Cell. Mol. Life Sci. 63 (2006) 540–551
1420-682X/06/050540-12
DOI 10.1007/s00018-005-5505-5

Cellular and Molecular Life Sciences

Review

Clostridial neurotoxins: structure-function led design of new therapeutics

J. A. Chaddock* and P. M. H. Marks

Health Protection Agency, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, Wiltshire SP4 0JG (United Kingdom), Fax: +44 (0)1980 612731, e-mail: john.chaddock@hpa.org.uk

Received 21 October 2005; received after revision 10 November 2005; accepted 16 November 2005
Online First 16 January 2006

Abstract. The neurotoxins produced by various species of *Clostridia* are the causative agents of botulism and tetanus. The ability of the toxins, specifically those of the botulinum neurotoxin family, to disrupt neurotransmission has been exploited for use in several medical indications and now represents the therapeutic option of choice in a number of cases. Clostridial neurotoxins have been discovered to have a multi-domain structure that is shared between the various proteins of the family, and it has also been determined that each domain contributes a specific role to the holotoxin. The extensive use of recombinant expression approaches, along with solution of multiple crystallographic structures of individual domains, has enabled researchers to explore structure-function relationships of the toxin domains more closely. These advances have facilitated a greater understanding of the potential use of individual domains for a wide variety of purposes, including the development of new therapeutics.

Key words. Botulinum toxin; recombinant; fusion protein; *E. coli*; pain; chronic obstructive pulmonary disorder; protein structure.

History of neurotoxins

From deadly food poison to a front-line medicine, the discovery, understanding and utilisation of clostridial neurotoxins is an intriguing story. Few cases of botulinum-derived food poisoning can be confirmed before the late 18th century, although there is little doubt that botulinum neurotoxins have been causing disease for thousands of years. The first well-documented cases of botulinum intoxication occurred in Württemberg during the Napoleonic wars between 1795 and 1813, where an increase in the cases of fatal food poisoning was linked to the consumption of blood sausages [1]. It was as a result of these 'sausage poisoning' outbreaks that Justinus Kerner published the first case studies of botulinum poisoning and accurately described all the neurological symptoms of botulism. In the final chapter of his 1822 monograph, Kerner also hypothesised about the potential for the use of the botulinum neurotoxin as a therapeutic agent, used in minimal doses to reduce or block the hyperactivity and hyperexcitability of the motor and autonomic nervous system [2]. The cause of botulism remained obscure for almost 70 years after the first description of the disease. It took until the last years of the 19th century to identify a toxin produced by an anaerobic bacterium (at that time known as *Bacillus botulinus*) as the causative agent, and to determine that this toxin could be neutralised by antibodies. Subsequent developments in understanding the associated microbiology determined that, whereas tetanus toxin (TeNT) is produced by a single clostridial species (*Clostridium tetani*), botulinum neurotoxin (BoNT) is produced by a range of species, including *Clostridium argentinese*, *Clostridium butyricum* and *Clostridium baratti* [3].

* Corresponding author.

Biomedical applications

The first documented use of BoNT for the treatment of disease was in the 1970s, about 150 years after Kerner's initial observations about the potential use of BoNT as a therapeutic. The initial development and assessment of BoNT for therapeutic purposes evolved out of a collaboration between Alan Scott and Edward Schantz. Alan Scott, an ophthalmologist, used BoNT injected into the extraocular muscle to block neurotransmission, thereby reducing muscle hyperactivity as an alternative to surgery for the treatment strabismus [4]. Edward Schantz, a biochemist working initially at Fort Detrick, and later at University of Wisconsin, developed the expression and purification process that was able to supply Dr. Scott with the purified toxin necessary for injection into patients [5]. From these initial findings a wide variety of treatments have been pioneered for alleviation of neuromuscular conditions resulting from involuntary muscle contraction, reviewed in [6–8].

It was during the treatment of one such debilitating neuromuscular condition, blepharospasm, that frown lines were observed to disappear after the application of BoNT. This observation sparked the explosion in usage of BoNT for cosmetic treatment, such that cosmetic use now makes up the largest market of BoNT treatments [9]. In yet another twist on the path from toxin to treatment, it was the usage of BoNT for the cosmetic treatment of facial wrinkles, which coincidentally resulted in a decrease in the frequency and intensity of headaches in migraine sufferers, that has opened up another avenue of potential exploitation for the toxin [10]. Though in its relative infancy compared with other treatment types, research into the efficacy of BoNT in reducing pain in a variety of different applications is now ongoing [11]. Along with the application in pain alleviation, BoNTs have also been found to act not only at the cholinergic neurones at the neuromuscular junctions but also on acetylcholine-releasing neurons in the autonomic nervous system, thereby facilitating treatment of some hypersecretory diseases and smooth muscle conditions [7, 8]. A summary of the key indications for which regulatory approval has been provided for the use of BoNT is provided in table 1, but it should be stressed that the number of neuromuscular indications for which BoNT has a potential use is far greater. It should also be noted that, possibly contrary to an individual's initial interpretation when considering therapeutic use of nature's most potent toxin, adverse events with BoNT is very low [12].

Given the ever increasing number of licensed uses for the BoNT, and also the increasing number of licensed botulinum toxin products on the market, it is clear that toxin-based treatments are a valuable source of patient care and will be successful for years to come. But will the story of exploitation of the neurotoxins be limited by the properties of the holotoxin, or are there further opportunities that currently lie unexplored? Within the remainder of this review, a number of such opportunities will be described that are likely to succeed because of the excellent protein framework that nature has provided within the toxins. To begin the process of understanding how domain fragments of clostridial neurotoxins (CNTs) can be utilised in the design of new therapeutics, it is necessary to introduce the basic CNT structure.

Table 1. Commercially available botulinum toxin products with approved indications.

Product	Serotype	Approved[1] indications
BOTOX®	BoNT/A	strabismus, blepharospasm, cervical dystonia, severe primary axillary hyperhidrosis
BOTOX® Cosmetic	BoNT/A	glabellar lines
Dysport®	BoNT/A	spasmodic torticollis, blepharospasm, hemifacial spasm, focal spasticity in paediatric cerebral palsy patients
Myobloc®	BoNT/B	cervical dystonia

[1]Products are approved for use in specific territories. This table does not indicate that a specific product is approved for use worldwide.

