of SNAP-25, transport to its presynaptic location and incorporation in the active zone membrane can exceed the residual rate of SNAP-25 cleavage [97].

Based on the above, the currently available BoNT LC protease inhibitors would not be expected to reverse paralysis after intoxication: Problems include low potency, limited membrane permeability, high toxicity and rapid metabolic clearance relative to that of BoNT LC, especially that of the more persistent serotypes A, C1 or B. Several peptide inhibitors have attained submicromolar K_I values for their LC targets [209], but the SMIs discovered to date have not generally reached comparable potencies. The peptide inhibitors, however, cannot readily access LC in the nerve terminal, so that they too are unable to antagonize the action of BoNT in the target tissues [188, 279]. For competitive inhibitors, an additional requirement for high potency stems from the relative abundance of SNAP-25 in the intracellular surface of the presynaptic membrane. It has been shown that BoNT/A LC, once internalized, is localized to the cytosolic plasma membrane within the same subcellular compartment as its target, SNAP-25 [93]. The K_m for SNAP-25 as a substrate for BoNT/A LC is in the range of 0.1–1 μM, depending on experimental conditions. If the local concentration of SNAP-25 within the confined two-dimensional space of the plasma membrane is $10 \times K_m$, the IC_{50} will occur at approximately tenfold higher concentrations than the K_I, since the inhibitor must overcome the high local substrate concentration. This further suggests that competitive inhibitors of the LC will need to be of significantly higher affinity than those currently in existence.

13.4.5.4 Peptide-Based Inhibitors

Peptide-based inhibitors were the first to be examined and remain the most potent in vitro inhibitors of BoNT LC catalytic activity. Shortly after the SNARE protein targets of the BoNTs and specific toxin cleavage sites were elucidated [36, 203, 204, 205, 271], the requirements for substrate recognition by the LCs were systematically investigated. Schmidt and Bostian [208] synthesized a series of short peptides based on the SNAP-25 sequence flanking the BoNT/A cleavage site (residues

197–198) and determined the minimal amino acid sequence required for enzymatic activity. These authors found that short peptides corresponding to residues 187–203 of the 206 amino acid SNAP-25 were sufficient for cleavage by BoNT/A LC; truncations past the P7-Arg or the P5′-Met led to significant reductions in activity. Additional work revealed that certain amino acid modifications, especially at the P2, P1′, and P2′ positions of the peptide, disrupted substrate cleavage [208]. These efforts led to the development of a series of peptide inhibitors based on the P1 through P6′ residues of the native SNAP-25 sequence in which four different amino acids were changed to Cys [212]. The most potent of these inhibitory peptides, Ac-CRATKML-amide, was found to have a K_I of 2 μM and has served as the template for subsequent peptide-based BoNT/A LC inhibitors.

The effect of Cys residue replacement at the P1 position on activity suggests that the high affinity of Ac-CRATKML-amide was a result of the thiol group forming a complex with the active site Zn^{2+}, a suggestion later verified by crystallographic data [224]. In subsequent studies, Schmidt and colleagues modified the Cys group to unnatural thiol-containing side chains [209]. The first observation made was that a single carbon separation between the thiol and the carbonyl group resulted in approximately tenfold higher affinity relative to that found with a two-carbon separation. The resulting 2-mercapto-3-phenylpropionyl (mpp)-RATKML-amide peptide had a K_I of 330 nM.

A second structure–activity relationship (SAR) study using this same mpp group coupled to amino acid chains of different lengths showed that mercapto-peptides, when truncated from the C-terminus of mpp-RATKMLGSG, underwent a tenfold decrease in binding affinity if the C-terminal five residues (MLGSG) were removed. An additional tenfold reduction was observed when a Lys residue was removed, thus confirming the importance of the P5′ and P6′ residues in both inhibitor and substrate binding [209]. The success of the thiol peptides as inhibitors of BoNT/A LC led to a similar approach for the development of inhibitors for BoNT/B [176] and for BoNT/F [211].

Following publication of the seven residue inhibitory peptides by Schmidt and colleagues, the scaffold was modified extensively by various investigators. It was concluded from the crystallographic and biochemical studies of peptides–LC interactions that the active site of BoNT/A is large and flexible, and capable of binding peptides with significant sequence modifications, albeit with affinities ranging from mid-μM to mid-nM. The most potent peptide inhibitor of BoNT/A thus far published was synthesized by Axel Brunger's group [278], and was also based on the Ac-CRATKML-amide scaffold. In their SAR study, 13 peptides were assayed for inhibitory activity with systematic modifications at the P1, P2′, and P4′ positions. The greatest increase in affinity occurred when the P2′ Ala residue was changed to the larger aromatic amino acid Trp or to the unnatural benzothien-3-yl-alanine moiety. Less significant increases in affinity were observed when the P1 residue was changed to the large dinitrophenyl-2-aminobutanoic acid, and the side chain or P4′ Lys residue was shortened to 2,4-aminobutanoic acid. The effect of the modifications was essentially additive, resulting in a K_I value of 41 nM [278]. Co-crystals of the peptide with BoNT/A LC demonstrated a partial helical structure of the peptide

within the active site of BoNT/A LC. Comparable binding orientations were observed in a subsequent study with three additional peptides [279]. Swaminathan and colleagues made similar observations in their co-crystals of the weak 6-mer inhibitors QRATKM and RRATKM ($IC_{50=}$133 μM and 95 μM, respectively), i.e., that the inhibitor peptides adopt a more ordered, slightly helical structure within the active site of BoNT/A LC [136].

