Handbook of Experimental Pharmacology 263

Scott M. Whitcup
Mark Hallett *Editors*

Botulinum Toxin Therapy

Springer

Handbook of Experimental Pharmacology

Volume 263

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Botulinum Toxin Therapy

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ISSN 0171-2004 ISSN 1865-0325 (electronic)
Handbook of Experimental Pharmacology
ISBN 978-3-030-66305-6 ISBN 978-3-030-66306-3 (eBook)
https://doi.org/10.1007/978-3-030-66306-3

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

Preface

In 1989, the US FDA first approved botulinum toxin for the treatment of strabismus and blepharospasm. Today, there are five commercially available botulinum toxins in the USA and for therapeutic indications from head (chronic migraine) to toe (lower limb spasticity). Importantly, botulinum toxins have been approved for multiple indications globally. This tremendous progress in the development of botulinum toxins for medical uses has been fostered by both increased understanding of the molecular mechanisms of how these substances interact and affect the tissues in the body and by astute clinicians and researchers observing the effects in patients. Despite being one of the most potent and potentially deadly substances in nature, our ability to reliably manufacture botulinum toxins and to deliver extremely small quantities locally to the site of action has allowed safe treatment of thousands of patients.

Botulinum Toxin Therapy is divided into two parts: a section on the basic science and a section on clinical practice. The basic science section starts with a chapter on the history of botulinum toxins in medicine (Dr. Whitcup), from the recognition of food-borne illnesses over a thousand years ago, to the regulatory approval of botulinum toxins for medical therapy over the last 3 decades. Drs. Dong and Stenmark then review the structure and classification of botulinum toxins, and Dr. Rossetto and colleagues highlight the progress we have made on understanding the molecular biology of the mechanism of action. Of course, the therapeutic use of botulinum toxins requires the manufacture of material that meets good manufacturing practices (GMP) criteria to ensure a safe and reliable source of the drug for patients, and the science of toxin production is discussed by Dr. Hasan. There are currently two botulinum toxin serotypes approved for human use (type A and type B); however, other serotypes and novel botulinum neurotoxins are in development. These novel, native, and engineered botulinum toxins are discussed by Dr. Steward and collaborators.

Part II of the book focuses on the use of botulinum toxins in clinical practice. This section starts with a chapter by Dr. Dressler on the general pharmacologic principles for clinical use including dosing and pharmacokinetics. The rest of the section consists of reviews of the major clinical uses of botulinum toxins by experts in the field. Although over a hundred of clinical uses of botulinum toxins have been attempted or discussed, these chapters predominantly cover approved indications

and major indications currently in randomized clinical trials. Drs. Berardelli and Conte discuss the use of botulinum toxins in dystonia, and Drs. Hunter and Wan review uses in ophthalmology; two important clinical areas where botulinum toxins were first studied in humans. Dr. Sheng describes botulinum toxin use in spasticity. Dr. Wang and colleagues review uses in dermatology including aesthetic indications and hyperhidrosis. Dr. Chancellor and Dr. Smith review uses in the genitourinary system, and Dr. Cariati et al. review gastrointestinal uses. The clinical part of the book ends with three chapters on the use of botulinum toxins for neurological and psychiatric diseases. Drs. Yuan and Silberstein review headache disorders and Dr. Lackovic discusses pain. Finally, Dr. Wollmer and colleagues cover the use of botulinum toxin for the treatment of depression.

Our primary goal in putting this book together is to provide an updated review of the science of botulinum toxin therapy to help basic scientists, clinical researchers, and practitioners in the study and use of currently available and future neurotoxins. Our hope is that these chapters provide both a detailed scientific description of the field and a practical guide to applying science. But there are a couple of subplots to *Botulinum Toxin Therapy* that make the story both fascinating and applicable to other medical therapies. One is that an incredibly potent and lethal substance can be studied and applied for beneficial purposes. The potential medical uses of botulinum toxin were recognized well over a century before their potential use as biological weapons. Fortunately for the patients who have benefited from their medical use, biological weapons programs have been largely abandoned or curtailed allowing the manufacture and use of botulinum toxins in research labs and medical practices for societal good. The second interesting part of the story is the importance of clinician scientists in the progress of medical science. Justinus Kerner was a physician and poet who studied cases of food poisoning in the early 1800s in Germany. He not only published the first case history of botulism but also hypothesized on the potential medical uses of the toxin including movement disorders and hypersecretion of body fluids, both approved medical indications for botulinum toxins today. Advancement in the field of medical toxin therapy will require both skilled basic scientists and dedicated and astute clinicians, and hopefully, as potential patients, we may all benefit.

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Part I

Basic Sciences

The History of Botulinum Toxins in Medicine: A Thousand Year Journey

Scott M. Whitcup

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Abstract

Botulinum toxin is one of the most potent and deadliest substances on earth. Because of its unique mechanism of action at the synaptic junction and the ability to precisely deliver the toxin locally to where it is needed, botulinum toxin has been used as an effective treatment for a plethora of diseases from head to foot, from chronic migraine to ankle spasticity. Unlike systemic drugs, botulinum toxin is delivered by injection to the site of disease. As we will see from the history of botulinum toxin, the ability to deliver the drug locally to minimize the amount of botulinum toxin needed and thereby minimizing systemic exposure has been key to its medical utility. Botulinum toxin was first approved by the US Food and Drug Administration in 1989 for the treatment of blepharospasm and strabismus, but the history starts long before this, with outbreaks of food poisoning in the tenth century. Importantly, the development of botulinum toxins for medical use continues today with the engineering of novel toxins to treat disease.

Keywords

Alan Scott · Botulism · History · Justinus Kerner · Strabismus

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S. M. Whitcup, M. Hallett (eds.), *Botulinum Toxin Therapy*,
Handbook of Experimental Pharmacology 263, https://doi.org/10.1007/164_2019_271

1 Introduction

Ancestors of modern bacteria probably appeared on earth approximately 3–4 billion years ago. A recent study presented direct fossil evidence life on land 3,220 million years ago in the form of terrestrial microbial mats in South Africa (Homann et al. 2018). Today it is estimated that there are about 5×10^{30} bacteria on earth (Whitman et al. 1998). Botulinum toxin is produced by *Clostridium botulinum*, a rod-shaped, gram-positive, anaerobic bacterium. There are seven serotypes (A–G), but types A, B, and E are the serotypes commonly involved in human disease and are also the three serotypes approved or being developed to treat human disease. Biological activity of the toxin occurs at approximately 1 ng, a billionth of a gram. As a result, about a tablespoon of toxin could supply the world for all the medical and cosmetic uses for a year.