Clostridial neurotoxin structure

It is important to acknowledge that the structural organisation of these potent toxins has been the result of millions of years of evolution, during which time the relationship between structure and function has been developed to the extent that the final product is considered to be the most potent natural toxin known to man. As mentioned earlier, BoNTs are produced by a number of species of *Clostridia*, and these BoNT products have been classified into seven families based on their reactivity to antisera. The seven serotypically different BoNTs are labelled as BoNT/A through to BoNT/G and, along with TeNT, constitute the eight members of the CNT group. Given their range of origins, it is not surprising that CNTs exhibit a range of biological activities, the details of which will be discussed later in this review. Briefly at this point, CNTs block neurotransmission by specific proteolysis of components of the vesicular fusion machinery, with BoNT/A, BoNT/C and BoNT/E having a different protein target from BoNT/B, BoNT/D, BoNT/F and BoNT/G. Proteolysis can lead to inhibition of neurotransmission for many days (in the case of BoNT/E) to many months (in the case of BoNT/A), and it is this extended duration of effect that has driven the adoption of BoNTs as a treatment of choice for multiple therapies.

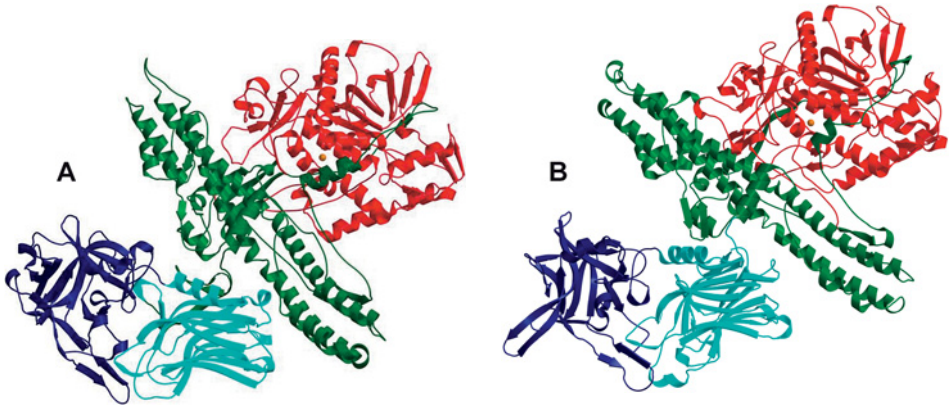

Figure 1. Tertiary structure of BoNT/A and BoNT/B. BoNT/A (3bta) and BoNT/B (1EPW) tertiary structural information prepared using Molscript [99]. The LC (red), H_N (green), H_{CN} (light blue) and H_{CC} (dark blue) domains are highlighted of BoNT/A (*A*: left image) and BoNT/B (*B*: right image).

Despite the range of targets and the range of biological effects, CNTs have many common structural elements. CNTs are synthesised as single-chain polypeptides of ~150 kDa and are subsequently cleaved to form di-chain molecules, in which the light (LC) and heavy chains (HC) are linked by a single disulphide bond (see fig. 1). The 50-kDa LC acts as a zinc-dependent endopeptidase. The heavy chain contains two functional domains, each of ~50 kDa. The N-terminal half (H_N) is the translocation domain, known to form ion channels in lipid bilayers, and the C-terminal half (H_C) is the ganglioside binding domain, which has a key role in binding to the target cell membrane and subsequent internalisation of the toxin molecules into cholinergic neurons. The H_C binding domain consists of two subdomains, H_{CN} and H_{CC}, the latter domain containing key residues responsible for the binding activity of the neurotoxins [13]. Though dissected in terms of multi-domain organisation, CNTs are representative of the classical A-B type toxin structure, in which the holotoxin is the result of an association of two individual protein domains, each of which in itself is not toxic The three functional CNT domains are structurally distinct and arranged in a linear fashion, such that there is no contact between the LC and H_C domains. This is an important point from the protein engineering aspect, since it facilitates manipulation of the domains at the extremities (i. e. the LC or the H_C) with a predicted minimal negative effect on the remaining H_N partner. Overall, BoNTs and TeNT share ~35% sequence identity [14]. The BoNT catalytic LC domains share up to 36% sequence identity [14], and the LC domains of BoNT/B and TeNT have over 50% identity [15]. As often the case with a protein family, the primary sequence similarity is nominal when compared with the similarity in tertiary structure. Figure 1 illustrates this by side-by-side comparison of the tertiary structures of BoNT/A and BoNT/B. The gross structural features of the two neurotoxins are clearly very similar; only isolated significant differences are observed such as the orientation of the H_N region that encircles the LC and the H_{CC} domain [16].

Before publication of the BoNT/A structure [17], structural analysis of the neurotoxins had been achieved by a variety of biochemical and genetic analysis approaches and much of the domain organisation had been predicted and discussed [18, 19], such that the functional roles of LC, H_N and H_C were essentially understood. Once the crystal structure had been identified, the domain organisation of the neurotoxin could be visualised, and the potential for rational utilisation of individual domains was clear. In the following seven years since the report of the BoNT/A structure, only the equivalent holotoxin structure of BoNT/B has been solved, though the majority of the activity in the structural biology field has been to examine the structures of the various domains in isolation (table 2).

Analysis of the various structural information has, among other aspects, facilitated analysis of structural interactions with the soluble NSF accessory protein receptor (SNARE) substrate [20], and proposed that the role of the active site zinc is predominantly catalytic rather than

Table 2. Selected tertiary structure information.

Toxin	Domain	PDB code	Recombinant	Reference
BoNT/A	whole toxin	3BTA	no	[17]
BoNT/B	whole toxin	1EPW	no	[16]
BoNT/A	LC	1XTG	yes	[20]
BoNT/A	LC	1E1H	yes	[91]
BoNT/B	LC	1F82	yes	[92]
BoNT/B	H_C	1Z0H	yes	[93]
BoNT/E	LC	1T3A	yes	[94]
BoNT/F	LC	2A8A	yes	[95]
BoNT/G	LC	1ZB9	yes	[49]
TeNT	LC	1YVG	yes	[96]
TeNT	HC	1AF9	yes	[97]
TeNT	H_C	1D0H	yes	[98]

structural [21]. The overall LC structures are very similar between the different serotypes, probably reflecting their common function: each must bind and hydrolyse a specific protein target. Structural information has suggested conformational variability in a number of surface loops that are proposed to play a role in substrate binding. In addition to tertiary sequence information, investigation of the LCs at the amino acid level has led to a suggestion of plasma membrane localisation and trafficking sequences being present at the N- and C-terminus of the protein, respectively [22].

