Chapter 5

Botulinum Neurotoxin: A Multifunctional Protein for the Development of New Therapeutics

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Additional information is available at the end of the chapter

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Abstract

Botulinum neurotoxin (BoNT) is a major therapeutic agent licensed in neurological indications such as dystonia and spasticity. In recent years, its use has steadily increased in other neurological areas and new therapeutic areas and also in the aesthetic setting. Paradoxically, BoNT is also the causative agent of the disease botulism and a potential bioterrorism toxin. The BoNT family of toxins comprised more than 40 individual members, classified into 7 serotypes and are produced by Gram-positive obligate anaerobic bacteria. BoNTs are enzymatic multi-modular proteins with a complex multistep mechanism of action. Their target site is at peripheral neurons, particularly the neuromuscular junction, at which they inhibit acetylcholine neurotransmission. Despite intense activity in the BoNT field, today there are still gaps in knowledge both in clinical practice and in basic research. The discovery of the structure-function of BoNT and its domains has allowed rational design of new features using molecular engineering. The diversity of BoNT molecules, both natural and engineered, is an invaluable pool from which to design future new therapeutics with unique pharmacological properties for current and novel indications.

Keywords: botulinum neurotoxin, therapeutic agent, botulism, recombinant protein, targeted secretion inhibitor

1. Introduction

There are currently four botulinum neurotoxin (BoNT) clinical products available in the Western hemisphere: abobotulinumtoxinA (Dysport®, Ipsen, Paris, France), incobotulinumtoxinA (Xeomin®, Merz Pharmaceuticals GmbH, Frankfurt, Germany), onabotulinumtoxinA (Botox®, Allergan, Irvine, CA, USA) and rimabotulinumtoxinB (Myobloc®, Solstice

Neurosciences, Louisville, KY, USA) [1]. Several other products are available for use in other countries, in particular in the Asian markets, and new formulations and products are under-development [2]. By 2022, it is expected that the market size for botulinum products will reach $6.6 billion, driven by the expansion of their therapeutic uses and also the appetite for non-invasive aesthetic applications [3].

Around 200 years ago (between 1817 and 1822), a German medical officer, Justinus Kerner, published a series of papers to provide the first accurate and complete description of the symptoms of food-borne botulism, which led to the discovery of BoNT as the causative agent and the prediction by Kerner of its potential clinical utility [4]. This fascinating class of proteins present a modular molecular architecture with distinct binding, translocation and enzymatic domains. The different structural and functional domains can be regarded as 'building blocks' and have facilitated a number of engineering approaches aimed, amongst other purposes, at extending the therapeutic applications of BoNTs to other cell types beyond their natural target of the neuromuscular junction [5].

The aim of this chapter is to (1) provide an overview of the current clinical uses and a historical perspective of botulinum neurotoxin discovery, the disease it causes and the threats and opportunities that it poses and (2) present the current understanding of the structure-function of the toxin and its application in the development of new therapeutics.

2. Clinical uses

BoNT products are neuromuscular blocking agents which exert their effect through inhibition of acetylcholine release. BoNTs are amongst the most tissue-selective drugs known in clinical pharmacology and are characterised by high potency, high specificity and long duration of action of around 3–6 months following a single injection [6]. These characteristics have made BoNTs highly successful and effective therapeutic agents for the management of several chronic and debilitating diseases of neuronal hyperactivity. Although initially thought to inhibit acetylcholine release only at the neuromuscular junction, BoNTs are recognised to also inhibit release of neurotransmitters from autonomic nerve terminals, for example, in glands (e.g. in hyperhidrosis), and nociceptive neurons in pain states [6, 7].

Currently, there are four formulations of BoNTs approved by the US Food and Drug Administration (FDA) for several clinical applications (see **Table 1**). Cervical dystonia, also known as spasmodic torticollis (disorder characterised by involuntary contractions of neck and upper shoulder muscles resulting in abnormal postures and/or movement of the neck, shoulder and head and that may be associated with neck pain), is the only condition for which all four formulations are approved. Other neurological conditions include spasticity (disorder characterised by tight or stiff muscles and an inability to control those muscles), with approved formulations both for adult and paediatric populations, migraine and blepharospasm (dystonia that can cause disabling eye closure). Other non-neurological therapeutic FDA-approved uses are strabismus (eye misalignment), overactive bladder, urinary incontinence and hyperhidrosis (excessive sweating).

FDA-approved indication	Treatment population	AbobotulinumtoxinA (Dysport®)	IncobotulinumtoxinA (Xeomin®)	OnabotulinumtoxinA (Botox®)	RimabotulinumtoxinB (Myobloc®)
Cervical dystonia	Adult	Approved	Approved	Approved	Approved
Upper limb spasticity	Adult	Approved	Approved	Approved	na
Lower limb spasticity	Adult	na	na	Approved	na
Lower limb spasticity	Children ≥ 2 years of age	Approved	na	na	na
Migraine	Adult	na	na	Approved	na
Blepharospasm	≥12 years of age	na	Approved	Approved	na
Strabismus	≥ 12 years of age	na	na	Approved	na
Glabellar lines	Adult	Approved	Approved	Approved	na
Overactive bladder	Adult	na	na	Approved	na
Urinary incontinence	Adult	na	na	Approved	na
Hyperhidrosis	Adult	na	na	Approved	na

na = indication not FDA-approved.

Table 1. Food and Drug Administration (FDA)-approved indications for the use of marketed botulinum neurotoxins products [1].

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Historically, BoNT products have been considered as a single pharmacological class [8]. However, the existing BoNT products vary in the identity and amount of toxin present, their formulations, the manufacturing processes and the potency methods used to determine the strength of the products [9, 10]. As a result, the different products are not considered to be interchangeable, and their respective clinical efficacy and safety are unique to each specific product [11].

In 2016, the American Academy of Neurology (AAN) published updated guidelines for the clinical use of BoNT [12]. The 2016 AAN recommendations for BoNT use, based on evidence from clinical trials, do not fully match the FDA-approved indications or AAN's previous guidelines from 2008, which is a reflection of the expanding uses of BoNTs [8]. Multiple clinical trials are being conducted to investigate the efficacy and safety of BoNTs for various clinical conditions and, in addition, pilot studies are being conducted to test the efficacy of BoNTs for new indications [9, 13, 14]. A summary of not approved new indications for which botulinum toxins are under investigation is presented in **Table 2**.

The use of BoNTs has been extended to aesthetic applications for the reduction of facial lines. According to recent statistics, BoNT injections are now the most popular of all cosmetic procedures worldwide, both surgical and non-surgical [15], and, in the US, more than 6.6 million injections were performed in 2014 alone for aesthetic reasons [16]. There are currently three BoNT products approved by the FDA for use in glabellar lines (wrinkles that appear between

Achalasia	Dysphonia	Neuromyotonia	Rhinorrhoea and/or rhinitis
Alopecia	Endometriosis	Nystagmus	Sialorrhea
Anal fissure	Esophageal spasm	Obesity	Spasmodic dysphonia
Anismus	Exotropia, esotropia, entropion	Orbital atrophy	Stiff person syndrome
Atrial flutter	Eyelid-opening apraxia	Oscillopsia	Stuttering
Autonomic dysreflexia	Facial flushing	Osteoarthritis	Synkinesis
Benign prostatic hyperplasia	Fecal incontinence	Some forms of pain	Temporomandibular joint syndrome
Bruxism	Frey's syndrome	Palatal myoclonus	Tennis elbow
Carpal tunnel syndrome	Gastroparesis	Paratonia	Tension headache
Cleft lip repair	Gustatory sweating	Peyronie's syndrome	Tetanus
Club foot	Hemifacial spasm	Piriformis syndrome	Tremor
Constipation	Hyperlacrimation	Plantar fasciitis	Trigeminal neuralgia
Cystitis	Lateral epicondylalgia	Protective ptosis	Vaginismus
Depression	Myofascial pain	Psoriasis	Ventricular arrhythmias
Diabetic polyneuropathy	Myokymia	Restless leg syndrome	Vocal tics

Table 2. Not approved new indications for which botulinum toxins are under investigation [9, 13, 14].

the eyebrows): abobotulinumtoxinA (Dysport®, Ipsen as the marketing authorisation holder with Galderma as distributor in the aesthetic indication), incobotulinumtoxinA (Xeomin®/Bocouture®, Merz) and onabotulinumtoxinA (Botox®/Vistabel®, Allergan) (see **Table 1**). The facial aesthetic uses of BoNTs are extensive, mainly not approved and under investigation, and patient satisfaction with treatment is very high, with significant improvement in patient-reported outcomes. Rhytides (skin wrinkles) regions for treatment include forehead, brow, region between the eyebrows, around the eyes (crow's feet) and nose (bunny lines), smile (gummy smile), upper lip, corners of the mouth, jaw, chin and neck area [16, 17].