2 History

The modern, written history of botulinum toxin begins with the recognition of food poisoning and probably starts over a millennium ago in Byzantium. Sausage plays a prominent role in the story of the medical use of botulinum toxin. Sausage has been a delicacy for centuries, and in the Byzantine era, blood sausage was commonly made by taking animal blood, fat, and organs, cooking them for varying amounts of time, and then stuffing them into "cleaned" animal stomach or intestine. Although the bacterial etiology of food poisoning was centuries away, the Byzantine emperor Leo VI embraced the association of blood sausage with food-related illness and later signed an edict that forbid the making and eating of blood sausage prepared in pig stomachs.

Further advancements into understanding the relationship between sausage and illness came during the Napoleonic War which took place from 1795 to 1813. The war leads to poor sanitary conditions in rural food production, and many deaths in Europe were associated with eating smoked blood sausages. These sausage-related deaths were studied, and in the early 1800s, the Department of Internal Affairs of the Kingdom of Wurttemberg attributed this food poisoning to a substance they called prussic acid. However, it was the physician and poet, Dr. Justinus Andreas Christian Kerner (Fig. 1), who made strong scientific inroads into our understanding of food poisoning, the role of botulism, and even the potential medical uses of botulinum toxin (Erbguth and Naumann 1999). Kerner was born in 1786 in Ludwigsburg, Germany. He studied at the University of Tubingen and received his medical degree in 1808. Kerner was a practicing physician who went on to publish the first case study of botulism and on the presumed fatty toxin from sour sausages. He described experiments, including those he performed on himself by eating small amounts of so-called sour sausage, and documented the signs and symptoms of botulism including vomiting and intestinal spasms, mydriasis, ptosis and strabismus, dysphagia, flaccid paralysis, and respiratory failure. He also noted that sausage poison develops under anaerobic conditions, interrupts motor signal transmission in the

Fig. 1 Justinus Kerner 1786–1862. From: Wikimedia Commons; adapted from Lee Byron Jennings: Justinus Kerners Weg nach Weinsberg. Die Entpolitisierung eines Romantikers. Camden House, Columbia, SC 1982, ISBN 0-938100-00-9, frontispiece

peripheral and autonomic nervous system, and is lethal in small doses. Incredibly, Kerner also proposed that the toxin could be used for therapeutic purposes. He hypothesized that this toxin could be used to lower sympathetic nervous system activity associated with movement disorders and decrease the hypersecretion of body fluids.

The bacteria that produces botulinum toxin was finally isolated around 1895 by Emile Pierre-Marie van Ermengem, a bacteriologist at the University of Ghent (van Ermengem 1897). This advance occurred again as a result of a recognized food-poisoning epidemic. However, this time the culprit was not sour sausage but bad ham.

The epidemic occurred on December 14, 1895, in a small town in Belgium where 34 musicians had a meal following a funeral where they played (Devriese 1999). Following the meal, musicians developed signs and symptoms of botulism, and three of the musicians died. Ham served at the meal was suspected as the cause of the illness. The ham was sent to Van Ermengem who performed a detailed scientific analysis where he isolated an anerobic bacteria and injected small pieces of the ham into animals leading to an illness similar to that experienced by the musicians. Van Ermengem named the bacterium *Bacillus botulinum* which stems from the Latin word botulus meaning sausage.

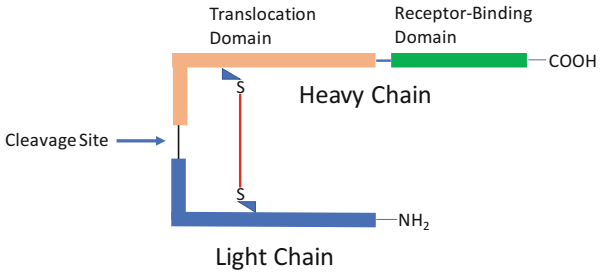

Fig. 2 Schematic drawing showing the structure of botulinum toxin A. The heavy chain and light chain are linked by a single disulfide bond. The heavy chain (approximately 100 kD) contains a receptor-binding domain and a translocation domain. The light chain (approximately 50 kD) acts as an endopeptidase and proteolytically cleaves protein involved in vesicle fusion at the inner cell membrane

Subsequent work leads to the early classification of botulinum serotypes (Burke 1919), and the bacterial exotoxin was first purified and crystalized in the 1920s (Snipe and Sommer 1928). Scientists then began to focus on the mechanism of action of botulinum toxin. Edmunds and colleagues showed that the toxin of botulism caused a complete curare-like action on the endings of the motor nerves to the voluntary muscles producing a paralysis (Edmunds and Keiper 1924). They also noted that the respiratory muscles were affected early and could lead to respiratory depression and death. Guyton and colleagues expanded on these studies describing the peripheral site of action of botulinum toxin (Edmunds and Keiper 1924). Experiments in the late 1940s then showed that the toxin acted by blocking neuromuscular transmission (Edmunds and Keiper 1924). Over the last 50 years, continued progress has been made into understanding the molecular biology of botulinum toxin activity. We now know that botulinum toxin consists of a heavy and light chain held together by a single disulfide bond and that the heavy chain has both a binding domain and a translocation domain Fig. 2. After the toxin binds to the cell, the light chain is internalized into the cell where it binds to a complex of proteins involved in neurotransmitter release (Rizo and Sudhof 1998). As an endopeptidase, the light chain then cleaves proteins involved in transmitter vesicle fusion to the inner cell membrane leading to chemical denervation. More recently scientists have imaged the crystal structure of botulinum toxin (Lacy et al. 1998) and identified the receptor for the toxin (Edmunds and Keiper 1924). The mechanism of action of botulinum toxin will be described in further detail in other chapters in this book.

Of course, the development of botulinum toxin as a medical therapy required highly pure and good manufacturing practices level material. Much of the work on the manufacture of botulinum toxin was spearheaded by Dr. Edward Schantz, a biochemist who worked in the Department of Defense laboratories at Fort Dietrich before continuing his career at the University of Wisconsin in Madison (Schantz et al. 1960). Schantz and colleagues not only worked out a manufacturing process for botulinum toxin but also supplied toxin to researchers. This included both basic

scientists and clinicians who first used the toxin to treat disease in humans (Schantz and Johnson 1992).