The Arg residue of the P1′ position is critically important for substrate cleavage as well as peptide inhibitor binding affinity for BoNT/A LC, a property unique to this serotype. Kumaran et al. [136] reported that poly-Arg peptides were able to inhibit the BoNT/A LC. This led to a set of four peptides with the sequence RRGx, where x was C, I, L, or M. The peptides all inhibited the BoNT/A LC with K_I values slightly < 1 μM [136]. Crystal structures of the inhibitors with BoNT/A LC revealed that the N-terminal Arg occupied the P1 position and the second Arg occupied the P1′ position, i.e., the expected location of the P1′ Arg in wild-type SNAP-25.

When the peptide–BoNT LC crystallographic data are considered as a whole, an additional noteworthy observation can be made: The orientation of the Ac-CRATKML-amide peptide within the active site is not the same as in the other peptide crystal structures [224]. In the former, a complex is formed between the active site Zn^{2+} and the thiol group of the P1 Cys residue, or more accurately, the sulfenic acid group [224]. For all other peptide inhibitor structures, the complex is produced by the backbone nitrogen and carbonyl oxygen forming the amide bond between the P1 and P1′ residues. Thus, although the Ac-CRATKML-amide and the other peptides adopt a partial helical secondary structure within the LC binding site, the Ac-CRATKML-amide is oriented one amino acid out of register with respect to the other peptide inhibitors (cf. [279]).

Other methods for discovering higher affinity peptides have included phage and mRNA display. Phage display represents the earliest method for obtaining directed libraries of BoNT inhibitors [88]. More recently, the same technique has been used for the development of camelid antibodies targeting BoNT holotoxin [106]. The related mRNA display method achieved similar results, although the selection process was performed entirely in vitro and was thus not dependent on expression on the surface of a biological entity. This technique identified peptide inhibitors with a five amino acid N-terminal extension on the parent CRATKML peptide, possessing lower IC_{50} and K_I values for BoNT/A LC than the parent peptide [273].

13.4.5.5 SMIs: Hydroxamates

Although potent peptide inhibitors were successfully developed for several BoNT serotypes, the general lack of stability and poor membrane solubility of peptides [258] has directed the majority of BoNT inhibitor efforts toward SMIs. The hydroxamic acid, or hydroxamate moiety, is a simple chemical structure that can form stable complexes with Zn^{2+} and is frequently found in metalloprotease inhibitors. The small size of hydroxamates permits them to be added to more complex structures. In addition, hydroxamates are uncharged at physiological pH, allowing them

potential access to the intracellular environment. The first study using a hydroxamate for mitigating BoNT intoxication was performed by Deshpande et al. [74] and revealed no antagonism of hemidiaphragm paralysis following BoNT/A or BoNT/B exposure. A decade later, Janda and colleagues performed a series of systematic studies on hydroxamates. In a first step, Arg-hydroxamate was synthesized and found to be a weak inhibitor of BoNT/A LC (K_I = 60 μM) using a 66-mer SNAP-25 substrate and a truncated recombinant BoNT/A LC [38]; further derivations of Arg-hydroxamate did not improve potency.

A second study was carried out using a recently developed process to convert a series of off-the-shelf carboxylic acids into hydroxamates [121]. This resulted in several compounds that were able to inhibit BoNT/A LC-mediated cleavage of the substrate SNAPtide® measured using Förster resonance energy transfer (FRET) spectroscopy [39]. One of the lead compounds, *para*-chloro-cinnamic hydroxamate, was further derivatized to yield 12 additional compounds. The most active of these was *ortho-para*-dichloro-cinnamic hydroxamate (or 2,4-dichloro-cinnamic hydroxamate, DCH), with an IC_{50} of 410 nM.

An additional hydroxamate, 1-adamantane-*N*-hydroxyacetamide, was also identified using similar techniques [54] In an attempt to displace an active site water, as well as to determine the importance of chirality, a hydroxyethyl moiety was attached to the central carbon chain of DCH in a stereoselective manner, thus introducing a chiral center in this hydroxamate [245]. The (R)-enantiomer of the molecule gave a K_I of 160 nM, fourfold lower than the (S) compound and twofold lower than the unsubstituted DCH. Additional hydroxamate-based inhibitors with markedly different structures yielded K_I values of 5–6 μM using a similar SNAPtide assay and truncated recombinant BoNT/A LC from List Biologicals [253]. A caveat for enzymatic studies using truncated BoNT/A LCs is that the absence of residues 425 through 437 at the C-terminus can make the truncated LCs more susceptible to SMIs than the full-length LC and also more sensitive to variations in assay conditions. For example, although DCH was initially reported to have an IC_{50} of 410 nM, [39] was subsequently found to have a much higher IC_{50} of 59 μM [49] or 81 μM [187] under different assay conditions.