Opportunities that emerge from the domain structure

Whilst detailed understanding of the mechanism of action of the neurotoxin has emerged from the advances made in understanding the structural relationships, an exciting consequence of this enhanced knowledge is the realisation that the domains may have sufficient independent functionality to be exploited individually. In essence, the three major activities of the neurotoxins, cell binding and internalisation, protein translocation and enzymatic modification of an intracellular substrate, are all provided by independent protein domains; the H_C, H_N and LC, respectively. The biological activities of these individual domains, and the theoretical opportunities for exploitation, will now be discussed in greater detail.

The LC domain as a warhead

CNT light chains are globular 50-kDa proteases with a mixture of both α-helix and β-strand secondary structure. The active sites tend to be buried deep in the protein accessible by a channel, and full LC activity is only achieved following reduction of the single disulphide bond that covalently attaches the LC to the H_N domain. In addition, LCs are one of the most specific classes of proteases so far discovered. Though all the LCs share the HExxH active site motif that is characteristic of zinc proteases such as thermolysin, the LCs have evolved to only cleave specific proteins of the SNARE complex. The level of specificity of the proteolysis is even more remarkable in that each CNT, with the exception of BoNT/C_1, cleaves just one of the SNARE proteins at a single peptide bond, and that the precise site of cleavage is specific to the particular neurotoxin. BoNT/C_1 is unique amongst the CNTs in cleaving a single peptide bond in each of two substrate proteins. By selective proteolytic cleavage of one of the three SNARE proteins (syntaxin, SNAP-25 or synaptobrevin) that form this complex in neuronal cells, CNTs prevent formation of a functional SNARE complex and so prevent neurosecretion [19]. Given that the SNARE complex represents a universal mechanism for vesicle fusion and secretion in eukaryotic cells [23], the endopeptidase activity of CNTs is potentially capable of SNARE protein cleavage and inhibition of vesicle fusion and secretion in a wide range of cell types, not just neuronal cells. Since the neuronal selectivity of clostridial endopeptidase results from specificity of the binding domain of the neurotoxin, the potential applications of the endopeptidase activity are not limited to neuronal cells if a suitable delivery method is established. Recently reviewed by Breidenbach and Brunger [24], the interaction between the LC and its substrate has become the subject of intense scrutiny. The crystal structures of several LCs have been determined, both in the presence and absence of substrate. At time of manuscript preparation, structural information for the isolated LC domains of serotypes A, B, E, F and G was available (table 2). Not intended to be an exhaustive list of the crystal structures that have been deposited, rather table 2 illustrates the progress that has been made in recent years to isolate structural information for the CNT domains. The advent of LC structural information in the presence of substrate has been instrumental in providing practical evidence for the presence of an array of substrate-binding sites remote from the active site (so-called exosites). In fact, rather than specific active site architecture, it is this multi-site binding approach that accounts for the selectivity of the CNTs, and the reason why enzymes with highly homologous active site regions, for example thermolysin, do not exhibit the ability to cleave SNARE proteins with any degree of specificity. The combination of a specific proteolytic activity and a substrate that has a key role in secretion and membrane protein insertion leads the LC to be considered as a valuable resource for utilisation in the development of novel agents to modulate a variety of conditions.

The H_N domain as a translocator

The N-terminal domain of the heavy chain is a fascinating polypeptide. A cylindrical shaped domain dominated by a pair of long α-helices, the H_N is structurally distinct from other pore-forming toxins. Designed to facilitate delivery of the LC from the endocytosed vesicle into the cytosol in order that the LC can access the SNARE substrate, the H_N domain is required to perform a complex task. Recent evidence from pH-induced conformational change studies would indicate that the toxin family has maintained key H_N residues to ensure that the conformational transition occurs within a narrow pH range [25]. It is fair to say that the translocation step is the least understood of the three key neurotoxin functionalities of binding, translocation and substrate cleavage. Even so, data have emerged that do help understand the potential, and the limitations, of the H_N domain as a translocator. The H_N domain is undoubtedly effective at transportation of

the LC, and may well play a chaperone-like role in this process. Evidence from recent BoNT/D work suggests that the H_N domain may also be able to translocate a limited population of non-clostridial proteins across the endosomal membrane. What is not clear at present is how widely applicable the H_N translocation function is, since structural restraints of the cargo protein are likely a significant limiting factor [26]. In addition to the paired α-helices, the H_N domain also consists of an unstructured polypeptide that wraps around the LC to partially shield the active site channel. This polypeptide, termed the belt, is an integral part of the H_N domain and raises engineering issues similar to the LC when one considers opportunities for utilisation of the individual domains for the creation of novel entities. Of major interest, however, is the observation that the H_N domain can function in the absence of the H_C domain, most significantly in the utilisation of the LH_N fragment (the heterodimer that results from association of the LC and the H_N) [27].

The H_C binding domain

In contrast to the H_N domain, for which little is known, much has been discovered about the structure-function relationships within the C-terminal heavy chain domain. Appearing as two distinct sub-domains of roughly equal size, the H_C domain consists predominantly of β-strands with a prominent α-helix to join the sub-domains together. Using a combination of site-specific mutagenesis and structural analysis, the sites for cell binding are now known to be located in the C-terminal sub-domain (H_{CC}). The role of the N-terminal domain, H_{CN}, remains unknown. As the H_C domain has been demonstrated to bind to target cells in isolation from the H_N domain [28], H_C has obvious applicability as a partner for targeting materials to the neuromuscular junction for the treatment of certain neurological diseases. Such materials could be of therapeutic or basic scientific value. In addition, as the precise nature of the binding domain-cell receptor interactions becomes understood in more detail, there is an engineering opportunity for manipulation of the H_C that could provide new selectivities of target.