Despite intense use of BoNTs in clinical practice, approval and labelling guidance does not exist to address key questions such as where BoNTs fit amongst various treatment options for a given condition, recommendations of one product over another for a given indication or clinical differences in potency and duration of action (see Refs. [8, 18]).

3. Disease and bioterrorism threat

Botulism is a rare but potentially fatal disease caused by BoNT intoxication. Botulism is characterised by a descending flaccid paralysis with symptoms of cranial nerve dysfunction such as diplopia (double vision), dysphagia (difficulty in swallowing), pupillary dilation and ptosis (drooping eyelids), progressing to respiratory failure and, in rare occasions if not provided with suitable intensive care and life support, ultimately death. Fever and altered mental status are absent. The diagnosis of botulism is largely clinical and is confirmed by laboratory tests, sometimes including the detection of BoNTs in contaminated materials, food or bodily waste [19]. Botulism in humans is classified according to the route of entry of the toxin: food-borne botulism occurs after the ingestion of BoNT-contaminated food that contains the preformed toxin; infant botulism is the result of bacteria colonising the immature gastrointestinal tract of infants which then produce and release the toxin in situ; wound botulism results from spore contamination into the tissue and is mostly associated with injection drug abuse; and iatrogenic botulism can occur as a result of excessive BoNT use either for therapeutic or cosmetic use [20]. Inhalation botulism is also a possibility, if the toxin were to enter through the respiratory tract. However, inhalation botulism is rare and does not occur naturally [21].

A stable number of cases of botulism have been reported in Europe (i.e. European Economic Area, comprised of 31 countries) in recent years. During the period 2007–2014, an average of 115 cases per year of confirmed botulism occurred, and 5% of those were fatal [22]. A very similar numbers in the US were reported by the Centres for Disease Control and Prevention (CDC) for the same period (2007–2014), with an average of 143 confirmed cases per year, with 2% of those being fatal. According to the CDC, the most numerous cases were of infant botulism, but wound and food-borne botulisms were also presented yearly, plus a minor percentage of cases of unknown aetiology [23].

There is currently no approved pharmacological treatment for BoNT intoxication in humans, and recent efforts have focussed on the development of (1) vaccines from partially purified toxins, (2) use of specific antitoxin antibodies and (3) small molecule inhibitors [20, 24]. Once an

outbreak occurs, medical treatment includes treatment with the botulin heptavalent antitoxin and consideration of admission to an intensive care unit with mechanical ventilation until recovery. Botulism is not contagious, and standard precautions are sufficient for infection control [19].

Botulism also occurs in animals and begins with the growth of the BoNT-producing bacteria in decaying carcasses followed by the release of the toxin into the environment. Both toxin and bacteria can spread via transmission of BoNT-insensitive animals such as maggots and other invertebrates that are consumed by healthy BoNT-sensitive animals, which eventually die and allow the growth of the bacteria and the subsequent production of the toxin to self-amplify the cycle [20].

Partly due to the fact that no effective treatment is available for BoNT intoxication in humans and the perceived ease in which it could be used in a bioterror attack, BoNT is classified as a potential bioterrorism weapon by the US CDC. BoNT belongs to the category A, the highest level of concern regarding public health and need of preparedness. Only five other agents are classified as category A agents, those being anthrax (*Bacillus anthracis*), bubonic plague (*Yersinia pestis*), smallpox (*Variola major*), tularemia (*Francisella tularensis*) and arenaviruses causing viral hemorrhagic fevers [19]. A contentious paper from 2005 regarding ease of BoNT intoxication through cow's milk destined for human consumption calculated if would take only 4 g of BoNT, e.g. roughly equivalent to 1 teaspoon of granulated sugar, to poison over 400,000 people [25]. The publication of that research opened a public safety debate within the scientific community regarding BoNT dual-use research [26], which was reopened when the allegedly new BoNT/H type was originally reported [27]. However, it should be noted that BoNTs are much more toxic (in the range of 100–1000 times) when injected than when administered orally; and delivery by aerosols is considered inefficient [20].

4. Historical overview of BoNT discovery

BoNT/A is the most potent toxin known to man, with a reported estimated human lethal dose of 1.3–2.1 ng/kg intravenously or intramuscularly and 10–13 ng/kg when inhaled [4]. Not surprisingly, its effects have been known throughout history long before the molecular identity of the toxin was elucidated. Botulism-like symptoms were known by ancient Greeks and Egyptians, and the Byzantine emperor Leo IV (886–911 AD) banned 'blood sausage' as it caused a fatal illness. It was not until around a thousand years later that following a number of sausage poisoning outbreaks in Germany the first accurate and complete description of the symptoms of food-borne botulism was described between 1817 and 1822 by J. Kerner. The extracted causative agent was named 'sausage poison' and was believed to be a 'fatty acid'. Later, a German physician named Muller referred to the sausage poisoning as botulism from the Latin name for sausage, 'botulus' [4, 28].

The first isolation of the bacteria responsible for producing the toxic agent causing botulism was performed by the Belgian professor Emile Pierre van Ermengem and was termed *Bacillus botulinus*. Its name was changed to *Clostridium botulinum* when the aerobic *Bacillus* genus was separated from the anaerobic *Clostridium* genus [29]. To date, six different BoNT-producing bacterial groups are known; all have been taxonomically classified as clostridia. These clostridia

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produce seven different serotypes of botulinum toxin, termed BoNT/A to BoNT/G. BoNT/A, BoNT/B and BoNT/F were discovered following incidences of food-borne botulism, reminiscent of the original 'sausage poisoning', whereas BoNT/C, BoNT/D and BoNT/E were discovered following incidences of botulism in animals [27] (see **Figure 1**). BoNT/G, discovered in 1970, was reported in a sample extracted from soil, and to date there has not been reported cases of botulism caused by BoNT/G in the wild affecting either humans or animals [30]. A possible eighth type, initially termed BoNT/H was reported in 2013, but later reclassified as a BoNT/FA hybrid [31].