Computer-aided molecular design has generated several novel parent structures for BoNT/A LC inhibitors. Using a library of 2.5 million compounds and the crystal structure of the BoNT/A holotoxin complex [138], an in silico screen was performed using the cationic dummy-atom approach [179] to better estimate the BoNT/A active site Zn^{2+} binding affinity of the compounds [184]. The initial in silico screen yielded eight "hits," all of which were screened in an HPLC BoNT/A LC inhibition assay using a 17-mer SNAP-25 peptide as the substrate. One of the eight compounds, [5-(4-chlorobenzoyl)-2-phenylthiophene-3-yl]acetic acid, produced 15% inhibition at 100 μM and was selected for further derivatization. Seven additional derivatives, four of which possessed hydroxamate moieties, were synthesized and tested but showed little increase in activity. Replacing 4-chlorobenbenzoyl with a phenyl-indole-carbonyl group resulted in only 4% inhibition at 100 μM. However, subsequent addition of an amino group to the indole nitrogen of the latter compound connected by four, five or six methylenes resulted in 96% inhibition at

100 μM. The *N*-amino substituted phenyl-indole-carbonyl-phenylthiophene-3-yl-hydroxamate compounds were tested and found to have similar activity, with the best compound giving a K_I of 12 μM [184]. Addition of a hydroxyl to the phenyl of the phenylthiophene-3-yl group resulted in a threefold decrease in the K_I, as well as complex inhibition patterns at high concentrations [250].

Using this latest structure as a scaffold for derivatization, additional changes were made: In two compounds, the hydroxyl on the phenyl-thiophen-3-yl was changed to an amine, and in a third compound, the hydroxyl was moved from the *meta* to the *para* position [181]. At 20 μM, the three compounds designated as H3H, F3A, and F4H inhibited BoNT/A LC activity by 78, 47 and 82 %, respectively, in an HPLC assay. Interestingly, these three compounds showed some protective effect against BoNT/A intoxication in vivo as will be discussed in Sect. 13.4.5.6.

Based on their success in the development of the phenylthiophenyl hydroxamates, additional inhibitors were synthesized by Pang and colleagues using a system termed synthesis-based computer-aided molecular design (SBCAMD). In this process, computer-aided design of novel derivatives is integrated with existing organic synthesis capabilities, resulting in molecular design of compounds that can be readily synthesized and tested for inhibition of BoNT/A LC [180]. After synthesis and assay of nine novel compounds of diverse structures, the most potent was a hydroxamate comprised of bis-7-aminoheptyl, diphenylpropyl, and phenylpyrole substituents with a K_I of 760 nM.

13.4.5.6 In Vivo Hydroxamate Studies

BoNT/A is known to have an extremely long duration of action [129], a property likely arising from the ability of the BoNT/A LC to evade intracellular degradation processes [255] ([137], Chap. 9). It is also known that upon systemic exposure to BoNT/A, the toxin can remain within the vasculature for several days [115, 139, 193, 249]. The protease inhibitors will typically be cleared from the body on a timescale of hours to days, while the target enzyme will in principle remain in nerve terminals for months. Thus, for SMIs directed against BoNT/A LC protease activity, a single dose administered at the same time as toxin would not likely result in complete reversal of effects, due to the considerable differences in pharmacokinetic timescale between the two entities. Once the inhibitor is eliminated, the LC will continue to cleave SNAP-25, and any observable improvement of paralysis would rapidly disappear.

The typical experimental paradigm used to determine in vivo efficacy of BoNT/A inhibitors is to administer a high dose of compound into the tail vein of mice. Immediately following, or up to 30 min after the inhibitor is given, animals are challenged with a 5–10-MLD_{50} dose of BoNT/A. Assuming that the pharmacokinetics of a hypothetical SMI follow simple first-order clearance with a hypothetical half-time of 6 h, < 10 % of the compound would be expected to remain after 24 h and < 1 % after 48 h. If a 10 μM (i.e., $10 \times K_I$) intracellular concentration were achievable for the hypothetical compound, this concentration would only exist transiently, becoming

reduced to its K_I value within 24 h. This would be followed by recurrence of SNAP-25 cleavage and paralysis.

With these concepts in mind, several studies evaluated compounds for protection against supra-MLD_{50} doses of BoNT/A with paradoxical results. An early example of this was a study performed on the DCH compound. Mice were injected i.v. with 0.1 ml of a 1-mM DCH solution into the lateral tail vein followed immediately with an i.p. injection of a 0.5-ml solution of a 5–10-MLD_{50} dose of BoNT/A. Of 31 mice injected, 5 survived the BoNT/A challenge indefinitely with no observable ill effects [89]. The other 26 mice died with a time course similar to that of vehicle-treated BoNT/A-injected mice. This apparent protective effect of DCH would not have been predicted from cell-based assays because DCH had been reported to be cytotoxic in cellular models. An additional nonhydroxamate compound described in the study prolonged the time-to-death by 36%, with no subpopulation of mice seemingly unaffected by the toxin, a result much more in line with expectations.