Isolation of clostridial neurotoxin domains and fragments

Simultaneous with the emerging structural understanding have been advancements in isolation techniques that have supplied individual domains or neurotoxin fragments for analysis. It is understandable that for the development of therapeutics other than using BoNT holotoxin, the component parts of the toxin need to be purified. However, production of the active holotoxin is problematic due to the working practises required to handle a material with such intrinsic toxicity, and this naturally limits the number of organisations that can work with this protein. Production of the component parts, LC, H_N and H_C, can be achieved by limited proteolysis of the holotoxin and subsequent purification of the desired domains. However, this approach requires significant quantities of holotoxin as raw material and is complicated by the inevitable difficulty in removing all remaining holotoxin from the final product. By way of example, the LH_N of BoNT/A was purified from the full-length BoNT [29] using classical techniques and was of suitable purity to explore the functionality of the H_N domain. However, to enable utilisation of this fragment for assessment of cell entry properties using sensitive *in vitro* cell culture assays, it was necessary to employ immunoaffinity techniques to eliminate the residual BoNT [30]. Though ultimately successful, such techniques are time consuming, costly and utilise significant amounts of holotoxin to achieve the final product. Due the problems of native BoNT fragment purification and the limitations of gene manipulation within *Clostridia*, any development of therapeutics based on BoNT fragments will almost certainly require recombinant production of the individual domains or fragments of the holotoxin in heterologous hosts.

Advancements in understanding the toxin structure have been valuable to assist in the design process for recombinant expression fragments, and as a result, over the last 5 or so years, there has been a wide range of recombinant clostridial fragments expressed and purified. These have typically been isolated for use as tools for deciphering the mechanisms of membrane exocytosis and prevention of cellular release or to investigate the structure and function of the neurotoxins as a means to further understand their therapeutic potential. There is also a significant body of work on the production of recombinant fragments, particularly of the binding domain, to produce non-toxic fragments for use in the development of new vaccines to protect against the effects of neurotoxin exposure.

Expression of clostridial genes in heterologous hosts: impact of codon usage

The first important lesson to be learnt for the work on the recombinant expression of clostridial fragments in simple bacterial hosts such as *Escherichia coli* is that expression can often be restricted by differences in codon usage between the species. Clostridial DNA tends to be rich in A+T nucleotides, whereas a typical *E. coli* expression host exhibits a slight G+C bias. Therefore, one of the issues highlighted by the desire to express recombinant clostridial fragments is the source of the DNA: should the DNA be polymerase chain reaction (PCR) amplified from a clostridial source or synthesised with a host-specific codon bias? Or are there expression host modifications that can be performed to enhance expression from DNA

with an alternative codon bias? All approaches have been taken. By using host stains which have been supplemented with transfer RNAs (tRNAs) for ATA, AGA and CTA codons that are frequently utilised in clostridial genes but are rarely used in *E. coli*, BoNT and BoNT fragment expression levels have been increased. However, because other genes with similar rare codon usage have shown little improved expression with tRNA supplementation, it may not be just the use of rare codons that impact on the translation efficiency but also their location within the gene [31, 32]. Synthetic genes with a codon distribution optimised for *E. coli* can also improve expression levels when compared with the native gene, but this does not lead to enhanced expression in all cases [personal observations]. These conflicting data would suggest that the codon usage problems can be very domain and serotype specific [31] and could possibly be influenced by other factors such as expression conditions and the nature of the protein being expressed. The current understanding of gene design for optimal protein production is still quite limited, and as such it is difficult to draw conclusions about the requirements for functional protein production. However, it is clearly an important factor in the expression of individual domains and potential new therapeutics.

Recombinant expression of fragments containing the protease function

The largest body of work on the expression and purification of recombinant fragments containing the enzyme activity has been carried out on the A serotype, and a wide range of LC/A fragments have been purified from the soluble fraction and using refolding techniques [33–43]. From these various reports, it is apparent that the resulting LC/A proteins differ considerably in stability and retained protease activity. These variations can be explained to some degree by the differences in LC length and gene codon bias, but even with comparable genes the result can vary from laboratory to laboratory. This suggests that the folding of the LC is sensitive to slight variations. Recent reports implicate the C-terminus of the LC as contributing to solubility and stability issues [35]. Soluble expression of LC/B [44], LC/C [33, 45, 46], LC/E [33, 45, 47, 48] and LC/G [49] have also been reported. In the case of LC/E, low temperature was necessary for soluble expression, and a variety of protease inhibitors, low levels of detergents and also iodoacetamide were needed in the process [47, 48]. The composition of the lysis buffer seems to be an important variable which affected both the yield and functionality of the final protein. It should also be noted that the LC/A has been expressed in a transient transfected cell line to study insulin release [50] and to demonstrate a method for the production of a stably transfected cell line for extended studies on synaptosome-related exocytosis [51].

The only report of recombinant expression of the H_N region of any serotype was achieved with H_N/A [52] and used a refolding approach to isolate the protein. This single case of poor recombinant H_N solubility and the many reports of sub-optimal LC solubility would suggest that these domains as individual components are lacking important interactions/associations required to produce stable proteins. Perhaps this is not unexpected when one considers that, in addition to the LC:H_N interface, the contacts made within the 50+ amino acids of the H_N 'belt' region would not occur when the domains were expressed individually. However, once the H_N is fused to its LC partner (the 'LH_N' fragment), soluble expression and purification become possible and the stability seem to be greatly improved. Purification of recombinant LH_N fragments of A, B and C serotypes have been reported [30, 41, 53]. Though advantageous in terms of stability of the protein, expression of the LC + H_N heterodimer is necessarily achieved as a single polypeptide expression product that requires subsequent treatment with protease to facilitate release and 'activation' of the LC. This was initially achieved using the method for purification of the LH_N/A from the native BoNT/A [29, 30] in which the recombinant LH_N/A was activated by digestion with trypsin [30, 41]. This method was improved by engineering a protease cleavage site between the LC and H_N domains allowing specific activation without the protein losses seen with tryptic digestion and improved activity compared with the trypsin-treated samples [53]. Incorporation of a specific cleavage site into the LC-H_N junction has also been used during the preparation of LH_N/B, which is of particular significance because trypsin treatment for the production of the LH_N from native BoNT/B or for the activation of a recombinant LH_N/B did not result in a stable fragment. A specific proteolytic activatable LH_N/C [53] has also been expressed in a soluble form. Interestingly, the sequence within the native activation region between the LC and H_N domains of the C serotype contains the recognition site for cleavage by Factor Xa protease, which was efficient for activation. Thus, no activation region engineering was required for specifically activating the LH_N/C.