Despite Kerner suggesting the potential of BoNT as a therapeutic agent in conditions of muscular hypercontraction and glandular hypersecretion, it was not until around 150 years later

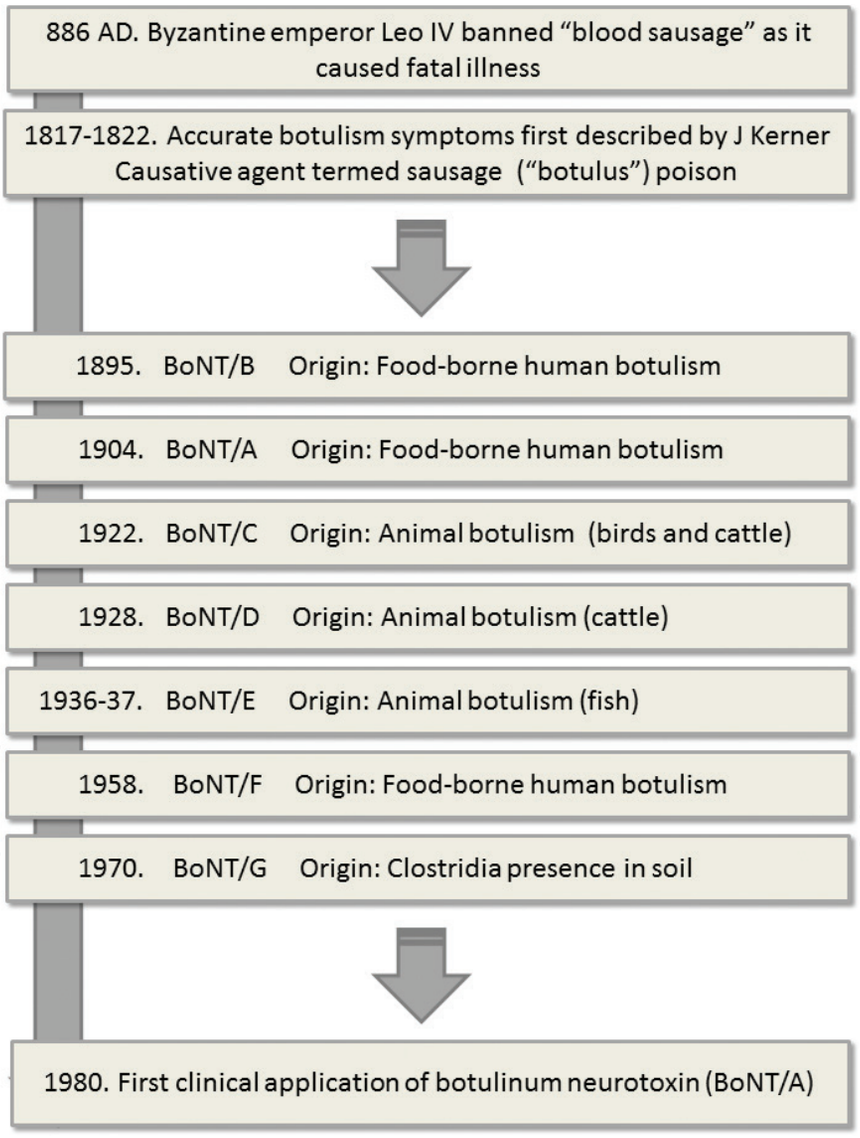

Figure 1. Timeline of the discovery of the seven botulinum toxin types. For context, also depicted are the dates of the first accurate description of botulism and the first use of botulinum toxins as therapeutic agent. A proposed eighth type was initially reported in 2013, now classified as an F/A hybrid toxin. For details see Refs. [2, 27, 28].

that the first clinical application was made. In 1981, Dr. Alan B. Scott at what was formerly known as the Smith-Kettlewell Institute of Visual Science, San Francisco, California, USA, used BoNT/A for the treatment of strabismus as an alternative to surgical intervention. The original name of the drug was Oculinum®, and its rights were later acquired by Allergan Inc., which changed the name of the drug to Botox®.

5. The producing bacteria: types of toxin and nontoxin proteins

Upon the first description of the botulism symptoms (see above), the initial hypothesis was that botulism was caused by a toxin produced by a single bacterial organism, as is the case for the closely related toxin tetanus toxin and its producing bacteria *Clostridium tetani* [32]. However, it soon became apparent that different types of toxin and different producing bacteria existed for BoNT [29].

In 1910–1919, serological methods were introduced for categorisation of the toxin-producing bacteria and for the toxins themselves, that are still in use today [33]. Biochemical and molecular techniques have complemented those initial classifications and have confirmed the presence of multiple species of BoNT-producing clostridia and multiple species of BoNT proteins. BoNT-producing bacteria are Gram-positive, anaerobic, spore-forming and rod-shaped organisms and are commonly found in any soil or water environment. The seven distinct serotypes differ by 37–70% in amino acid sequence [34]. Early observations pointed to a level of intratypic serological diversity that led to variants within serotypes to be called sub(sero)types and a proposal that new subtypes would differ by 2.6% at the protein sequence level. However, this rule is not consistently applied today throughout all the subtypes [35]. It is considered that 41 individual toxins exist and the various toxin subtypes are given a letter designation for the toxin serotype followed by a sequential number in order of discovery, e.g. BoNT/A1 and BoNT/E11. Only 4 serotypes currently present subserotypes, namely BoNT/A (8 subtypes), BoNT/B (8 subtypes), BoNT/E (12 subtypes) and BoNT/F (7 subtypes) (see **Figure 2**). Interestingly, BoNT/C and BoNT/D occur naturally as well as hybrid toxins, termed BoNT/CD and BoNT/DC. A third naturally occurring hybrid, BoNT/FA, was initially proposed as the new serotype BoNT/H following its discovery in 2013 but later reclassified as a hybrid toxin [36].

Current classification of BoNT-producing clostridia is according to group designation based on metabolic biochemical criteria (see **Table 3**). The metabolic groups represent distinct species of *Clostridium botulinum* (Groups I to III) and *Clostridium argentinense* (Group IV), and these species include non-toxigenic as well as neurotoxigenic members. In addition, *Clostridium baratii* and *Clostridium butyricum* are also known to produce BoNTs (Groups V and VI). To add to the confusion, some *Clostridium botulinum* strains do not produce BoNT, in particular if subcultured repeatedly in the laboratory; and some additional toxins are produced by the neurotoxigenic *Clostridium botulinum*, such as C2 toxin, C3 exoenzyme and botulinolysin. However, no alternative nomenclature for this group of organisms has been accepted [32].

Clostridial strains in different groups can produce the same toxin (e.g. Groups I, II and V produce BoNT/F), and bivalent toxin combinations within the same strain have been identified.