The first human use of botulinum toxin was for strabismus. Alan Scott, an ophthalmologist, was looking for a surgical alternative to the treatment of strabismus. He started by injecting local anesthetic into the extraocular muscles but later decided to look for something with a longer duration of action. Dr. Scott stated that he initially put this at the bottom of the list because of concerns about toxicity and the thought that it would never be approved by the FDA. Dr. Scott then became aware of the work of Daniel Drachman, who injected small amounts of botulinum toxin into the hind limbs of chick embryos and noted atrophy of skeletal muscle consistent with denervation (Drachman 1964). Dr. Drachman also told Dr. Scott that he received the toxin from Ed Schantz, who was now at the University of Wisconsin. This allowed Dr. Scott to start work on the use of botulinum toxin in experimental models and paved the way to start clinical trials in patients with strabismus and blepharospasm. Dr. Scott was first to describe the beneficial effects of botulinum toxin type A in patients, publishing on its use in strabismus in 1980 (Scott 1980a, b).

These studies paved the way for the first regulatory approval of botulinum toxin for a therapeutic use. Dr. Scott's original product was called Oculinum and was approved by the US FDA on December 29, 1989, for the treatment of strabismus and blepharospasm associated with dystonia including benign essential blepharospasm or (seventh) nerve disorders in patients 12 years of age and above. The product was initially marketed and later sold to Allergan who changed the name to Botox in 1991. The nonproprietary name, onabotulinumtoxinA was given in 2011.

Clinicians from around the world heard about Dr. Scott's research and flew to California to learn how to inject the extraocular and periorbital muscles with botulinum toxin. A group of physicians at the Moorfields Eye Hospital in London received some of the Dr. Schantz botulinum toxin and studied its use in 85 adults with strabismus (Elston et al. 1985). This research fostered a collaboration with the Centre for Applied Microbiology and Research (CAMR) at Porton Down in the United Kingdom who began providing their own botulinum toxin, produced with a different manufacturing process to the researchers, and lead to the creation of Porton International, a biotechnology company that was later purchased by Ipsen. Their commercialized product called Dysport comes from combining dyes from dystonia and port from Porton Down (Monheit and Pickett 2017).

Since the US FDA approval of onabotulinumtoxinA for strabismus and blepharospasm in 1989, there have been many additional FDA approvals for the use of onabotulinumtoxinA for other indications and for other botulinum toxin type A products and a botulinum toxin type B product. In December 2000, the US FDA approved the type B serotype, rimabotulinumtoxinB, under the brand name of Myobloc by Solstice Pharmaceuticals for the treatment of patients with cervical dystonia to reduce the severity of abnormal head position and neck pain associated with cervical dystonia. AbobotulinumtoxinA, under the brand name of Dysport by Ipsen, was initially approved in the United States in 2009 for the treatment of cervical dystonia. In July 2010, incobotulinumtoxinA, under the brand name Xeomin by Merz, was first approved by the FDA for the treatment of cervical

Table 1 US Food and Drug Administration (FDA) approval of botulinum toxins

Botulinum toxin (brand name)	Abbreviated indication[a]	FDA approval (year)
AbobotulinumtoxinA (Dysport)	Cervical dystonia	2009
	Glabellar lines	2009
	Adult upper limb spasticity	2015
	Pediatric lower limb spasticity	2016
	Adult lower limb spasticity	2017
IncobotulinumtoxinA (Xeomin)	Cervical dystonia	2010
	Blepharospasm	2010
	Glabellar lines	2011
	Adult upper limb spasticity	2015
	Sialorrhea	2018
OnabotulinumtoxinA (Botox and Botox cosmetic)	Strabismus	1989
	Blepharospasm	1989
	Cervical dystonia	2000
	Glabellar lines	2002
	Axillary hyperhidrosis	2004
	Adult upper limb spasticity	2010
	Chronic migraine	2010
	Urinary incontinence due to detrusor overactivity	2011
	Overactive bladder	2013
	Lateral canthal lines	2013
	Adult lower limb spasticity	2016
	Forehead lines	2017
	Pediatric upper limb spasticity	2019
PrabotulinumtoxinA-xvfs (Jeuveau)	Glabellar lines	2019
RimabotulinumtoxinB (Myobloc)	Cervical dystonia	2000

[a]Please read FDA labeling for the complete labeled indication

dystonia and blepharospasm. In February 2019 prabotulinumtoxinA, under the brand name Jeuveau by Evolus, was approved by the FDA for temporary improvement in the appearance of moderate to severe glabellar lines associated with corrugator and/or procerus muscle activity in adults.

Many of the botulinum toxins are approved for multiple uses in dozens of countries around the world. Table 1 lists all of the botulinum toxins currently approved in the United States and the indications they are approved to treat. When people hear about the use of botulinum toxin in patients, they usually think of wrinkles. New therapeutic uses of botulinum toxins have been predominantly driven by astute clinicians who understood the science behind both the treatment and other potential disease states or who recognized beneficial effects in a second condition in patients being treated for a separate disease. In 1989, botulinum toxin received FDA approval for strabismus. That same year, Clark and Berris reported on the use of botulinum toxin as a treatment for facial asymmetry caused by a facial nerve

paralysis (Clark and Berris 1989). The patient was noted to experience satisfactory relief of the asymmetry caused by one-sided forehead wrinkling and brow elevation. Jean and Alister Carruthers, an ophthalmologist and dermatologist practicing in Canada, noticed that their patients treated for blepharospasm had resolution of their frown lines and published on the treatment of glabellar frown lines with botulinum toxin in 1992 (Carruthers and Carruthers 1992). A number of other clinicians also noted the aesthetic use of botulinum toxin injections and began conducting randomized clinical trials to study the safety and efficacy of this approach for facial wrinkles (Keen et al. 1994). Botulinum toxin was first FDA approved for the treatment of moderate to severe glabellar lines in April 2002.

Fortunately for patients, the history of botulinum toxin for medical use does not stop here. Academic laboratories, pharmaceutical companies, and clinicians continue to conduct research on the use of botulinum toxins in medicine, both on additional indications for currently approved botulinum toxins and for new botulinum toxins in development. We continue to learn more about the molecular structure of botulinum toxin and its mechanism of action. These advancements should allow us to better achieve the goal to provide additional therapeutic options for patients with the hope of improving efficacy and minimizing adverse effects.