Recombinant expression of fragments containing the binding function

Much of the recombinant expression effort expended in pursuit of the H_C and heavy chain fragments has been directed at preparation of material that is suitable for use in a vaccine. Bacterial expression of the H_C/A domain has typically been problematic, with mostly insoluble protein and low expression levels [31, 54, 55]. However, because of the promising immunogenicity of the H_C domain, recombinant expression was transferred into *Pichia pastoris*, a yeast system which showed significant improve-

ments in solubility and expression levels [56] and has been produced at pilot scale [57, 58]. The H_{CC} carboxyl terminal region of A and B has also been purified [28], but little data on the stability and yields were reported.

Expression of the H_C domain has also been achieved for serotypes B, C, D, E and F. Similar solubility and expression issues as seen with the A serotype were seen with the B, leading to a transfer of the H_C/B into *P. pastoris* also [56, 59]. H_C/C and /D have been purified from the insoluble fraction with low yields [60] and less than satisfactory recovery of binding activity, suggestive of incorrect refolding [61], whereas soluble H_C domain can be produced by low-temperature expression [31]. Recent reports of the scale-up of the H_C/E expression and purification process have confirmed the successes of using *P. pastoris* as the expression host [62]. The H_C/F has been expressed in both bacterial [63] and yeast [64, 65] hosts, which provides an interesting comparison between the two systems for the expression of the H_C domain. The *E. coli* expressed H_C was insoluble with a GST tag but soluble with an MBP tag, although the H_C was highly unstable and the yields were low [63]. The same synthetic gene was used for yeast expression but failed before it was re-engineered for expression in *P. pastoris*. H_C/F purified from both the soluble and insoluble fractions resulted in a functional antigen [64] and has also been produced at pilot scale [65].

Expression of the complete heavy chain has been achieved for HC/B [66]. Little soluble expression was achieved, though the heavy chain purified by refolding showed receptor binding equal to that of the BoNT control. HC/C and /D have been expressed in the soluble fraction and have been partially purified from bacterial cultures [61].

Development of CNT domain conjugates

The enzymatic activity of the LC domain has exciting potential for use in SNARE protein modification, which in turn leads to modulation of cellular events that depend on vesicle-plasma membrane fusion. However, unless the LC can be introduced by an artificial delivery mechanism directly into the cytosol, there is no structural information inherent to the LC to effect translocation across an internal membrane. To combine the SNARE modification function and the membrane translocation function, it is necessary to use both the LC and H_N domains which, though retaining 65% of the amino acid content of the parent neurotoxin, is effectively non-toxic because it lacks the necessary H_C domain with which to bind to acceptors on the neuronal surface. First identified following proteolytic breakdown of TeNT [67], the LH_N heterodimer consists of the LC and the H_N domain functionalities coupled together by a single disulphide bond. Possessing both the exquisite proteolytic activity of the LC, and the inherent cell membrane transporting function of the H_N, the LH_N has much potential for the design of new therapeutic molecules.

To provide LH_N with a cell-binding activity, a variety of cell-binding ligands have been chemically coupled using hetero-bifunctional coupling agents such as SPDP. Though coupling of one protein to another for the design of novel therapeutics has been commonplace in the anti-cancer field [68], one of the initial demonstrations that this was achievable with fragments of botulinum neurotoxin was achieved in 1984. In this study, the LH_N of TeNT was coupled to either ricin toxin B-chain or wheat germ agglutinin [69]. Subsequently, the majority of this chemical coupling work has been achieved with the LH_N/A. By coupling alternative protein domains to the LH_N/A fragment, the LH_N/A endopeptidase has been retargeted into a range of neuronal and neural-crest derived cells, where it inhibited secretion via cleavage of its substrate SNARE protein, SNAP-25 [27, 70]. These *in vitro* data support the concept of retargeting LH_N to a cell of interest and achieving inhibition of stimulated secretion. These data also supported the hypothesis that the functional activity within the domain structures of the LC and the H_N would be effective when delivered to a cellular environment outside of the motorneuron. Though it was anticipated that the LC activity would be retained in a variety of cellular models (since, ultimately the LC is 'merely' a metalloprotease that happens to be derived from *C. botulinum*), the activity of the H_N was less predictable, in particular its ability to effectively translocate the LC in an intracellular membrane environment that was not derived from the motorneuron.

Demonstration of retargeted LH_N/A efficacy in a range of cells motivated the search for *in vivo* proof-of-principle in a condition that would benefit from inhibition of secretion by a clostridial endopeptidase, particularly chronic conditions that would be most suited to treatment with an agent that had an extended duration of action. The initial condition that met these criteria was chronic pain, with the primary nociceptive afferent as the cellular target for development of a retargeted protein. Galactose-containing carbohydrates are selectively present on nociceptive afferents in the central and peripheral nervous system relative to other neurons, and lectins from *Erythrina* species have been identified to bind such galactose-containing carbohydrates [71, 72]. Therefore, a chemical conjugate of *Erythrina cristagalli* lectin (ECL) and LH_N/A, ECL-LH_N/A, was created using chemical conjugation approaches. This conjugate was demonstrated to inhibit the release of both substance P and glutamate from embryonic dorsal root ganglion (DRG) neurons in culture, but it was relatively ineffective at inhibiting release from a control cell population of *in vitro* cultured embryonic spinal cord neurons [73]. Coupled with the observation

that the inhibition of substance P release from DRG neurons was maintained for at least 25 days, the ECL-LH_N/A conjugate provided *in vitro* proof-of-principle for the retargeting concept in an *in vitro* model that is relevant to pain. The properties of this conjugate were subsequently tested in an electrophysiology model [73] and *in vivo* analgesia models [74] with the result that clinically relevant analgesia was observed over extended periods. Thus, *in vivo* proof-of-principle was achieved with the ECL-LH_N/A conjugate, and the generalised concept of targeting LH_N-endopeptidase to selected cells to inhibit exocytosis was proven.