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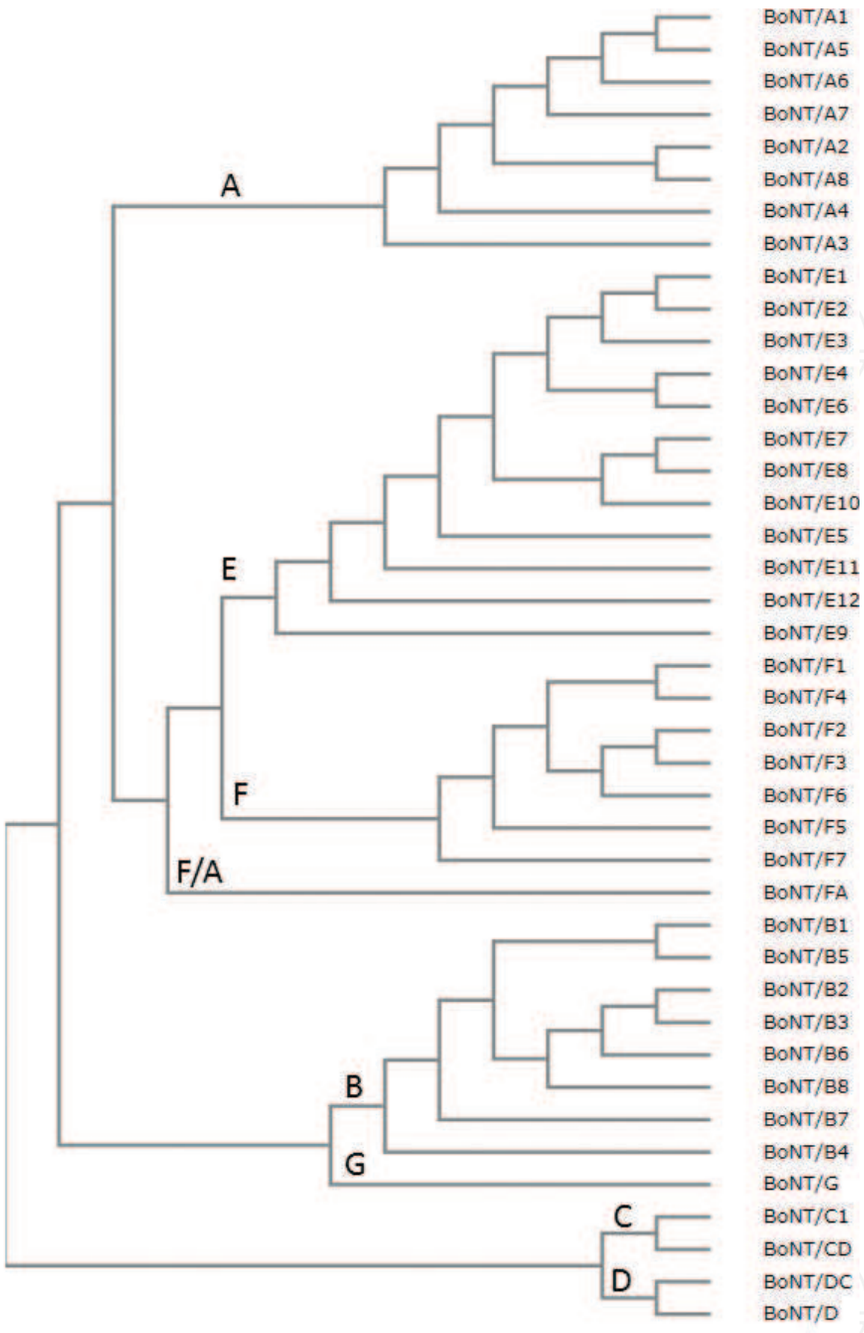

Figure 2. Phylogenetic tree depicting the relationship of the 41 known botulinum neurotoxins. Individual FASTA files were accessed through the NCBI portal (NIH, USA), and the protein alignment and phylogram were constructed using the online software Clustal Omega (EMBL-EBI, Germany).

When more than one toxin is produced by a single strain, such as Ba or Bf, the capital letter designates the toxin produced in greater amounts. If a gene is present but not expressed, it is denoted between brackets, for example, A(B); and if a gene is present but truncated, it will have an apostrophe to indicate this fact, such as A(B′). This diversity in BoNT-producing bacterial strains is the result of toxin gene associations with transposases such as insertion sequence elements, recombinases, the acquisition of plasmids or infection by phage [37], within and between the

Clostridial bacteria	Group	BoNT serotype(s) produced	Mixture of serotype(s) produced by a single strain	Nontoxinogenic bacteria belonging to the same group
Clostridium botulinum	I	A, B, F	A(B), A(B′), Ab, Af, Ba, Bf, Bf/a	*Clostridium sporogenes*
Clostridium botulinum	II	B, E, F	–	*Clostridium taeniosporum*
Clostridium botulinum	III	C, D, CD hybrids DC hybrids	–	*Clostridium novyi*
Clostridium argentinense	IV	G	–	*Clostridium argentinense* *Clostridium subterminale* *Clostridium hastiforme*
Clostridium baratii	V	F	–	*Clostridium baratii*
Clostridium butyricum	VI	E	–	*Clostridium butyricum*

Table 3. BoNT-producing clostridial species, see Refs. [20, 29, 38].

groups and species. Groups IV–VI have the toxin genes located in the chromosome, considered less mobile, whereas Group III has the toxin genes in highly mobile elements such as plasmids and bacteriophages. Groups I and II have a mixture of chromosome and plasmid localisation [38]. A recent genetic study of *C. botulinum* strains causing human botulism in France showed that the genetic diversity of the BoNT-producing organism appeared as a result of multiple and independent genetic rearrangements and not from a single evolutionary lineage [39].

All seven BoNT serotype toxins are released from the producing bacteria as large protein complexes with a number of neurotoxin-associated proteins (NAPs) to become highly potent oral toxins, often ingested in contaminated foods [38, 40]. The NAPs are encoded together with the *bont* gene in one of two different gene clusters, the hemagglutinin (HA) cluster or the orfX cluster. Both clusters encode the nontoxic non-hemagglutinin (NTNHA) protein, which assembles with BoNT to form the smaller of the progenitor toxin complexes. BoNT/A, BoNT/B, BoNT/C and BoNT/D complexes contain HA, whereas BoNT/E and BoNT/F complexes do not contain HA. The components of the BoNT complex vary with neurotoxin serotypes and the *Clostridium* strain producing them. BoNTs are produced in three progenitor forms: M (medium), L (large) and LL (extralarge) complex. The M form consists of the neurotoxin (of 150 KDa) with NTNHA and has a total weight of ~ 300 KDa. The L and LL complexes consist of several HA proteins besides the BoNT and NTNHA, and its molecular weight is ~ 500 KDa for the L form and ~ 900 KDa for the LL form. The function of the proteins encoded in the orfX genes remains unknown [41]. NAPs are known to protect BoNTs against the proteases of the gastrointestinal tract and the acidic conditions of the stomach and to facilitate the intestinal trans-epithelial delivery to the toxin into the lymphoid and general circulation [38]. The role of NAPs in the producing bacteria is not known. Recently, it has been proposed that the primary role of NAPs and in particular that of NTNHA is to protect BoNTs from damage in the decaying biological material where the toxin is mostly produced in the wild [20].

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Until recently, BoNTs were believed to be produced exclusively by clostridia organisms. In 2015, the first homologue of BoNTs was described within the genome of the rice fermentation bacteria *Weissella oryzae* SG25 [42]. Bioinformatic analysis of the genomic sequences of *W. oryzae* SG25 revealed one gene with a very similar structure to BoNTs, whereas a second gene showed partial similarity with the BoNT-associated NTNH proteins [42]. Recombinant expression of the BoNT-like protein revealed that it shares similarities with BoNT/B regarding its targeting profile and it is also expected to block neurotransmitter release. The new BoNT-like protein showed no serological cross-reactivity with the seven known BoNT serotypes, and it was dubbed BoNT/Wo by the authors [43].

6. Structure-function of BoNT toxins

BoNTs are zinc metalloproteases consisting of three major domains. Produced as a single polypeptide of 150 KDa, BoNTs require activation by cleavage of the polypeptide post-translationally resulting in the so termed heavy chain, of ~ 100 KDa, and a light chain (LC), of ~ 50 KDa, held together by a disulphide bridge between the two chains [44]. Functionally, the light chain hosts the metalloprotease domain, and the heavy chain comprises both the binding domain (H_C) and the translocation domain (HN). The producing bacteria in Groups I, III and IV (see **Table 3**) are proteolytic strains and will release the cleaved active product, whereas the products of the other producing bacteria are believed to be activated by proteases of the intoxicated organism [38].

The neuromuscular junction is the natural target of BoNTs, and intoxication follows an intricate multistep mechanism [20, 45], in which the toxin-associated proteins of the progenitor toxin complex play a crucial role. For an overview of the routes of entry and mechanism of action of the toxin, see **Figure 3**.

Unintentional BoNT entry into the organism occurs mainly through ingestion of contaminated foods leading to food-borne botulism (see above) or through wounds [20]. Alternatively, and in particular in cases of infant botulism, the producing bacteria can colonise the immature gastrointestinal tract and produce the exotoxin in situ. The progenitor complex allows BoNTs to effectively cross the intestinal trans-epithelial barrier and reach the lymphoid and general blood circulation. Under neutral and alkaline environments, such as in the bloodstream, the complex dissociates and the naked toxin is able to target neuromuscular junctions [46]. In clinical applications, the toxin is delivered locally to the site of action. BoNT entering the body undergoes a relatively short distribution phase which sees the toxin selectively targeting peripheral nerve endings, and an elimination phase that comprises both (1) an interneuronal metabolism following cellular entry and (2) systemic metabolism and elimination which are assumed to be through the liver [47].