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The Structure and Classification of Botulinum Toxins

Min Dong and Pål Stenmark

Contents

Abstract

Botulinum neurotoxins (BoNTs) are a family of bacterial protein toxins produced by various *Clostridium* species. They are traditionally classified into seven major serotypes (BoNT/A-G). Recent progress in sequencing microbial genomes has led to an ever-growing number of subtypes, chimeric toxins, BoNT-like toxins, and remotely related BoNT homologs, constituting an expanding BoNT superfamily. Recent structural studies of BoNTs, BoNT progenitor toxin complexes,

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S. M. Whitcup, M. Hallett (eds.), *Botulinum Toxin Therapy*,
Handbook of Experimental Pharmacology 263, https://doi.org/10.1007/164_2019_342

tetanus neurotoxin (TeNT), toxin-receptor complexes, and toxin-substrate complexes have provided mechanistic understandings of toxin functions and the molecular basis for their variations. The growing BoNT superfamily of toxins present a natural repertoire that can be explored to develop novel therapeutic toxins, and the structural understanding of their variations provides a knowledge basis for engineering toxins to improve therapeutic efficacy and expand their clinical applications.

Keywords
Bacterial toxins · BoNT · BoNT-like toxins · Botox · Botulinum neurotoxin · Botulinum toxin · Tetanus neurotoxin · X-ray crystal structure

Botulinum neurotoxins (BoNTs) are a family of bacterial protein toxins that cause the human and animal disease botulism (Fig. 1) (Dong et al. 2019; Pirazzini et al. 2017; Montal 2010; Rossetto et al. 2014). Together with the related tetanus neurotoxin (TeNT), they are known as clostridial neurotoxins. These toxins are composed of two chains and three functional domains (Fig. 2a): the light chain (LC, ~50 kDa), which is a zinc-dependent metalloprotease that cleaves the target proteins in neurons, and the heavy chain (HC), which can be further divided into the N-terminal membrane translocation domain (H_N, ~50 kDa) and the C-terminal receptor-binding domain (H_C, ~50 kDa). These toxins are initially produced as a single polypeptide known as the pro-toxin. The linker region between the LC and HC needs to be

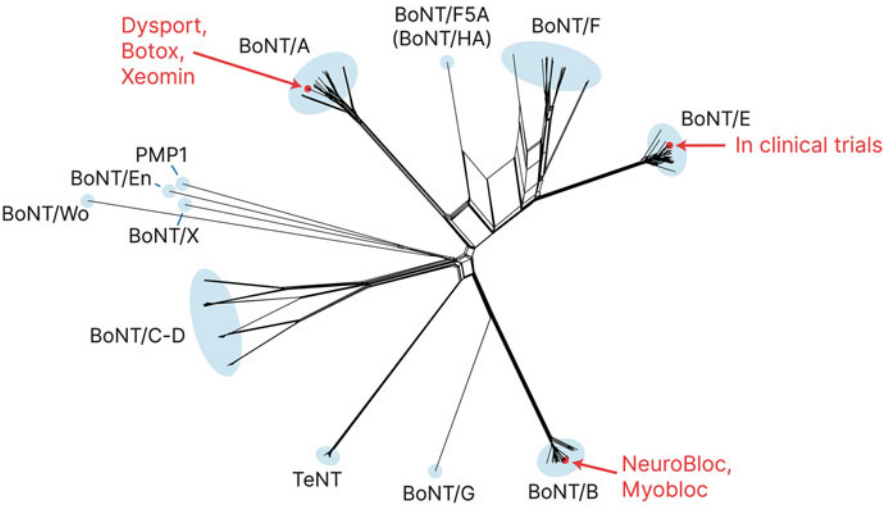

Fig. 1 A phylogenetic split network of BoNT and BoNT-like toxins. The diagram illustrates the potential evolutionary relationships based on comparing protein sequences of all known BoNT subtypes, chimeric toxins, BoNT-like toxins, and BoNT/Wo. BoNT/A1 (with such brand names as Dysport, Botox, and Xeomin from different companies) and BoNT/B1 (with the brand names NeuroBloc or Myobloc) have been approved by the FDA for medical and cosmetic uses, while BoNT/E1 is under clinical trials. These three toxins are marked in red

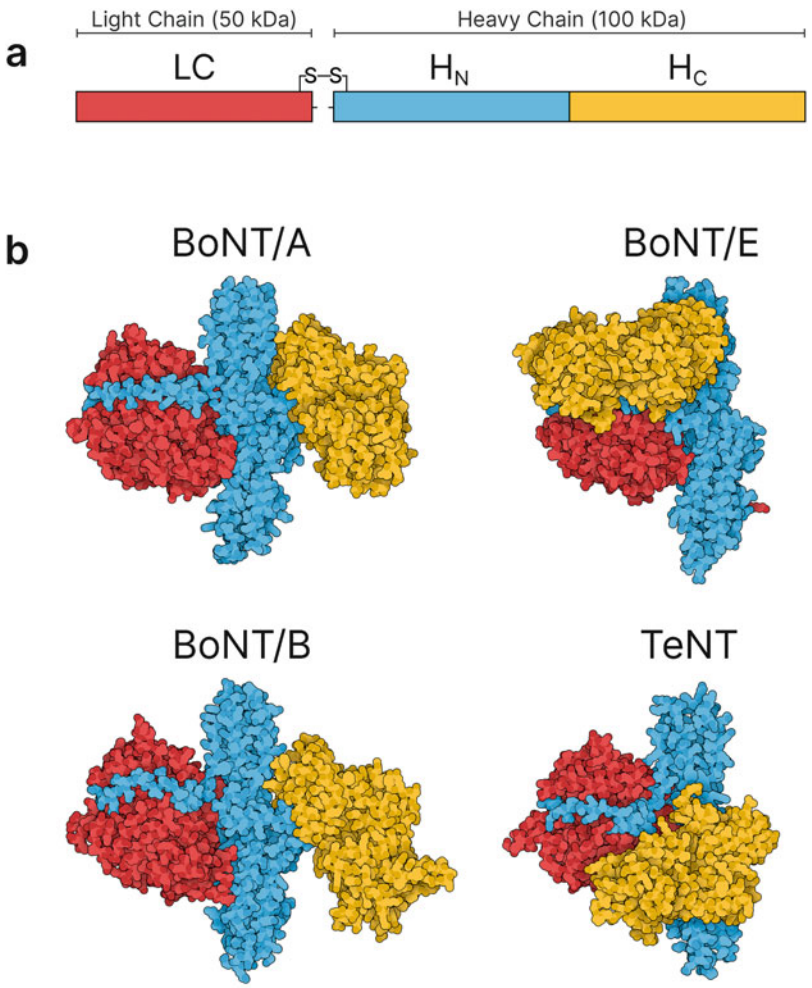

Fig. 2 The three-domain architecture of BoNTs. (**a**) A schematic drawing of the di-chain and three-domain architecture of BoNTs. The light chain (LC) is colored red, the translocation domain (H_N) blue, and the receptor-binding domain (H_C) yellow. The two chains are connected via a disulfide bond. (**b**) The crystal structures of full-length BoNT/A, BoNT/B, BoNT/E, and TeNT. LC is colored red, the H_N blue, and the H_C yellow. The protein structures are shown in a space filling representation. BoNT/A and BoNT/B display a linear arrangement for the three domains, with the LC and the H_C on each side of the H_N. BoNT/E and TeNT have their LC and H_C located on the same side of the H_N. PDB: 3BTA, 3FFZ, 1S0D, and 5N0B