A comparable finding was reported by Pang et al. [182] for the indole phenylthiophen-3-yl hydroxamates described earlier. Increased survival was observed in mice pretreated with 2 mg/kg of inhibitor followed 30 min later by i.p. challenge with 5 MLD_{50} of BoNT/A; five of ten animals were alive 24 h after BoNT/A, whereas none of the five vehicle-treated animals (DMSO) were able to survive 5 MLD_{50} of BoNT/A. As with DCH, one of ten mice in each compound-treated group survived BoNT/A challenge with no signs of intoxication for up to 5 days posttreatment.

In the study of Pang et al. [181], the pharmacokinetics of the compounds were also examined. Following a 2-mg/kg i.p. injection of each compound, plasma half-lives were observed to range from 4.4 to 6.5 h for the three compounds. Maximum plasma concentrations (C_{max}) ranged from 256 to 738 ng/ml (0.46–1.32 μM). Although the C_{max} does not necessarily reflect the concentration inside the nerve terminal, it is doubtful that the latter would accumulate the inhibitor at concentrations significantly higher than the C_{max} in the absence of an active transport or retention mechanism. Since these compounds were reported to inhibit 47–82% of BoNT/A LC activity at 20 μM (a concentration >20-fold higher than the C_{max}), it is unlikely that the compounds exerted their effects in vivo by inhibition of BoNT/A LC activity in the nerve terminal; the more plausible hypothesis is that these hydoxamates are acting on toxin in the extracellular compartment, i.e., by inactivating or increasing clearance of the toxin before BoNT/A LC internalization.

These results suggest a complex mechanism of action for BoNT/A LC inhibitors in vivo. Despite the demonstrated inhibition of BoNT/A LC protease activity in vitro, the pharmacokinetic and pharmacodynamic data are difficult to reconcile with protease inhibition as the dominant mechanism. The duration of action of the BoNT/A LC is orders of magnitude longer than the plasma half-life for any of compounds examined, so that long-term survivors following a single pretreatment with a protease inhibitor would not be expected in animals challenged with multiple MLD_{50} doses of BoNT/A. Furthermore, the concentrations of drug required for inhibition of BoNT/A LC in vitro do not appear to be reached in vivo. Additional studies in a true postexposure model in which the inhibitor can be applied after

intoxication over an extended time frame via multiple injections or continuous minipump infusion may help to unravel the paradox.

13.4.5.7 Mercaptoacetamides

In an effort to develop additional scaffolds for SMIs of BoNT/A, a mercaptoacetamide structure–activity study was performed [163]. These compounds were synthesized as an extension of the work on Ac-CRATKML-amide, which utilizes the free thiol group of the N-terminal Cys to form a complex with the active site Zn^{2+}. As was observed by Schmidt and Stafford [209], a single carbon between the thiol and carbonyl groups was optimal for inhibition. Derivatives of the phenyl-pyrazole ring resulted in multiple compounds with IC_{50} values < 100 μM, with the three best compounds ranging from 3 to 7 μM. These compounds were all similarly substituted at the 4-phenyl position and were active both in rat primary cerebellar neurons and, to a limited degree, in a mouse phrenic nerve-hemidiaphragm preparation [163].

13.4.5.8 Bis-Imidazoles

Based on the idea that imidazole moieties readily form complexes with Zn^{2+}, Merino et al. [158] performed a series of experiments exploring bis-imidazoles linked by carbon chains of varying lengths. A preference of linker chains comprising 13 methylenes was observed, with molecular modeling showing the dual imidazole groups spanning the active site Zn^{2+} and Glu54. Methylene chain lengths of 12–16 gave essentially the same percent inhibition (maximum 61 % at 100 μM), with chain lengths of 9–11 giving weaker inhibition. Furthermore, the study found that imidazoles linked by bis-amide-methlylene chains had essentially no activity, suggesting that inhibition may have resulted from hydrophobic interaction within the binding site rather than from efficient spanning of the Zn^{2+} and Glu54 by the imidazole.

13.4.5.9 Multi-Zone Pharmacophore Models

The initial attempts at structure–activity studies in developing inhibitors to BoNT LCs were hampered by a lack of high-resolution structural data for the LCs complexed with either native substrate or inhibitor. Initial molecular models were built using crystallographic data for the BoNT/A holotoxin [138]. In an effort to incorporate existing and emerging biochemical data into a structural model for BoNT/A LC inhibitors, Burnett and colleagues have developed a continually evolving pharmacophore comprised of multiple zones in which a chemical moiety is positioned in a three-dimensional (3D) scaffold [50, 51]. This model has led to the development of several distinct compounds with related structural motifs.