Upon reaching the neuromuscular junction, BoNTs are able to specifically target nerve terminals using their Hc-binding domain and internalise through endocytosis. Once in the acidic environment of the endosome, the BoNT HN domain translocates the LC domain into the cytosol, allowing the Zn^{+2} metalloprotease enzyme to cleave target soluble N-ethylmaleimide-sensitive

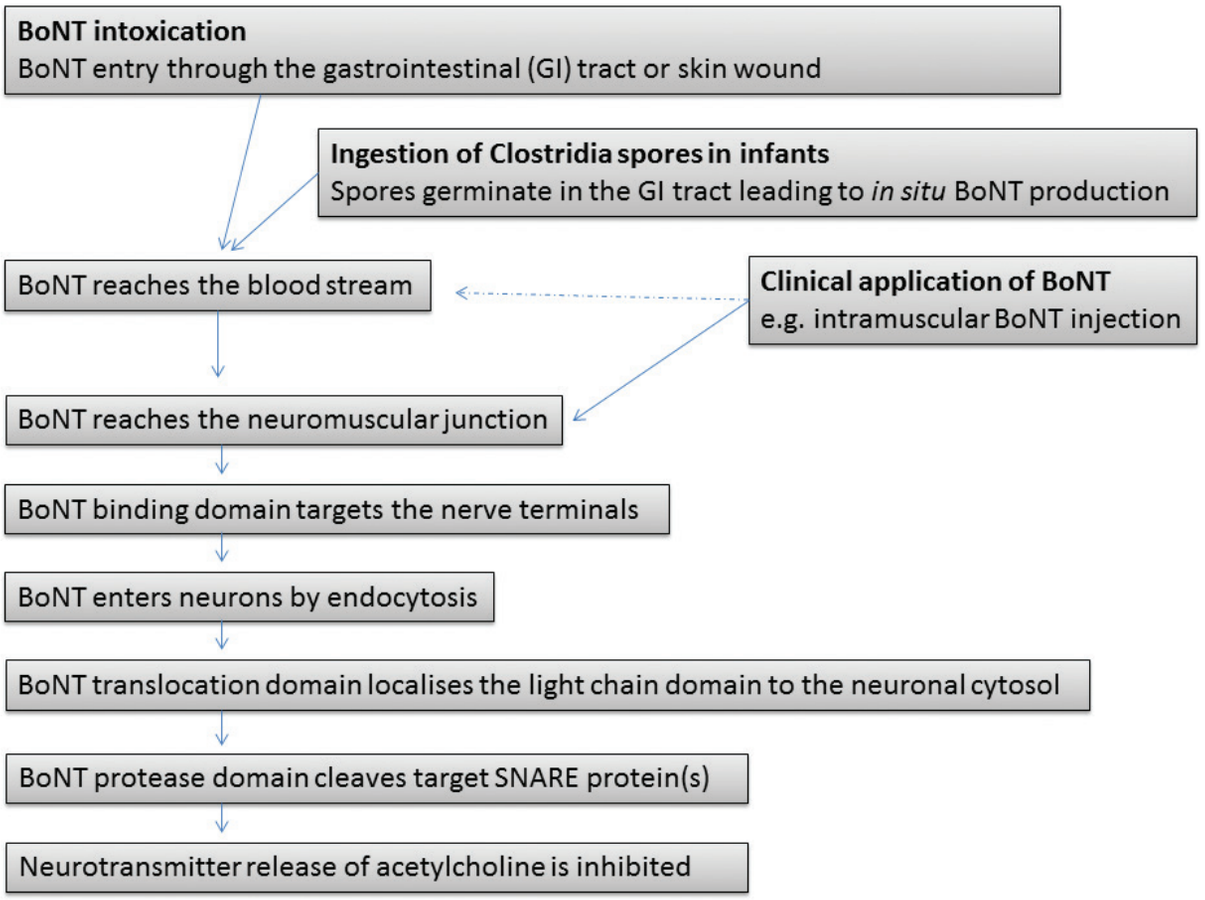

Figure 3. Mode of action of botulinum toxins; for details in each step, see Refs. [20, 45].

factor attachment protein receptor (SNARE) proteins. SNARE proteins constitute an essential part of the machinery for neurotransmitter release in eukaryotic cells, and once their function is compromised by BoNTs, release of acetylcholine in the neuromuscular junction is prevented [19].

Although all serotypes, and even the most recently described BoNT-like protein BoNT/Wo [43], share a multidomain structure, crystallographic data has revealed that the molecular arrangement in the 3D space varies. BoNT/A and BoNT/B present an 'open butterfly' structure, whereas BoNT/E has a 'closed butterfly' organisation when viewed taking the HN translocation domain as a sagittal axis [48, 49]. In **Figure 4**, three different representations illustrate the organisation of BoNT/A and BoNT/E. This differential 3D topology has been credited to confer particular characteristics to BoNT serotypes, such as a faster way of entry for BoNT/E compared to BoNT/A [50].

6.1. Binding domain

BoNTs belong to the family of AB exotoxins, consisting of an 'A' toxic domain and a 'B' binding domain. AB toxins such as cholera toxin, lethal factor from *Escherichia coli* and Shiga toxin use gangliosides as their cellular receptors; whereas anthrax toxin and ricin have protein receptors identified as their targets [51].

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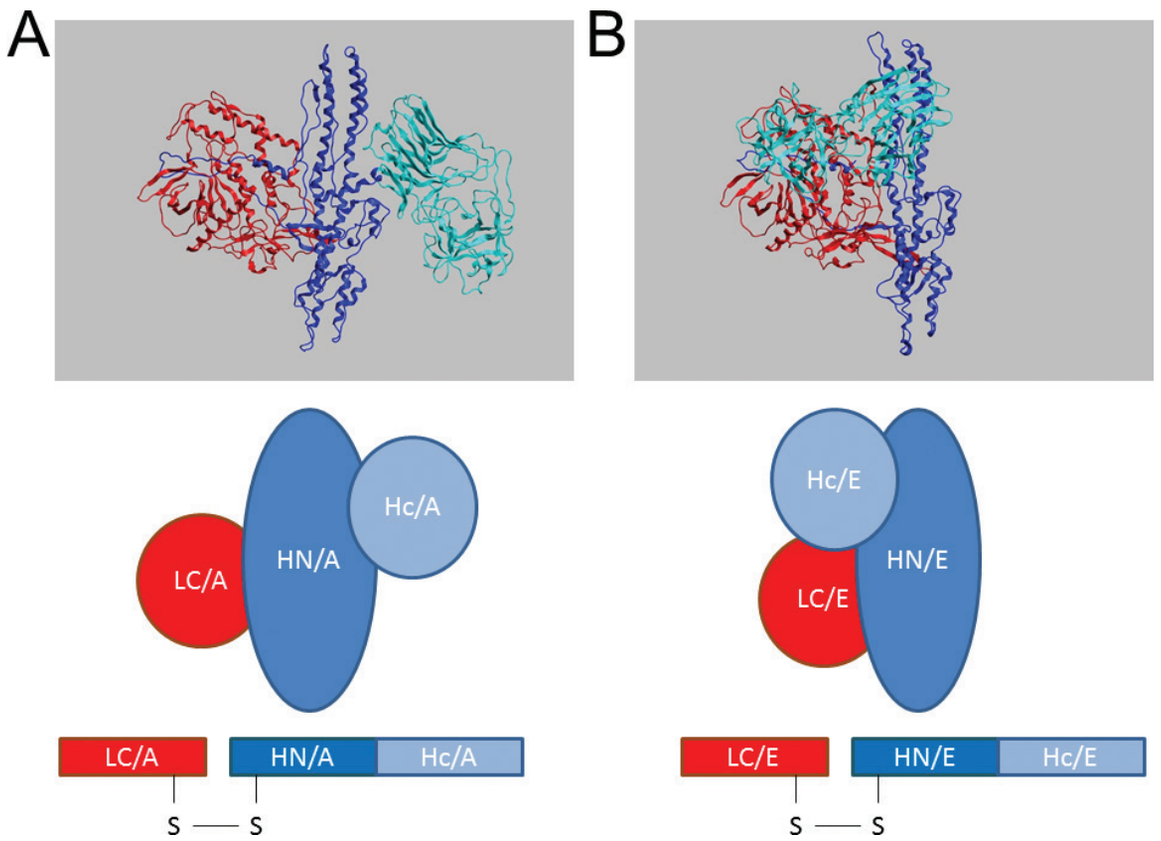