cleaved by bacterial or host proteases, which converts the inactive pro-toxin to a di-chain active form. The LC and HC remain connected via a single disulfide bond. Once the H_C recognizes the receptors on nerve terminals, the toxin enters neurons via receptor-mediated endocytosis. The H_N then mediates translocation of the LC across endosomal membranes into the cytosol. The LC cleaves neuronal substrate

proteins, including Syntaxin 1, SNAP-25, and VAMP1, 2, 3, which are required for neurotransmitter release (Jahn and Scheller 2006; Sudhof and Rothman 2009), thus blocking neurotransmission.

1 BoNT Serotypes, Chimeric Toxins, and Subtypes

The classifications of BoNTs are traditionally based on their antigenicity and are known as serotypes, meaning that anti-sera generated against one toxin cannot recognize and neutralize another toxin (Fig. 1). The first BoNT was identified in 1897 (van Ermengem 1897). A serologically distinct BoNT was recognized in 1904, and hence serotypes A and B were designated to differentiate these two toxins (Burke 1919a, b; Leuchs 1910). This was followed by recognition of serotypes C in 1922, D in 1928, E in 1936, and F in 1960. The latest serotype, BoNT/G, was isolated from soils in Argentina and reported in 1970 (Gimenez and Ciccarelli 1970). This traditional serological classification has provided a way to distinguish diverse BoNT members and played a key role in developing vaccines and neutralizing antibodies against BoNTs.

The DNA and protein sequences for the prototypes of the seven BoNTs, as well as related TeNT, were resolved by the early 1990s, revealing ~37–70% variation in protein sequences among different serotypes. A phylogenic tree can be constructed based on protein sequences (Fig. 1). The two pairs BoNT/B versus BoNT/G and BoNT/E versus BoNT/F show the highest sequence identity (57% and 63%, respectively) among BoNTs. BoNT/A and BoNT/B have been approved by the FDA for use in humans (Schantz and Johnson 1992; Johnson 1999), and BoNT/C and BoNT/F have been investigated for potential medical use (Eleopra et al. 1997, 2006). BoNT/E is currently under clinical trials, which has a faster onset and shorter paralysis duration than BoNT/A (Fig. 1) (Eleopra et al. 1998).

The limitations of serological classification were recognized as early as the 1920s, when inconsistent neutralization efficacy was observed while serotyping "type C" toxins from different bacterial strains. Sequence information later revealed that this was due to the existence of naturally occurring chimeric toxins. For instance, there is a chimeric BoNT/CD with its LC-H_N derived from BoNT/C and its H_C from BoNT/D (Moriishi et al. 1996b). Anti-sera raised against BoNT/CD can neutralize BoNT/C, but anti-sera against BoNT/C are not effective in neutralizing BoNT/CD (Pfenninger 1924). There is also a chimeric toxin BoNT/DC, which is composed of a LC-H_N that is 98% identical with the corresponding region in BoNT/D and a H_C that shares 77% identity with BoNT/C-H_C (Moriishi et al. 1996a). Serologically, because this toxin can be recognized and neutralized by anti-sera against BoNT/D, it has been considered a BoNT/D (the bacteria strain is known as the strain D-5995, D-SA, or D-4947) and has been supplied as BoNT/D by a commercial vendor (Metabiologics Inc. Madison, WI, USA); however, its H_C is clearly distinct from BoNT/D-H_C.

Sequencing toxin genes has also revealed a growing number of subtype toxins with significant protein sequence variations from known toxin sequences (Hill et al. 2007; Peck et al. 2017). These variations could significantly reduce the efficacy of the standard anti-sera. For instance, there are at least eight BoNT/A subtypes (A1-A8).

The prototype is referred to as BoNT/A1, which is the only FDA-approved BoNT/A type for use in humans (Peck et al. 2017). Among BoNT/A subtypes, BoNT/A3 contains the greatest sequence variations from BoNT/A1 (15.4%). BoNT/F subtypes contain the most variation among all seven serotypes from their prototype BoNT/F1: as high as 30.2% for BoNT/F5 and 26.3% for BoNT/F7. The difference between BoNT/F5 and BoNT/F7 is 36.2%, the highest variation among all subtypes. These sequence differences help explain the significant variations in neutralization efficacy observed when BoNT/A and BoNT/F were serotyped from different bacterial strains.

The limitations of the traditional serotyping approach are further illustrated by the recent controversial naming of BoNT/H. This BoNT was identified in 2013 from a bacterial strain isolated from an infant botulism case (Dover et al. 2014; Barash and Arnon 2014). This toxin was not neutralized by anti-sera against other BoNTs following the established serotyping protocol. Thus, it was proposed as a new serotype. However, sequencing the toxin gene revealed that its LC shares ~80% identity with the LC of BoNT/F5, while its H_C shares ~84% identity with BoNT/A1-H_C (Maslanka et al. 2016). As the LC of BoNT/F5 (F5-LC) has a relatively high sequence variation from BoNT/F1-LC (only ~47% sequence identity) (Kalb et al. 2012), it is not surprising that anti-sera against BoNT/F1 failed to neutralize this toxin. Later studies showed that this toxin can be neutralized by antibodies against BoNT/A1, albeit a higher antibody titer is required than the standard serotyping protocol (Maslanka et al. 2016). Thus, this toxin is also considered a chimeric toxin BoNT/FA or more precisely BoNT/F5-A. To make the matter even more complicated, the H_N of this toxin does not appear to be close to either BoNT/F5-H_N or BoNT/A1-H_N. Thus, it has also been speculated that the LC-H_N of this toxin might be derived from a yet-to-be-identified BoNT.