Though highly successful in establishing the potential for retargeting the functional domains of the LC and H_N, this work highlighted some of the drawbacks to the approach when considering development of materials into a pharmaceutical product. First, there is the source of the endopeptidase and cell binding domains. Both of these materials were initially sourced from native material, which can be inconvenient in terms of availability (in the case of BoNT) or cost, but processes were subsequently developed to obtain each component from a recombinant source [30, 75]. Second, there is the chemical coupling process, which leads to an inevitable inherent heterogeneity of the species that are produced. The activity of the heterogenous product is likely to represent an underestimate of the true activity achievable with a homogeneous material, and additionally is challenging to progress through validation. In some cases it has been possible to establish protein purification techniques that can preferentially isolate mono-derivatised components which can then be used in the conjugation [76]. However, fully recombinant expression approaches are the preferred route for development of a therapeutic protein. The requirement is, therefore, to develop a fully recombinant chimera protein incorporating the translocation and endopeptidase domains of a CNT and a targeting ligand. Again, comparisons can be drawn with the immunotoxins field, where techniques have been developed for the expression, refolding and purification of a variety of anticancer hybrid agents based on the catalytic domain of *Pseudomonas* exotoxin or diphtheria toxin [77]. Given the size and complexity of an LH_N-ligand hybrid fusion protein, this is a challenging task, but has recently been achieved by the creation of a fully recombinant fusion of the LH_N fragment of BoNT/C_1 and epidermal growth factor (EGF) [78]. To create the hybrid fusion protein, a gene encoding EGF was inserted to the C-terminus of the H_N domain in the location usually occupied by the H_C. Clearly, EGF (~6 kDa) and the H_C domain (~50 kDa) are very different in terms of mass and structure, but the EGF was sufficiently tolerated by the LH_N/C structure to ensure soluble protein could be isolated from *E. coli*. One important observation from the creation of a successful fusion protein is the confirmation that the H_N domain structure is not limited to interactions with the H_C and will fold satisfactorily in the fusion protein context.

There have been a number of other BoNT hybrid molecules developed as research tools for neuronal cell targeting and as delivery molecules for potential therapeutic moieties. In one study the C-terminal fragments of the BoNT A and B serotypes were engineered for either chemical or biotin conjugation with molecules for neuronal targeting and expressed in *E. coli*. Unfortunately, in contrast to the soluble hybrid fusions created with the LH_N fragment, problems were encountered with the refolding of the BoNT fragments [79]. Another study demonstrated that a full-length BoNT/D enabled cytosolic delivery of a limited set of cargo proteins into neurons [26]. Of particular interest was the delivery of a functional LC/A which could provide an alternative method for the delivery of the long-acting A and B serotypes but without the highly immunogenic portion bypassing the immuno response which some patients develop [26]. The H_C domain has also been demonstrated to transcytose across human epithelial cells [80], thereby opening up the possibility of delivery of cargo proteins across epithelial cells.

Opportunities for therapeutics development based on fragments

There are multiple opportunities for the development of therapeutics based on clostridial neurotoxin fragments which, broadly speaking, fall into one of four main categories (fig. 2). First, there is the ability to use the fragments as vaccine components. Second, there is the modification of intracellular processes by delivery of materials into the cell using the neurotoxin (or binding domain fragment) as a transporter. The possibilities for cellular modification are many, with the main limitation being the focussed target cell population of the binding domain. Third, there is the opportunity to manipulate how the cell interacts with the extracellular environment by prevention of membrane channel/receptor/transporter insertion using a specifically targeted LC. Finally, there is the opportunity to use the retargeted LC domain as a method of inhibition of secretion for extended periods from a specific cell population.

Considering the opportunities surrounding development of clostridial fragment vaccine components, the possibilities for utilisation of the toxin subunits has been discussed [81–83]. Most extensively based on expression of H_C binding domains, a number of vaccine candidates have been developed with a view to replacement of the current pentavalent vaccine that protects against types A–E. Recently, it has also been proposed that the LH_N fragment is worthy of exploration as a complementary strategy to the H_C program, and early data have supported the potential efficacy of this approach [30, 41].

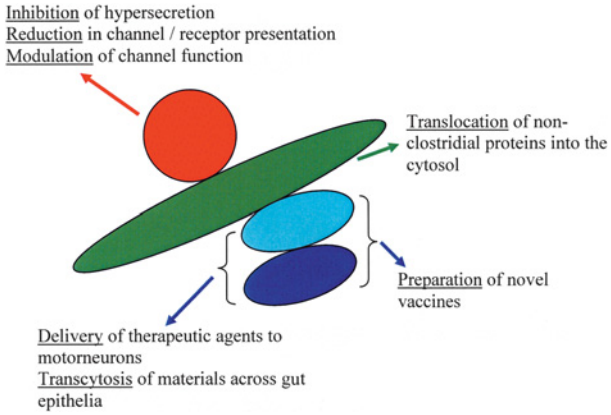

Figure 2. Opportunities for exploitation of clostridial neurotoxin domains. The LC (red), H_N (green), H_{CN} (light blue) and H_{CC} (dark blue) domains of clostridial neurotoxins are illustrated.

Considering the second opportunity category, a range of therapeutics can be envisaged that are focussed on delivery of materials to the motorneuron. The self-contained organisation of the heavy chain, and the opportunity for site-specific coupling of materials through the single free cysteine, are attractive attributes when considering the development of delivery vehicles for therapeutics materials. However, the reconstitution approaches that are necessary to couple reduced heavy chain and other materials are traditionally of low efficiency. The experience gained with recombinant domain expression has facilitated the creation of binding domain hybrids, for example in the creation of a tetanus toxin H_C hybrid with the free-radical detoxifying enzyme superoxide dismutase (SOD) [84]. Though of undoubted potential utility in the delivery of therapeutic materials to damaged/diseased motorneurons, it is not a given that all materials can be transported into the correct intracellular compartment for warhead function. For example, the delivery of human survival motor neuron (SMN) protein into motor neurons by an engineered delivery vehicle consisting of the binding domain of tetanus toxin and the catalytic and translocation domains of diphtheria toxin was ineffective [85]. In addition to delivery of therapeutic entities, the binding domain may also have utility for delivery of botulinum toxin antagonists. Using the simple but extremely valid logic that botulinum toxin inhibitors, for example those that interfere with the endopeptidase, need to be delivered into the same cells that are intoxicated, successful delivery of a small molecule carrier protein into the cytosol of a botulinum toxin-sensitive cell has been reported [86].