Figure 4. Structural and functional domains of (A) BoNT/A (PDB 3BTA) and (B) BoNT/E (PDB 3FFZ). Hc = binding domain, HN = translocation domain, LC = light chain protease domain. Upper panels: ribbon diagram of the respective crystal structures. Middle panels: diagram depicting the three-dimensional organisation of the domains within the structure. Lower panels: simplified two-dimensional block diagram in which the HN and LC can be seen being connected by a conserved disulphide bridge. Structural image created from crystallographic using the MOE software (Molecular Operating Environment 2013.08; Chemical Computing Group Inc., Montreal, Canada).

In the case of BoNTs, a dual receptor theory was postulated [52]. This dual-binding anchorage is credited for the high affinity and specificity by which BoNTs target neurons. All serotypes share a similar binding site for the interaction with the oligosaccharide portion of a polysialoganglioside. For BoNT/A, BoNT/B, BoNT/E, BoNT/F and BoNT/G, the conserved ganglioside-binding site SXWY has been reported, whereas BoNT/C, BoNT/D and BoNT/DC have analogous sites for ganglioside binding at a similar position [53]. A second, non-conserved binding site that binds a protein receptor has been identified in several BoNTs [54]. BoNT/A, BoNT/E and BoNT/F bind the family of synaptic vesicle protein SV2, whereas BoNT/B, BoNT/D and BoNT/G recognise a short peptide sequence in the luminal domain of the family of synaptic vesicle protein synaptotagmin. A protein receptor has not yet been identified for BoNT/C, which uses a dual ganglioside mechanism [55]. A second protein receptor has been identified for BoNT/A, namely, FGFR3 [56]. Crystal structures of the H_C domains in complex with their receptors, where available, have contributed a major advance in the understanding of BoNT-cell interactions.

Recently, glycan motifs in both gangliosides [53] and protein receptors [57, 58] have emerged as key players in the targeting of BoNTs to the neuronal membranes, albeit glycosylation is not required for binding for all BoNTs [38].

6.2. Translocation domain

Neuronal internalisation triggers the translocation of the LC domain to the cytosol, separating it from the HN and H_C domains and thus allowing it to cleave the cytosolic target SNARE proteins. In neurons in vitro, internalisation of BoNT/A and translocation of the LC into the cytosol occur rapidly, with estimates either side of ~ 60 minutes [59]. Following entry into the synaptic vesicle, the proton pumping action of the v-ATPase present on the synaptic vesicle membrane, responsible for the loading of neurotransmitters into the vesicle, will acidify the organelle and produce the necessary environment for the LC to translocate. Treatments that inhibit internalisation, synaptic vesicle recycling, or acidification also inhibit BoNT action [60].

Of the various steps of the cellular mechanism of intoxication, membrane translocation for the LC is least understood at the molecular level, and several models have been suggested [48]. The first model proposes that upon acidification of the lumen of the synaptic vesicle, HN penetrates the membrane and forms an ion channel assisting a partially unfolded LC to pass through it. This model has been revised and a new proposed mechanism includes binding of the toxin domains to the luminal membrane of the synaptic vesicle, and following acidification both HN and a partly unfolded LC will destabilise and penetrate the membrane. LC will move to the cytosolic side, refold and be released upon reduction of the disulphide bond. At the same time, segments of the HN insert in the membrane and assemble an ion channel. The main difference between these models is that in the first model, channel formation by HN is an early event and translocates LC, whereas in the second model, the channel formed by HN occurs as a consequence of the LC translocation. In both models, the reduction of the disulphide bond is essential to free the LC at the end of the translocation step, and the enzyme thioredoxin and its regenerating enzyme thioredoxin reductase have been identified as the cellular system responsible for the reduction of the disulphide bridge [61]. Following translocation, another key protein recently identified is Hsp 90, which may act as a chaperone assisting the refolding of the LC once in the cytosol [62].

Different models have been proposed for the mechanism by which BoNT domains approach the membrane, which may have physiological consequences. BoNT/E is thought to owe its rapid translocation to its 'closed butterfly' three-dimensional structure in which the Hc and LC are in close proximity [50], whereas BoNT/A and BoNT/B, which in principle share the 'open butterfly' configuration, would approach the membrane differently [63].

6.3. Protease domain

The LC domain is a metalloprotease that cleaves SNARE proteins within the nerve terminal cytosol, resulting in the inhibition of the acetylcholine release which causes a reversible neuroparalysis [44].

SNARE proteins are membrane-associated proteins and comprise a large family of proteins that are responsible for the binding and fusion of vesicles to membranes. In humans, there are 38 different types of SNARE proteins [64]. SNARE proteins that mediate the exocytosis of neurotransmitter vesicles with the plasma membrane of neurons are the target substrates of BoNTs [65]. In addition to inhibiting neurotransmitter release, SNARE cleavage by BoNT also

affects trafficking of proteins, for example, TRPV1 and TRPA1 receptors to the neuronal surface [66]. BoNT/A, BoNT/C and BoNT/E target SNAP-25, whereas BoNT/B, BoNT/D, BoNT/F and BoNT/G target VAMP1, VAMP2 and VAMP3 proteins. BoNT/C is unique amongst BoNTs in targeting two different SNARE types, as it targets syntaxin 1 and syntaxin 2 besides also targeting SNAP-25. Hydrolysis of the SNARE proteins occurs at a unique cleavage site specific to each BoNT [35].

No additional target substrates have been reported for BoNTs beyond SNARE proteins. This may be due to the extensive interaction that BoNTs make with the target proteins, including the cleavage site, which may be responsible for the exclusive specificities to SNARE isoforms in a species-specific manner [48].

The length of BoNT-induced intoxication may depend on (1) how long the cleaved SNARE proteins remain in the cytosol and the ability of the cleaved SNARE proteins to maintain the block to exocytosis, (2) how long the BoNT protease remains in place to cleave newly synthesised SNARE proteins, (3) the rate at which the neuron is able to replenish uncleaved SNARE proteins relative to ongoing cleavage, and (4) the ability of the presynaptic terminal to remodel in order to overcome the temporary paralysis. There is preclinical evidence for all these hypotheses [67]. The ubiquitination pathway has been proposed as a main mechanism responsible for degradation of the LC in the cytosol, thus terminating BoNT activity [68].

6.4. Three domains and four functions

Despite intense activity in recent years towards understanding the basic mechanism of action (MOA) of BoNTs, currently known structure-activity relationships of the four BoNT functions (binding, internalisation, translocation and SNARE cleavage at the nerve terminals of the neuromuscular junction) within three domains (Hc, HN, and LC) are not fully understood. Current gaps in basic understanding include molecular details of the specificity of the binding of each BoNT to neurons, entry into the nerve terminal and translocation of the LC, the correlation between SNARE cleavage and neuroparalysis and the length of BoNT-induced neuroparalysis. For example, it is known that the length of paralysis varies with BoNT type, dose, animal species and type of nerve terminal (from 3 to 4 months for skeletal nerve terminals to 12–15 months for autonomic cholinergic nerve terminals) [54, 69]. Furthermore, there are emerging functions that do not fall within the canonical intoxication pathway.