The initial data for the development of the pharmacophore model began with a screening of the National Cancer Institute (NCI) Diversity Set consisting of 1,990

compounds composed of a wide range of structures. Compounds were screened for inhibitory activity using the full-length recombinant BoNT/A LC [15] and a short peptide-based fluorescent substrate [210, 213], with the initial hits verified by an HPLC-based assay. A total of 21 compounds were studied [47]. Two compounds were 8-quinolinols, a known Zn^{2+} chelating compound, and the inhibitory capacity of these compounds was found to be reversed by 20 μM $ZnCl_2$, thus disqualifying them from further study.

Subsequently, Burnett and colleagues examined existing libraries of *N*,*N*-bis(7-chloroquinolin-4-yl)alkanediamines and heteroalkane diamines developed initially as antimalarial agents [257]. In addition, several clinically used antimalarial compounds were tested. Although most were weak inhibitors of BoNT/A LC at 20–50 μM, several of the "hits" from both screens were used to develop their first pharmacophore model. In this model, flexible linkers of seven or more intervening methylenes or amines allowed the two bisquinoline moieties to fold into a structure consistent with the other compounds, forming a common pharmacophore [47]. The compounds Q2–15 and michellamine B were the most potent, and an additional series of molecular dynamics and docking simulations was performed using the existing crystal structures of the BoNT/A holotoxin [138, 214]. The dynamics simulations showed little change in the secondary structure of the BoNT/A LC, but did reveal large changes in the surface loops, referred to as loop 1 (residues 48–78), loop 2 (residues 167–180), and loop 3 (residues 232–258). The contact points between the individual inhibitor compounds and the structural model of the LC were used to modify the pharmacophore model [48].

The progression of a molecular model is dependent on continual refinement with biochemical data. To this end, the previously described peptide mpp-RATKML was docked into the active site of the BoNT/A LC crystal structure [214]. However, the authors found that the docking orientation did not satisfy SAR experiments previously published [209]. In particular, the dramatic loss in activity when the Arg was changed to a Lys could not be explained unless the loop 1 residues 48–78 were reoriented. When the pharmacophore was expanded to incorporate chemical moieties not included in the initial model, several new inhibitors were found, with four possessing K_I values of 3–10 μM. Three compounds were observed by autofluorescence to be rapidly taken up by primary cultures of chick neurons, although two of the compounds were cytotoxic at low micromolar concentrations. One compound, NSC240898 (2-(4-(4-(aminoiminomethyl) phenoxy)phenyl)-1H-indole-6-carboximidamide), had a K_I value of 4.6 μM as determined by isothermal titration calorimetry and was less cytotoxic than the other compounds. In addition, NSC240898 showed dose-dependent protection of endogenous SNAP-25 cleavage. Chick primary neurons were incubated for 30–45 min with 5–40 μM NSC240898, then for an additional 3.5 h with 10 nM BoNT/A in the continued presence of inhibitor. Densitometric analyses showed a small dose-dependent decrease in cleaved SNAP-25. However, since the toxin and inhibitor were co-applied to the cultured neurons, the mechanism of action of the inhibitor cannot be differentiated among blockade of entry, disruption of intracellular translocation or inhibition of the protease activity. Furthermore, the authors used a

much higher dose of BoNT/A (10 nM) than is typical for intoxication of primary neurons. This could lead to a very high intracellular copy number of BoNT/A LC requiring an inhibitor concentration significantly higher than its K_I value to adequately block the LC activity [49].

The pharmacophore model was used to reexamine the 4-amino-7-chloroquinolines identified previously from bis-quinoline libraries [47], with the refined pharmacophore model used to develop additional derivatives. The authors replaced one of the chloroquinoline moieties with a congeneric series of cholate acetates and tris-chloroquinoline, identifying three new inhibitors with IC_{50} values ranging from 3.2 to 17 μM. As with previous molecular models, the chloroquinolines fit into the BoNT/A LC subsite S1′, while the cholate portion occupied the LC substrate cleft and positioned functional groups into subsite 2′ [50]. An additional series of 4-amino-7-chloroquinolines coupled to steroidal and adamantane constituents provided compounds with IC_{50} values of 12–50 μM for inhibition of BoNT/A LC. Linking the cholate or the adamantine groups to the chloroquinoline moiety via two- or three-carbon linkers resulted in compounds with inhibitory activity, whereas longer linkers or linkers possessing an amide bond had no activity, as was also found for compounds with a second adamantane moiety [240].

Continuing to expand on the three-zone pharmacophore model, a 4-amino-7-chloroquinoline group was added to each end of the existing aminoiminomethyl-phenoxy-phenyl-indole carboximidamide of the compound NSC240898, resulting in an analog with a K_I of 600 nM [51]. The improved K_I resulting from addition of the chloroquinoline group to the existing amino-phenoxy-phenyl indole structures led to the hypothesis of a four-zone pharmacophore in which chloroquinoline groups were added to each end of an additional structure identified via the 3D database screen. These compounds possessed IC_{50} values of 600–900 nM [175].

In a departure from the pharmacophore model, two compounds identified by Burnett et al. [50] were used as query structures to perform a 3D database search of the ~ 270,000-compound NCI Open Repository. Twenty "hits" were mapped to the search query, of which ten were available for testing; three inhibited BoNT/A LC in the standard HPLC assay. Two of the compounds were congeners of the query compounds, as would be expected in a 3D database search. A third compound possessed a fused four-ring diazachrysene scaffold reminiscent of the cholate structure used in their previous work, although the diazachrysene is aromatic and more structurally rigid than the cholates.