In terms of the third opportunity category, manipulation of cellular function by modulation of membrane protein insertion, it is well known that the endopeptidase activity of the clostridial toxins can inhibit the presentation of membrane proteins and that this can lead to modification of cellular function. For example, stimulation of DRG neuron cultures *in vitro* results in increased expression of the vanilloid receptor-1 (TRPV1) at the neuronal surface, and this increase in TRPV1 expression is blocked by BoNT/A [87]. Such a blockade might represent a mechanism by which the toxin could reduce inflammatory pain, particularly the development of hyperalgesia. In addition, BoNT/B has been demonstrated to inhibit insulin-stimulated glucose uptake into 3T3-L1 adipocytes by causing a reduction in the presentation of the glucose transporter-isotype 4 (GLUT4) transmembrane protein [88]. BoNTs have also been shown to have an impact on channel function by affecting associations between SNARE proteins and transmembrane channels. For example, BoNT/C_1 has been reported to affect the regulation of, among others, pre-synaptic calcium channels [89] and delayed rectifier potassium channels [90]. Such observations pave the way for assessment of therapeutic opportunities across a wide range of diseases.

Already discussed in terms of a novel treatment for chronic pain, the fourth opportunity for development of therapeutics based on clostridial toxin endopeptidase fragments has potential applicability to a wide range of conditions that have a secretory component. *In vitro* proof of principle for inhibition of secretion following exposure to a retargeted endopeptidase has been obtained in both neuronal and non-neuronal cells, and also for a range of vesicular contents. In the case of agents for the treatment of chronic pain, the retargeting concept has also been proved *in vivo*, thereby establishing that this approach has real potential for the development of novel therapeutics. In addition to chronic pain, a range of conditions can be identified that would potentially benefit from modulation of cellular secretion pathways. For example, chronic obstructive pulmonary disorder, diabetes, inflammatory and immune disorders may all have a hypersecretory component that could be decreased or eliminated by retargeted LC action. Though secretory events contribute to a wide range of conditions, it will be chronic conditions that will initially be best suited to treatment with such agents, since it will be advantageous to use the extended duration of action of the endopeptidase action to modify cellular events over extended periods of time.

Conclusions

Clostridial neurotoxins, specifically botulinum neurotoxins, are effective therapeutics for a range of conditions. Though the advances made in understanding the structure-function relationships have had little impact on the current use of BoNT as a medicine, the potential for exploitation of this information in the design of novel clostridial-fragment based medicines is great. Understanding of the functionalities of the individual domains may be used to develop medicines and research tools that can affect a multitude of cellular functions. Supporting

this new level of understanding is the development of recombinant methods for the expression and purification of active sub-domains, a process that will also facilitate domain modification through the application of protein engineering techniques. This report has highlighted a number of the possibilities for utilisation of neurotoxin domains; the development of more efficacious vaccines, delivery of therapeutic agents to the motor neuron and modulation of intracellular processes in specific cells. The range of therapeutic indications for which the neurotoxin is considered to be suitable is already wide; the range of situations for which hybrid, toxin domain-based therapeutics will be suitable is vast.

Acknowledgements. The authors wish to thank Nethaji Thiyagarajan for provision of figure 1.