Regarding discrete functions of BoNT domains, there is increasing evidence that, in addition to their individual functions, each domain influences the other to work in concert to achieve BoNT intoxication. For example, the binding domain is not necessary for cell entry or LC translocation, but it determines the pH threshold for HN channel formation during the translocation step [70].

Entry of BoNTs has also been reported independent of synaptic vesicle recycling [71]; and retrograde transport within non-acidifying organelles, a characteristic of the related tetanus toxin, has been described for BoNT/A and BoNT/E [72]. Effects of BoNT in the central nervous system, such as in pain states, have also been reported, indicating actions beyond the neuromuscular junction that would involve retrograde transport of the toxin [73].

Furthermore, BoNTs, and in particular BoNT/A, are known to exert further actions unrelated to the cleavage of SNAP-25, at doses/concentrations that prevent SNAP-25-mediated neurotransmitter release. These activities include (1) increasing the proteosomal degradation of the protein RhoB in arachidonic-mediated neuroexocytosis, (2) induction of neuritogenesis, (3) reduction of cellular proliferation and (4) effects on gene expression, both in in vivo and in vitro settings [74]. The significance of these findings is not yet fully understood, but opens exciting opportunities to expand the use of BoNTs beyond their classical SNARE-cleaving MOA.

7. New therapeutics

7.1. Improvements on current products

The four FDA-approved formulations in the market for BoNT products are manufactured starting with the fermentation of the respective *C. botulinum* [1]. As a result, the manufacturing processes come with their own challenges, namely, (1) the anaerobic requirements mean that oxygen must be excluded from the first stages of the production system as the *C. botulinum* are obligate anaerobes, (2) the production of the toxin progresses from the first stages of growth, so health and safety measures are paramount throughout the manufacturing process, (3) sporulation of the bacteria can occur at low levels during the growth stages, but particularly when the bacterial life cycle ends and the bacteria die, and (4) the nutritional growth requirements of *C. botulinum* are not known in detail, which results in complex growth media adding extra degrees of complexity [75]. Recombinant production of BoNTs in non-obligate anaerobes and non-sporulating organisms, already widely used in the research setting (e.g. Ref. [76]), will simplify the manufacturing process enormously, as well as facilitate molecular engineering approaches that are state of the art in the protein field.

One aspect that is still contentious about the current BoNT drug products is the presence/absence of the ancillary non-toxic associated proteins (NAPs). In particular, it is not clear what role these proteins, which are critical to protect the toxin during entry through the gastrointestinal tract, are playing when the toxin is injected, as is the case for the current therapeutic and aesthetic uses. AbobotulinumtoxinA (Dysport®) and onabotulinumtoxinA (Botox®) present a complex of BoNT plus non-toxic associated proteins (NAPs), whereas incobotulinumtoxinA (Xeomin®) does not have NAPs present in its formulation [11]. RimabotulinumtoxinB (Myobloc®) is also a neurotoxin complex in which the BoNT is associated with hemagglutinin and non-hemagglutinin proteins [1].

Regarding distant spread, the FDA prompted an inclusion of a black box warning for all FDA-approved BoNT products, as follows: 'The effect of all botulinum toxin products may spread from the area of injection to produce symptoms consistent with botulinum toxin effects. These symptoms have reported hours to weeks after injection. Swallowing and breathing difficulties can be life-threatening and there have been reports of death' [1]. Ancillary proteins are not likely to play a role in distant spread since studies show that there were no differences in product diffusion when the same dose was injected with the same technique [15].

Triggering of immune responses by BoNT use, and possibly triggering non-responsiveness to treatment, is a controversial topic since, despite dissociation from the toxin NAPs, HA and NTNHA proteins form part of the protein load of the injection [77]. Following meta-analysis of clinical incidence of neutralising antibody immunogenicity is often revealed as a very minor issue with low, single-digit percent occurrence with the current main products [78]. Differences have been seen with an older product (which exhibited higher incidence of neutralising antibodies), dosing frequency and cumulative dose [79].

New products, produced using different manufacturing processes and with different final formulations, may help address the above issues and indeed as well for the existing natural products, which have not changed formulation or manufacturing process significantly in the last 20 years [75]. Alternative new products include Nabota® (Daewoong Pharmaceutical Co., Korea), which consists of BoNT/A obtained following a special purification process, and RT002 (Revance Therapeutics Inc., USA), which is an injectable formulation of BoNT/A containing a polycationic excipient developed to limit diffusion of the toxin into adjacent tissues and to be longer acting than the current BoNT products, amongst others [2, 9]. The use of hydrogels and liposomes, for example, in treatments for bladder or gastric disorders, has also been reported as novel BoNT formulations being investigated [80]. Liquid formulations for BoNT/A products, already in the market for BoNT/B, are actively being pursued, and their use would preclude the need of reconstitution of the products [75].

7.2. Molecular engineering of BoNTs

Given the natural diversity of BoNTs, with 7 serotypes and over 40 individual subtype proteins, it is surprising that the leading marketed products are restricted to only two serotypes, BoNT/A and BoNT/B. So far, anecdotal use of BoNT/C and BoNT/F was reported few years ago [81]. The current landscape of new therapeutics include, for example, the potential use of the short-acting BoNT/E1 as reported in WO2014068317 [82], the use of a BoNT/B toxin with increased binding affinity for its human cognate receptor synaptotagmin II as reported in WO2013180799 [83] as well as BoNT/A3 (WO2013049139 [84]). In particular, serotype BoNT/A2 has been extensively studied in Japan as an alternative BoNT/A with differentiated biology [85, 86].

Molecular engineering approaches facilitate the harnessing of inherent characteristics present in the already diverse natural BoNTs [87] but also allowing the introduction of new properties. When considering engineering approaches, all three BoNT domains offer exciting opportunities; for a recent review, see Ref. [5]. Firstly, engineering of the Hc domain could facilitate (1) alternative receptor targets to modify specificity, (2) allow immune epitope modification and (3) add/modify receptor-binding motifs and related structural regions to modify affinity. Secondly, engineering the HN domain could modulate cargo capacity and pH dependency of translocation of cargo. Finally, LC engineering could provide (1) substrate specificity, (2) desired intracellular localisation, (3) modification of immune epitopes and (4) modification/manipulation of self-proteolysis and degradative pathways.

An example of such engineering approaches is targeted secretion inhibitors (TSI), in which the Hc domain of BoNT is substituted by an alternative cellular targeting domain (e.g. see WO2006059093 [88]), which will be discussed in the next section.

7.3. Example of new therapeutics: targeted secretion inhibitors

Natural BoNT toxins target neuronal terminals, and their duration of action is often measured in months. These characteristics have made them very successful therapeutic and aesthetic agents (see Section 1 above), but it also limits their use to their specific target cells. Given that SNARE proteins underpin a universal mechanism of secretion in eukaryotic cells, an engineering approach that would lead to cleaved SNARE proteins in a wide range of (hypersecreting) cells would provide novel and exciting therapeutic opportunities. In TSI, the Hc-binding domain of BoNTs is substituted by an alternative cell-binding moiety, and the resulting proteins are not neurotoxins but a new class of biopharmaceuticals [89].