The three compounds produced 40–50 % inhibition of BoNT/A LC activity at 10 μM and thus would have presumed IC_{50} values of ~ 10 μM. The authors also identified several closely related compounds that were not active, demonstrating the specificity of active site fit required for inhibition and arguing against nonspecific effects of the compounds. A second query resulted in two additional compounds with similar activities and unique structures [117]. The 1,7-bis(alkylamino)-diazachrysene structure identified from the NCI screen was further derivatized via modification of the bis-alkylamino substituents. Thirteen derivatives inhibited BoNT/A LC 39–73 % at 20 μM. Interestingly, the compounds also showed activity as both antimalarials and antivirals [177].

13.4.5.10 Quinolinols

Using slightly different docking parameters, Roxas-Duncan et al. [200] also used the NCI compound database to perform an in silico screen for compounds capable of docking into the BoNT/A LC active site based on the unliganded BoNT/A LC crystal structure [214]. The database screen yielded ~ 500 candidate compounds, of which 100 compounds that fit best into the active site were chosen for biochemical screening. Of these, seven inhibited LC activity at 20–200 μM. An 8-quinolinol compound, NSC1010, was chosen for further study. Although NSC1010 was the most potent inhibitor of the group, it was found to be toxic to cultured cells and was instead used to perform a similarity search of compounds in the NCI, Sigma, and ChemBridge compound databases. An additional 55 compounds were identified and tested in the same biochemical assay, and the five most active compounds, all 8-quinolinols, were chosen for additional study. IC_{50} values for the group ranged from 1.5 to 5.0 μM when tested with either the full-length or truncated BoNT/A LCs. All five of the compounds had significantly less cellular toxicity despite being more potent than NSC1010 in inhibiting BoNT/A LC [200].

The authors tested their hits in cell-based and muscle-function assays. In the former, Neuro2A cells were completely protected from BoNT/A-induced cleavage of endogenous SNAP-25. However, the assay was performed by preincubating BoNT/A holotoxin with inhibitor for 30 min at 37 °C. Since incubations were carried out before BoNT/A was exposed to cells, it is difficult to distinguish whether the effects are occurring inside or outside of the cell, and thus it is not clear if the compounds have promise as post-intoxication treatments. Similarly, mouse hemidiaphragm muscle twitch studies showed an apparent antagonism of BoNT/A activity, but only when the compound and toxin were incubated in advance of applying to the muscle bath. The authors reported that pretreating cells or hemidiaphragm preparations with inhibitor before addition of toxin did not protect either from BoNT/A intoxication [200].

In a recent study, quinolinols were investigated using SNAP-25 from rat brain synaptosomes as substrate for BoNT/A LC [234]. SNAP-25 was considered to be more relevant than small synthetic peptides since the former, like the natural substrate, was full length and membrane bound. Three compounds from the NCI and ChemBridge libraries were found to be sufficiently potent to transition to in vivo studies. One compound (NSC 84087) provided substantial protection of SNAP-25 at 100 nM and extended survival from ~ 9 h to 48 h in mice challenged with BoNT/A (5 MLD_{50}). NSC 84087 was equally effective when co-administered with BoNT or applied 30 min after toxin. NSC 84087 was somewhat less effective when animals were pretreated with inhibitor for 30 min prior to BoNT challenge. These results are of considerable interest, since NSC 84087 is the first inhibitor that has been found to be effective when administered after intoxication, and greater than fivefold increase in survival time is the most dramatic in vivo protection reported to date.

13.4.5.11 Exosite Inhibitors

Recognition between BoNT/A and SNAP-25 involves interaction of substrate with two spatially distinct exosites (α and β) as well as with the active site [42]. Although small peptides such as SNAPtide (13-mer) or the 17-mer peptide used in inhibitor screens can be cleaved by BoNT/A LC, full activity requires the presence of ≥66 residues (141–206). This substrate requirement is unique for BoNT and generally not seen with other Zn^{2+}-containing endoproteases such as thermolysis [190]. The large interaction area between SNAP-25 and BoNT/A LC as seen in the co-crystallization studies, coupled with the biochemical data indicating that full catalytic efficiency only occur when nearly all of the interacting regions of SNAP-25 are present [201], suggests that inhibitors that bind tightly to exosites on the BoNT LC could potentially inhibit substrate binding from outside of the catalytic site [81]. These exosite inhibitors could be co-administered with an active site inhibitor to produce an additive effect that could potentially be more effective than either inhibitor alone. Alternatively, the combination of exosite and active site inhibitors may enable use of lower concentrations of each, allowing for a reduction in off-target effects.