Most subtypes likely cleave the same substrate protein at the same site and utilize the same receptors as their prototypes. However, exceptions with altered functional specificity have been reported. For instance, BoNT/F5 cleaves a site on VAMP1/2/3 that is distinct from all other BoNTs, indicating that its sequence variation is large enough to shift its cleavage site (Kalb et al. 2012). Similarly, sequence variations between the H_Cs of BoNT/DC and BoNT/C result in BoNT/DC utilizing a protein receptor that is not the receptor for BoNT/C (Peng et al. 2012).

Interestingly, even relatively low levels of sequence variations among subtypes, which may not alter the cleavage site on their substrates or switch receptors, could have measurable impacts on in vivo efficacy and pharmacological properties. For instance, BoNT/A2, which has 90% sequence identity with BoNT/A1, showed faster onset than BoNT/A1 on cultured neurons and in animal models (Torii et al. 2011; Pier et al. 2011; Whitemarsh et al. 2013; Pellett et al. 2015). It has been suggested that this faster onset time is because BoNT/A2 has an overall faster translocation process across the membrane than BoNT/A1 (Pier et al. 2011; Whitemarsh et al. 2013). As faster onset is clinically beneficial, BoNT/A2 has been explored for clinical uses. Additionally, it was recently reported that BoNT/A6, which has 4.3% sequence variation from BoNT/A1, also showed faster entry into neurons in culture (Moritz et al. 2018). Another example is that sequence variations in the LC of BoNT/A3 from BoNT/A1-LC result in a shorter duration of paralysis induced by BoNT/A3 compared with that induced by BoNT/A1 (Pellett et al. 2018). Thus,

sequence variations among the growing number of subtypes provide valuable resources for developing a new generation of therapeutic toxins and for optimizing toxin sequences to improve their pharmacological properties.

While traditional serotyping has value as a framework for categorizing toxins and identifying their distinct antigenic properties, describing toxins with seven serotypes is clearly insufficient to capture the growing diversity among BoNTs. Given the current ease of determining the exact toxin sequences, it will be important to note the specific subtype information when discussing a particular BoNT. To manage the naming of the growing number of subtypes, a guideline was proposed in 2017, which officialized the previous proposed threshold for a toxin sequence to be considered a new subtype as >2.6% variations at protein sequence levels from any known BoNT sequences (Peck et al. 2017). A few previous defined subtypes with <2.6% variations are grandfathered in, such as BoNT/B2, B3, and B6, which encompass only 1.5–1.9% variations, and BoNT/E2, E3, and E7, which differ from BoNT/E1 by only 1.0 to 2.1%.

To avoid duplication in numbering new subtypes, an email address has been set up at the Centers for Disease Control and Prevention (CDC, bontsubtype@cdc.gov) to receive requests for designation of new subtypes (Peck et al. 2017). There are also other efforts to develop a unified reporting system and database. One such a database, BoNTbase (https://bontbase.org) developed by Dr. Jonathan Davies in the laboratory of Prof. Stenmark, contains all reported BoNT subtypes as well as BoNT-like sequences along with associated research publications.

2 TeNT

TeNT, produced by *Clostridium tetani*, shares the same overall domain structures and mode of actions with other BoNTs; in fact, sequence alignment places TeNT in the middle of the family (Fig. 1). However, TeNT is not classified as a BoNT because it causes tetanus, a disease that is clinically distinct from botulism. TeNT and BoNTs both target and enter peripheral motor neurons. Unlike BoNTs, which block neurotransmitter release from motor neurons, thus causing muscle relaxation (flaccid paralysis), TeNT undergoes retrograde transport and transcytosis: it moves along the axons of motor neurons into the cell body in the spinal cord and is then released from motor neurons and enters the connecting inhibitor neurons where TeNT blocks neurotransmitter release (Lalli et al. 2003; Surana et al. 2018). Loss of inhibitory input leads to overactivity of motor neurons, resulting in spastic paralysis. Interestingly, it has been suggested that at least a small fraction of some BoNTs such as BoNT/A1 may also undergo long-range transport and transcytosis along peripheral neuronal axons into connecting neurons (Restani et al. 2011, 2012; Antonucci et al. 2008; Bomba-Warczak et al. 2016). The molecular basis for the different traffic pathways utilized by TeNT versus BoNTs remains unknown.

3 BoNT-Like Toxins

Rapid progress in sequencing microbial genomes in recent years has fundamentally changed how novel toxins are discovered. In 2015, a new toxin gene was recognized through bioinformatic analysis of the genome of a *Clostridium botulinum* strain. It encodes a protein containing the same three functional domains and key motifs found in BoNTs, with ~28–30% of sequence identity compared with the seven BoNTs (Fig. 1) (Zhang et al. 2017). This toxin was named BoNT/X because of varying opinions on what naming convention to utilize. Subsequent functional characterization confirmed that BoNT/X is capable of cleaving VAMP1, 2, 3 at a novel cleavage site distinct from all known cleavage sites for BoNTs. Interestingly, BoNT/X is a unique toxin that can also cleave VAMP family members VAMP4, VAMP5, and Ykt6, although the physiological consequences of these noncanonical cleavage events remain to be determined. Because BoNT/X was not recognized by antisera raised toward any of the seven BoNTs, it could be considered a novel serotype. However, unlike the seven classic BoNTs, BoNT/X showed only a low level of toxicity in mice. These findings suggest that BoNT/X may not naturally target mice and other vertebrates. The host species targeted by BoNT/X remains to be established.

In 2017, sequencing the genome of an *Enterococcus faecium* strain collected from cow feces revealed another BoNT-like toxin, designated BoNT/En (Zhang et al. 2018; Brunt et al. 2018). It too shares the same three-domain arrangement and key motifs found in BoNTs, with 24–27% protein sequence identity with the seven classic BoNTs. It is most closely related to BoNT/X, sharing 37% sequence identity. Functional validation demonstrated that BoNT/En is capable of cleaving VAMP1, 2, 3 and SNAP-25 in cultured neurons. Because BoNT/En is not recognized by any anti-sera against the seven BoNTs and BoNT/X, it too can be considered a new serotype, but BoNT/En showed no toxicity in mice. This is largely due to the lack of appropriate receptors in mice, as a chimeric toxin containing the LC-H_N of BoNT/En fused with the H_C of BoNT/A showed high neuronal toxicity and induced muscle paralysis. Thus, BoNT/En does not appear to target mouse motor neurons, and the host species naturally targeted by BoNT/En remains unknown.