The basis for the TSI platform development is a functional fragment from BoNTs comprising the LC and HN domain, termed LHN. LHN proteins are proteolytically cleaved during activation, and the two domains remain connected by a disulphide bridge, as is the case in the parental BoNTs. LHN/A, LHN/B, LHN/C and LHN/D are amenable to recombinant expression in *E. coli* and have all been described as functionally active, resembling the respective parental toxin [90, 91]. Examples of TSI include those where the targeting domain is comprised of wheat germ agglutinin, nerve growth factor, an epidermal growth factor receptor (EGFR) targeting ligand or a growth hormone-releasing hormone receptor (GHRHR) targeting ligand. These TSI have shown that it is possible to achieve internalisation of the active BoNT LC contained in their structure into non-neuronal cells otherwise resistant to the parental BoNT [92–94].

The structures of LHN/D and a GHRHR-targeted TSI/D, SXN101959, are shown in **Figure 5**. When compared with BoNT structures depicted in **Figure 3**, it is seen that the BoNT Hc domain is absent in the LHN structure and, in the case of the TSI a new targeting moiety takes the place of Hc. Often, the new targeting moiety is considerably smaller than the original Hc domain of the parental BoNT. That poses its own challenges regarding ligand accessibility, and so linkers and spacers are frequently used. Furthermore, in the case of this GHRHR ligand, a free N-terminus of the peptide is required for optimal activation of the GHRHR receptor [95], which has prompted the position of the ligand to be at the N-terminal end of HN when compared to the Hc (located at the C-terminal of HN in the natural structure). Functionally, this TSI has been shown to exert a powerful and reversible inhibitory action on the endocrine growth hormone and insulin-like growth factor-I axis [96].

Little is known about TSI intracellular trafficking, and it is generally assumed that the BoNT four-step MOA (binding, internalisation, translocation and SNARE cleavage) will apply. A study using a GHRHR-targeted TSI/D reported an intracellular, punctate, immune-staining pattern indicative of the presence of the TSI in endosomes [97]. In a recent paper, internalisation of an EGFR-targeted TSI/A and BoNT/A was assessed in the same cellular system [98]. The EGFR-targeted TSI/A partially internalised in an intracellular compartment consistent with endosomes, whereas BoNT/A did so in a different compartment consistent with synaptic vesicle recycling. Both proteins were able to cleave the cytosolic SNARE protein target SNAP-25. The study confirmed that BoNT domains are a versatile tool to extend the pharmacological effect of BoNTs beyond the natural target of the neuromuscular junction.

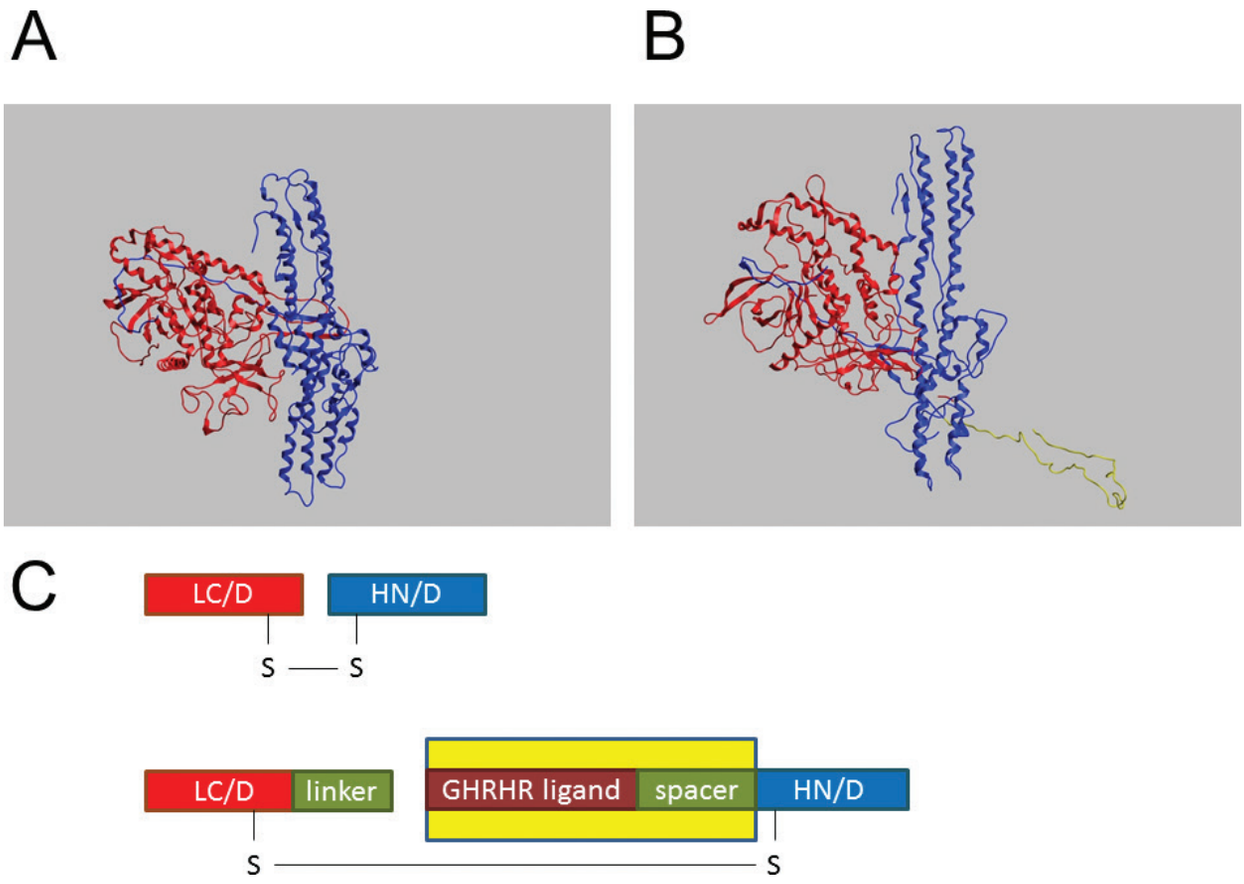

Figure 5. Structural and functional domains of LHN/D and a GHRHR-targeted TSI/D. (A) Crystallographic data of LHN/D (PDB 5BQN). (B) Crystallographic data for the GHRH-targeted TSI/D (PDB 5BQM) in which the targeting domain has been added using molecular modelling for illustration purposes. The ribbon to the right of the LC and HN domains corresponds to the GHRHR ligand plus the spacer, as illustrated in (C). (C) Simplified block diagrams of the structures presented in (A) and (B), respectively. The HN and LC domains in both structures can be seen being connected by a conserved disulphide bridge. Structural images created using the MOE software (Molecular Operating Environment 2013.08; Chemical Computing Group Inc., Montreal, Canada).

In addition to the delivery of SNARE cleaving activity into non-neuronal cells, TSI can also be used to provide alternative targeting to neurons with improved neuronal selectivity. One such example is neuronal targeting *via* the nociceptin receptor, which reached Phase II clinical trials for post-herpetic neuralgia and overactive bladder (WO2006059093) [88, 99].

8. Conclusions

BoNTs are key therapeutic agents with a seemingly ever-increasing list of new applications. The fascinating modular molecular architecture and natural diversity of BoNTs is the base for future therapeutics, being developed using recombinant technologies, new formulations and engineered new pharmacological properties.

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Appendices and nomenclatures

AAN	American Academy of Neurology
BoNT	Botulinum neurotoxin
CDC	Centres for Disease Control and Prevention, US
EGF	Epidermal growth factor
EMA	European Medicines Agency
FDA	Food and Drug Administration, US
GHRHR	Growth hormone-releasing hormone receptor
HA	Hemagglutinin
Hc	BoNT-binding domain
HN	BoNT translocation domain
LC	BoNT enzymatic domain
MOA	Mode of action
NAPs	Neurotoxin-associated proteins
NTNHA	Nontoxic non-hemagglutinin protein
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins
TSI	Targeted secretion inhibitors

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