Recently, several potential lead compounds for exosite inhibition have been identified. D-chicoric acid, a component of Echinacea, was found to be a noncompetitive inhibitor of BoNT/A LC [223]. D-chicoric acid was also determined to be a noncompetitive inhibitor of BoNT/B LC, but only if a larger FRET substrate was used (58-mer); no inhibition was observed with shorter substrates, suggesting that the longer substrate was required for the chicoric acid binding site. A similar observation was made when comparing SNAPtide with a 66-mer SNAP-25 peptide as substrates for BoNT/A LC; only the latter was inhibited by chicoric acid.

Lomofungin, a broad-spectrum antibiotic, was identified from the Johns Hopkins Clinical Compound Library of ~ 1,500 compounds, using a high-throughput fluorescence screen. As with chicoric acid, a secondary biochemical assay using SNAP-25 (141–206) demonstrated lomofungin to have a K_I of 6.7 μM, with classical noncompetitive inhibitor kinetics [90]. Additional experiments suggested that lomofungin was mutually nonexclusive in binding with both DCH (competitive inhibitor) and chicoric acid (noncompetitive inhibitor), thus revealing three distinct sites on BoNT/A LC targetable with inhibitors. These findings expand the potential armamentarium for attack on the BoNT/A LC.

13.4.5.12 Irreversible Inhibitors

Because of their extraordinarily high potency and long duration of action, irreversible inhibitors may be more effective in antagonizing BoNT intoxication than competitive inhibitors. Although inhibitors functioning via covalent modification have the potential for significant off-target effects, an antagonist of sufficient potency and specificity could be administered in lower doses for a shorter period of time to an intoxicated individual to mitigate any off-target complications.

With this in mind, Janda and colleagues have taken steps toward development of an irreversible inhibitor for BoNT/A LC by replacing the hydroxamate moiety of DCH with either a cyclopentenedione or a maleimide group [54]. To assay for inactivation of the LC, different concentrations of compounds were incubated with BoNT/A LC for fixed time intervals. The enzyme–inhibitor mixture was diluted 100-fold with 10 μM SNAP-25, and aliquots were assayed at regular time intervals. Catalytic rates were determined from the HPLC peaks of the cleaved 9-mer fragment at each time point.

Their results demonstrated enzyme inactivation in cell-free assays and in a primary neuron assay of SNAP-25 cleavage. In each case, high concentrations of inhibitor were required, and the primary neuron assay was performed as a co-incubation of the toxin with the inhibitor. Thus, it is still unknown if the compounds are capable of reversing the effects of BoNT/A after intoxication. The study, however, serves as a proof of concept for the potential role of irreversible inhibitors in the antagonism of BoNT intoxication.

13.4.5.13 Natural Product: Toosendanin

Toosendanin is a triterpenoid compound obtained from the bark of *Melia toosendan* and has been used in traditional Chinese medicine as an antiparasitic agent and agricultural pesticide. Toosendanin was reported to inhibit BoNT intoxication in animal models, including nonhuman primates, and to alter the action of a number of ion channels, including the BoNT/A translocation channel [143, 218]. Based on reports of its efficacy against BoNT in the Chinese literature, considerable interest has arisen in toosendanin as a potential BoNT antagonist [2, 78]. In our laboratory, we have found that toosendanin was able to antagonize BoNT/A intoxication in isolated mouse phrenic nerve-hemidiaphragm preparations when administered at the same time or 30 min before BoNT/A, but not when given 30 min after BoNT [2]. We have also found that toosendanin is equally effective against BoNT serotypes /A, /B or /E. The absence of serotype selectivity, coupled with its reported action on the BoNT translocation channel [96], suggests that toosendanin may be acting on a common step such as slowing translocation of the LC into the nerve terminal cytosol. Current efforts are focused on examining analogs of toosendanin to shed more light on its mechanism of action.

13.4.5.14 Insulin-Like Growth Factor

Intoxication by BoNT/A leads to flaccid paralysis that can last for many months, leading to extensive remodeling of neuromuscular junction. Recovery is delayed by the continued persistence of BoNT LC proteolytic activity [129], the inability of newly formed nerve sprouts to innervate the original endplate, and the extensive loss of muscle protein. We have demonstrated that injection of the insulin-like growth factor 1 (IGF-1) in BoNT/A-paralyzed rat *extensor digitorum longus* (EDL) muscles led to marked improvements in twitch and tetanic tensions (Fig. 13.3) and