In 2019, another BoNT-like toxin, PMP1 (paraclostridial mosquitocidal protein 1), was reported (Contreras et al. 2019). It was identified by screening and analyzing bacteria that can kill *anopheles* mosquito larvae. The toxin gene is located on a plasmid found in two strains with mosquitocidal activity: *Paraclostridium bifermentans malaysia* isolated from a mangrove swamp in Malaysia and *Paraclostridium bifermentans Paraiba* isolated in Brazil. PMP1 shares 36% sequence identity with BoNT/X and 34% identity with BoNT/En. These three toxins form a distinct branch in the BoNT superfamily (Fig. 1). Functional analysis showed that PMP1 is capable of cleaving mosquito Syntaxin 1 and has no toxicity in mice. PMP1 is the first known neurotoxin that naturally targets *anopheles* mosquito larvae. Its insecticidal toxicity and selectivity have the potential to be harnessed for developing novel mosquito control agents.

The crystal structure of the PMP1-H_C has been solved, revealing features distinct from the classic BoNT-H_Cs (Contreras et al. 2019). For instance, there are a dozen

aromatic residues exposed on the surface of PMP1-H_C, forming unique hydrophobic patches. Mutations at these hydrophobic patches reduced toxicity, suggesting that they may contribute to receptor binding. The receptors for PMP1 and other BoNT-like toxins remain unknown, which likely dictate the species targeted by each toxin, although other barriers may also exist. It is possible that BoNT/X and BoNT/En may also target insects or other invertebrates, and we expect that additional members of this group will continue to be discovered, which may form a group of neurotoxins targeting invertebrates. As the H_C can be switched between BoNT and BoNT-like toxins, chimeric toxins utilizing the LC-H_N part of BoNT-like toxins may provide an additional toolbox for designing new therapeutic toxins with unique properties.

4 BoNT Homologs

Bioinformatic analysis also revealed a growing number of sequences bearing various degrees of homology to BoNT, defined as BoNT homologs. The first was discovered in the genome of *Weissella oryzae*, a gram-positive anaerobe isolated from fermented rice in Japan (Mansfield et al. 2015). The protein was later named BoNT/Wo (Zornetta et al. 2016). The protein sequence of BoNT/Wo can be divided into LC, H_N, and H_C based on homology analysis with BoNTs, and it contains a few key conserved moieties found in BoNTs. However, BoNT/Wo is significantly different from BoNTs and BoNT-like toxins. First, the sequence identity of BoNT/Wo to other BoNTs and BoNT-like toxins is only 14–16% (Fig. 1). Second, there is no cysteine located at the linker region between BoNT/Wo-LC and HC. Third, while all BoNTs and BoNT-like toxins are located within a similar gene cluster (discussed in Sect. 6), BoNT/Wo is not in such a cluster. Thus, BoNT/Wo is only a distant homolog of BoNTs. It has been reported that BoNT/Wo-LC is capable of cleaving VAMP2 in vitro, but its physiological function remains to be established (Zornetta et al. 2016).

Three more BoNT homologs were recently reported in the genome of *Chryseobacterium piperi* (Mansfield et al. 2019). They showed low levels of sequence identity to BoNTs. For instance, one of these proteins, designated Cp1, shares ~17% identity with BoNT/A1. Cp1 can be divided into LC, H_N, and H_C based on homology analysis with BoNTs, and there are two cysteine residues located at the linker region between its LC and HC, suggesting an inter-chain disulfide bond. The function of these BoNT homologs remains to be fully characterized.

5 Three-Domain Architecture

The full-length crystal structures of BoNT/A, BoNT/B, BoNT/E, and TeNT have been determined, clearly demonstrating a three-domain architecture, composed of the LC, H_N, and H_C (Fig. 2b) (Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000; Kumaran et al. 2009; Masuyer et al. 2017). The overall fold of each domain is largely conserved across these toxins, despite their rather low levels of amino acid sequence identity. BoNT/A and BoNT/B both showed a linear domain arrangement,

with the LC and H_C located on each side of the H_N, while the LC and H_C in BoNT/E are located on the same side of H_N and interact with each other. Thus, BoNT/E has an overall more-compact globular shape than BoNT/A and BoNT/B. The structure of TeNT has been investigated using multiple approaches: small-angle X-ray scattering analysis showed that TeNT is in a linear domain arrangement (open state) at neutral pH and changes into a compact globular form (closed state) under acidic pH (Masuyer et al. 2017). An intermediate semi-open state was also observed by low-resolution cryogenic electron microscopy (Cryo-EM). The high-resolution X-ray crystal structure of TeNT showed a closed state, with all three domains interacting with each other. Within TeNT, the LC-H_N forms a relatively stable core, while the H_C alters its position under different experimental conditions. The physiological relevance of the domain rearrangement in TeNT and whether similar flexibility exists in BoNTs remain to be determined.

5.1 Translocation Domain

The crystal structures of BoNT/A, B, E, and TeNT all reveal that the LC forms extensive contacts with the H_N. Particularly, the N-terminal region composed of ~50 residues of the H_N, designated the "belt" region, wraps around the LC (Fig. 2b). Because the belt region partially covers the active site of the LC, the LC reaches its full activity only once it is dissociated from the H_N after the disulfide bond connecting the LC and HC is broken (reduced).

The H_N is responsible for translocating the LC across the endosomal membrane. It is well established that the low pH within endosomes triggers conformational changes in BoNTs, leading to translocation of the LC, but the molecular mechanism for this translocation process remains to be elucidated. The H_N domain prominently features two long α-helices of ~105 Å. It remains unclear how these helices may alter their conformations upon encountering the low pH within endosomes.

A potential transmembrane region has been proposed based on analyzing hydrophobicity (e.g., residues 659–681 in BoNT/A), and a similar region (residues 593–686) in BoNT/A has been suggested to contribute to forming a channel in membranes (Montal et al. 1992; Lebeda and Olson 1995; Fischer et al. 2012). Bioinformatic analysis comparing the H_N of BoNTs and BoNT-like toxins revealed similarities between this region and the proposed transmembrane helix of diphtheria toxins, and also identified a conserved K/R. . .PxxG motif (Mansfield et al. 2019).