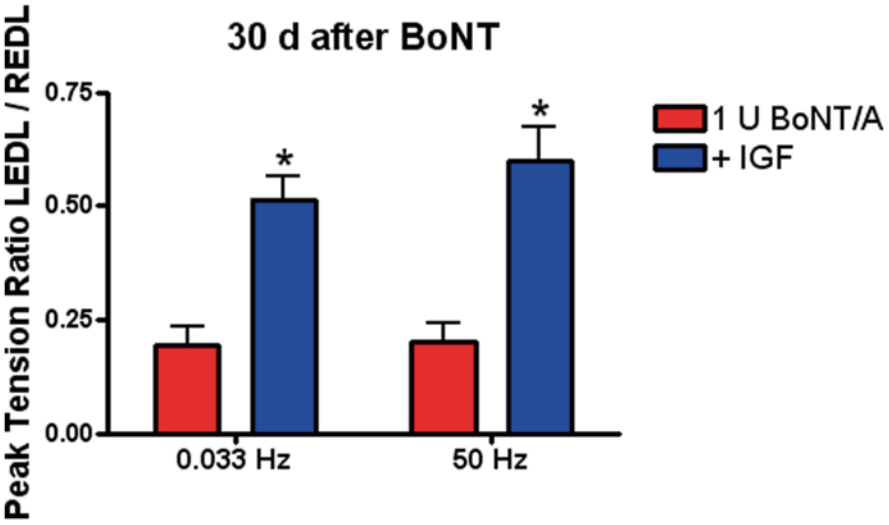

Fig. 13.3 *Extensor digitorum longus* (*EDL*) muscles were injected locally with 5 mouse intraperitoneal median lethal dose (MLD_{50}) of botulinum neurotoxin serotype A (*BoNT/A*) (15 μl) at day 0, followed by local injections of insulin-like growth factor 1 (*IGF-1*) (*blue*) or saline (*red*) twice per week for 30 days in the same muscle. At the end of this time period, muscles were tested in situ for twitch (0.033 Hz) and tetanic (50 Hz) tensions following stimulation of the peroneal nerve. Injection volumes of IGF-1 or saline were 50 μl. In the absence of IGF-1, muscle tensions (twitch and tetanic) recovered to only ~20% of control at 30 days, but increased to more than 50% of control in IGF-1-treated muscles. These differences were highly significant (*$p < 0.001$). The bars represent mean ± SEM, $n = 6$. LEDL/REDL is the ratio of tensions in the left EDL (BoNT/A-injected) to right EDL (control) muscles

muscle mass compared to BoNT-intoxicated muscles treated only with vehicle. The mechanism of IGF-1 in protecting muscles from BoNT-mediated paralysis is unknown. Possibilities include (1) enhanced sprouting of preterminal nerve fibers leading to hyperinnervation of muscles beyond the original intoxicated endplate, (2) increased rate of recovery of function at the original endplate or (3) direct actions of IGF-1 on muscle fibers to enhance muscle mass. The data in Fig. 13.3 were obtained using a locally injected model of BoNT intoxication [5]. Based on these results, IGF-1 may also be expected to accelerate recovery following systemic BoNT intoxication, especially when combined with an effective inhibitor of LC-mediated proteolysis.

13.5 Conclusions and Future Research

Substantial progress has been made in transitioning from the traditional toxoid vaccine for BoNT intoxication to the development of safe and effective recombinant products. The major challenge is no longer our ability to produce improved vaccine products but in selecting the appropriate population to be vaccinated and in meet-

ing the enormous cost of producing vaccines to cover all serotypes and relevant subtypes. During the past several years, HBAT, the new despeciated heptavalent equine antitoxin, has become available and achieved licensure by the US FDA in 2013. Compared to previous antitoxins, HBAT is expected to be safer and less reactogenic, and an adequate supply will be available in the SNS to meet all contingencies. Next-generation antitoxins derived from mixtures of mAbs are in development and represent a stable source with a superior biological half-life.

Efforts to develop pharmacological inhibitors of BoNT have increased substantially during the last decade. The major focus of the current research is the design and synthesis of specific metalloprotease inhibitors. Early drug discovery efforts were hampered by the lack of information on targets and the absence of the structural information on BoNT. Current research has been aided enormously by the availability of precise structural information and by knowledge of the mechanism of LC-mediated proteolysis of SNARE proteins [42, 60, 136]. Results to date indicate that a number of SMI and peptide inhibitors are effective in inhibiting BoNT LC-mediated protease activity in cell-free in vitro systems [38, 39, 49, 89, 184, 246].

Development of safe and effective metalloprotease inhibitors with in vivo efficacy will no doubt be difficult, but the data with new quinolinol compounds are encouraging [234]. Some of the challenges involve targeting of drugs to the nerve terminal, ensuring their access to the intracellular compartment and increasing the persistence of the drugs to match the duration of the toxin [89, 108, 276]. In addition, different inhibitors may be needed for each serotype, requiring multiple parallel efforts. A more complete characterization of BoNT receptors and a better understanding of the internalization process have recently become available and will aid in accomplishing these objectives by refining drug delivery methodologies [58, 123].

It may also be necessary to accelerate the removal of truncated SNARE proteins from the nerve terminal, to introduce noncleavable SNARE analogs for a more rapid recovery [178] and to accelerate degradation of BoNT/A LC by the ubiquitin–proteosome system [255] ([137], see also Chap. 9 this volume). The latter may be especially relevant for treatment of intoxication by persistent serotypes such as BoNT/A and BoNT/B [10, 97, 128, 129].

Other approaches such as combinations of K^+ channel blockers and Ca^{2+} channel activators appear to be promising for eliciting rapid reversal of paralysis following intoxication by BoNT/A [13]. Finally, growth factors such as IGF-1 can increase the rate of recovery and their use would be particularly useful following intoxication by the more persistent serotypes. Progress made during the last decade suggests that pharmacological treatments for BoNT intoxication may soon be a reality.

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