A recent study reported that the isolated H_N fragment of BoNT/A lacking the belt region can be produced as a soluble protein, and its crystal structure under acidic pH conditions has been resolved (Lam et al. 2018a). The structure highlights major conformational changes in the region from residues 620 to 667. This region is termed the BoNT-switch and contains disordered loops and short helices under neutral pH but switches to β-hairpins containing five β-strands under acidic pH. Interestingly, the sequence of this region, particularly the β2/β3 loop, is highly conserved across all BoNTs and bears an "aromatic-hydrophobic-glycine" tripeptide motif flanked by proline residues, which is similar to the lipid-binding peptide found in viral fusion proteins such as the internal fusion loop of Ebola virus glycoprotein 2. Thus, it was

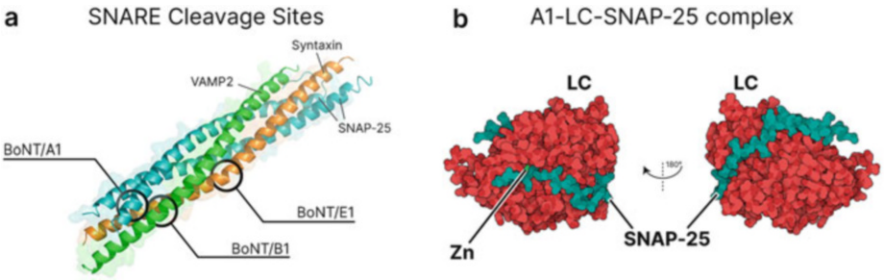

Fig. 3 BoNT-LCs cleave SNARE proteins. (**a**) The three SNARE proteins, Syntaxin 1, SNAP-25, and VAMP1/2/3 form a complex of four alpha helix bundles, which is essential for fusion of synaptic vesicle membranes to the plasma membrane of neurons. Cleavage of any one of these three SNARE proteins is sufficient to block vesicle exocytosis and neurotransmitter release. The cleavage sites for BoNT/A1, B1, and E1 are marked. PDB: 1N7S. (**b**) The crystal structure of a SNAP-25 fragment (colored dark green) in complex with BoNT/A1-LC (red), showing extensive interactions of SNAP-25 with the BoNT/A1-LC (right panel: rotated 180°). PDB: 1XTG

suggested that the BoNT-switch region is responsible for sensing the pH change and initiating membrane penetration via a mechanism similar to that used by viral fusion peptides. These results represent a major advance in our understanding of pH-induced conformational change in H_N. How the changes in the BoNT-switch region leads to further conformational changes in the rest of H_N and the eventual translocation process remains to be determined.

5.2 The Structure of the LC

By aligning the protein sequence of five BoNTs and TeNT, Giampietro Schiavo and Cesare Montecucco recognized a conserved HEXXH motif that is the key feature of metalloproteases, suggesting that BoNTs and TeNT act as proteases (Schiavo et al. 1992b). In their following seminal work published in 1992, they identified BoNT/B and TeNT as zinc-dependent proteases that cleave the synaptic vesicle protein VAMP2 (Schiavo et al. 1992a). Within a few years, it was fully established that BoNT/B, D, F, and G cleave homologous VAMP1, 2, and 3, while BoNT/A, C, and E cleave the peripheral membrane protein SNAP-25 (Fig. 3a). In addition, BoNT/C can also cleave the plasma membrane protein Syntaxin 1. BoNT/B and TeNT both share the same cleavage site on VAMP1, 2, and 3, while all other toxins have their own unique cleavage sites. These three toxin substrates are members of SNARE family proteins. They form the core complex that mediates fusion of synaptic vesicle membranes to plasma membranes, which is essential for releasing neurotransmitters (Jahn and Scheller 2006; Sudhof and Rothman 2009).

The crystal structures of all seven BoNT-LCs have been resolved, revealing an overall conserved globular fold (Jin et al. 2007; Arndt et al. 2005, 2006). The

catalytic site with the signature motif HEXXH is conserved in both composition and geometry across all BoNTs. BoNT-LCs are zinc-dependent proteases with remarkable substrate specificity. As their catalytic sites are similar, the specific recognition and cleavage of different substrates must involve regions outside the catalytic site. Indeed, co-crystal structure of an inactive form of BoNT/A-LC (A-LC, containing two-point mutations that abolish its protease activity) in complex with a fragment of its substrate SNAP-25 (residues 141–204) reveals that SNAP-25 wraps around A-LC, forming extensive interactions particularly via an α-exosite bound by the N-terminal region of the SNAP-25 fragment as well as a β-exosite bound by the C-terminal region of SNAP-25 (Fig. 3b) (Breidenbach and Brunger 2004). This requirement of "long stretch" of SNAP-25 to be properly docked into A-LC ensures specificity.

Each BoNT-LC likely possesses its own distinct exosites, whose location and composition determine the selection of the substrate SNARE proteins and the specific cleavage site. The co-crystal structure of BoNT/F-LC (F-LC) in complex with a VAMP2 fragment containing a point mutation that renders it resistant to BoNT/F is the only other toxin-substrate complex that has been resolved (Agarwal et al. 2009). This structure also demonstrated extensive interactions between F-LC and the VAMP2 fragment, with VAMP2 docked onto F-LC through at least three exosites distinct from the exosites in A-LC. The precise locations of exosites in other BoNT-LCs remain to be established. The crystal structure of BoNT/X-LC (X-LC) has been solved (Masuyer et al. 2018). Despite only ~30% sequence identity with other BoNTs, X-LC display a typical BoNT-LC fold with many conserved secondary structural features. The structure further demonstrates that X-LC is a bona fide member of the BoNT-LC family. The crystal structure of the BoNT/Wo-LC has recently been solved as well, showing that it shares a common core fold found in other BoNT-LCs but also revealing several distinct features including an unusually wide and open catalytic site (Kosenina et al. 2019).

Notably, A-LC has been shown to maintain its activity in cultured neurons for several months, which is the major reason for BoNT/A's ability to induce persistent paralysis that lasts several months in humans (Keller et al. 1999; Whitemarsh et al. 2014; Tsai et al. 2017; Pellett et al. 2015; Foran et al. 2003). This is a key pharmacological property that contributes to the success of BoNT/A as a therapeutic agent. Among the seven BoNTs, BoNT/E showed the shortest half-life, with only a few weeks in humans (Foran et al. 2003), a key feature differentiating it from BoNT/A. The molecular basis for the extremely long half-life of BoNT/A remains to be fully established. A-LC has been shown to bind the cytoskeleton protein septin complex, which may shield A-LC from degradation (Vagin et al. 2014). It has also been suggested that A-LC recruits deubiquitinase to reduce its ubiquitination (Tsai et al. 2017). The structural basis for those interactions remains to be solved.