1 Erbguth F. J. and Naumann M. (1999) Historical aspects of botulinum toxin: Justinus Kerner (1786–1862) and the 'sausage poison'. Neurology **53:** 1850–1853
2 Erbguth F. J. (2004). Historical notes on botulism, Clostridium botulinum, botulinum toxin and the idea of the therapeutic use of the toxin. Mov. Disord. **19 Suppl 8:** S2–S6
3 Henderson I., Davis T., Elmore M. and Minton N. P. (1997). The genetic basis of toxin production in *Clostridium botulinum* and *Clostridium tetani*. In: The *Clostridia*: Molecular Biology and Pathogenesis, pp. 261–294, I. Rood (ed.), Academic Press, New York
4 Ting P. T. and Freiman A. (2004). The story of Clostridium botulinum: from food poisoning to Botox. Clin. Med. **4:** 258–261
5 Schantz E. J. and Johnson E. A. (1997). Botulinum toxin: the story of its development for the treatment of human disease. Perspect. Biol. Med. **40:** 317–327
6 Mahant N., Clouston P. D. and Lorentz I. T. (2000). The current use of botulinum toxin. J. Clin. Neurosci. **7:** 389–394
7 Bhidayasiri R. and Truong D. D. (2005). Expanding use of botulinum toxin. J. Neurol. Sci. **235:** 1–9
8 Charles P. D. (2004). Botulinum neurotoxin serotype A: a clinical update on non-cosmetic uses. Am. J. Health. Syst. Pharm. **61:** S11–S23
9 Scott A. B. (2004). Development of botulinum toxin therapy. Dermatol. Clin. **22:** 131–133
10 Binder W. J., Brin M. F., Blitzer A., Schoenrock L. D. and Pogoda J. M. (2000). Botulinum toxin type A (BOTOX) for treatment of migraine headaches: an open-label study. Otolaryngol. Head Neck Surg. **123:** 669–676
11 Foster K. A. (2004). The analgesic potential of clostridial neurotoxin derivatives. Expert. Opin. Investig. Drugs **13:** 1437–1443
12 Cote T. R., Mohan A. K., Polder J. A., Walton M. K. and Braun M. M. (2005). Botulinum toxin type A injections: adverse events reported to the US Food and Drug Administration in therapeutic and cosmetic cases. J. Am. Acad. Dermatol. **53:** 407–415
13 Schiavo G., Matteoli M. and Montecucco C. (2000). Neurotoxins affecting neuroexocytosis. Physiol. Rev. **80:** 717–766
14 Lacy D. B. and Stevens R. C. (1999). Sequence homology and structural analysis of the clostridial neurotoxins. J. Mol. Biol. **291:** 1091–1104
15 Kurazono H., Mochida S., Binz T., Eisel U., Quanz M., Grebenstein et al. (1992). Minimal essential domains specifying toxicity of the light chains of tetanus toxin and botulinum neurotoxin type A. J. Biol. Chem. **267:** 14721–14729
16 Swaminathan S. and Eswaramoorthy S. (2000). Structural analysis of the catalytic and binding sites of Clostridium botulinum neurotoxin B. Nat. Struct. Biol. **7:** 693–699
17 Lacy D. B., Tepp W., Cohen A. C., DasGupta B. R. and Stevens R. C. (1998). Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nat. Struct. Biol. **5:** 898–902
18 Habermann E. and Dreyer F. (1986). Clostridial neurotoxins: handling and action at the cellular and molecular level. Curr. Top. Microbiol. Immunol. **129:** 93–179
19 Montecucco C. and Schiavo G. (1994). Mechanism of action of tetanus and botulinum neurotoxins. Mol. Microbiol. **13:** 1–8
20 Breidenbach M. A. and Brunger A. T. (2004). Substrate recognition strategy for botulinum neurotoxin serotype A. Nature **432:** 925–929
21 Eswaramoorthy S., Kumaran D., Keller J. and Swaminathan S. (2004). Role of metals in the biological activity of Clostridium botulinum neurotoxins. Biochemistry **43:** 2209–2216
22 Fernandez-Salas E., Steward L. E., Ho H., Garay P. E., Sun S. W., Gilmore M. A. et al. (2004). Plasma membrane localization signals in the light chain of botulinum neurotoxin. Proc. Natl. Acad. Sci. USA **101:** 3208–3213
23 Bonifacino J. S. and Glick B. S. (2004). The mechanisms of vesicle budding and fusion. Cell **116:** 153–166
24 Breidenbach M. A. and Brunger A. T. (2005). New insights into clostridial neurotoxin-SNARE interactions. Trends Mol. Med. **11:** 377–381
25 Puhar A., Johnson E. A., Rossetto O. and Montecucco C. (2004). Comparison of the pH-induced conformational change of different clostridial neurotoxins. Biochem. Biophys. Res. Commun. **319:** 66–71
26 Bade S., Rummel A., Reisinger C., Karnath T., Ahnert-Hilger G., Bigalke H. et al. (2004). Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates. J. Neurochem. **91:** 1461–1472
27 Chaddock J. A., Purkiss J. R., Duggan M. J., Quinn C. P., Shone C. C. and Foster K. A. (2000). A conjugate composed of nerve growth factor coupled to a non-toxic derivative of Clostridium botulinum neurotoxin type A can inhibit neurotransmitter release in vitro. Growth Factors **18:** 147–155
28 Rummel A., Mahrhold S., Bigalke H. and Binz T. (2004). The HCC-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction. Mol. Microbiol. **51:** 631–643
29 Shone C. C., Hambleton P. and Melling J. (1987). A 50-kDa fragment from the NH2-terminus of the heavy subunit of Clostridium botulinum type A neurotoxin forms channels in lipid vesicles. Eur. J. Biochem. **167:** 175–180
30 Chaddock J. A., Herbert M. H., Ling R. J., Alexander F. C., Fooks S. J., Revell D. F. et al. (2002). Expression and purification of catalytically active, non-toxic endopeptidase derivatives of Clostridium botulinum toxin type A. Protein Expr. Purif. **25:** 219–228
31 Lalli G., Herreros J., Osborne S. L., Montecucco C., Rossetto O. and Schiavo G. (1999). Functional characterisation of tetanus and botulinum neurotoxins binding domains. J. Cell Sci. **112:** 2715–2724
32 Zdanovsky A. G. and Zdanovskaia M. V. (2000). Simple and efficient method for heterologous expression of clostridial proteins. Appl. Environ. Microbiol. **66:** 3166–3173
33 Vaidyanathan V. V., Yoshino K., Jahnz M., Dorries C., Bade S., Nauenburg S. et al. (1999). Proteolysis of SNAP-25 isoforms by botulinum neurotoxin types A, C, and E: domains and amino acid residues controlling the formation of enzyme- substrate complexes and cleavage. J. Neurochem. **72:** 327–337
34 Vertiev Y. V., Zdanovsky A. G., Shevelev A. B., Borinskaya S. A., Gening E. L., Martin T. et al. (2001). Recombinant Listeria strains producing the nontoxic L-chain of botulinum neurotoxin A in a soluble form. Res. Microbiol. **152:** 563–567
35 Baldwin M. R., Bradshaw M., Johnson E. A. and Barbieri J. T. (2004). The C-terminus of botulinum neurotoxin type A light chain contributes to solubility, catalysis and stability. Protein Expr. Purif. **37:** 187–195

36 Li L. and Singh B. R. (1999). High-level expression, purification and characterization of recombinant type A botulinum neurotoxin light chain. Protein Expr. Purif. **17:** 339–344
37 Kadkhodayan S., Knapp M. S., Schmidt J. J., Fabes S. E., Rupp B. and Balhorn R. (2000). Cloning, expression and one-step purification of the minimal essential domain of the light chain of botulinum neurotoxin type A. Protein Expr. Purif. **19:** 125–130
38 Rigoni M., Caccin P., Johnson E. A., Montecucco C. and Rossetto O. (2001). Site-directed mutagenesis identifies active-site residues of the light chain of botulinum neurotoxin type A. Biochem. Biophys. Res. Commun. **288:** 1231–1237
39 Ahmed S. A. and Smith L. A. (2000). Light chain of botulinum A neurotoxin expressed as an inclusion body from a synthetic gene is catalytically and functionally active. J. Protein Chem. **19:** 475–487
40 Zhou L., de Paiva A., Liu D., Aoki R. and Dolly J. O. (1995). Expression and purification of the light chain of botulinum neurotoxin A: a single mutation abolishes its cleavage of SNAP-25 and neurotoxicity after reconstitution with the heavy chain. Biochemistry **34:** 15175–15181
41 Jensen M. J., Smith T. J., Ahmed S. A. and Smith L. A. (2003). Expression, purification and efficacy of the type A botulinum neurotoxin catalytic domain fused to two translocation domain variants. Toxicon **41:** 691–701
42 Ahmed S. A., McPhie P. and Smith L. A. (2003). Autocatalytically fragmented light chain of botulinum a neurotoxin is enzymatically active. Biochemistry **42:** 12539–12549
43 Ahmed S. A., Byrne M. P., Jensen M., Hines H. B., Brueggemann E. and Smith L. A. (2001). Enzymatic autocatalysis of botulinum A neurotoxin light chain. J. Protein Chem. **20:** 221–231
44 Rhee S. D., Jung H. H., Yang G. H., Moon Y. S. and Yang K. H. (1997). Cleavage of the synaptobrevin/vesicle-associated membrane protein (VAMP) of the mouse brain by the recombinant light chain of Clostridium botulinum type B toxin. FEMS Microbiol. Lett. **150:** 203–208
45 Glenn D. E. and Burgoyne R. D. (1996). Botulinum neurotoxin light chains inhibit both Ca(2+)-induced and GTP analogue-induced catecholamine release from permeabilised adrenal chromaffin cells. FEBS Lett. **386:** 137–140
46 Kiyatkin N., Maksymowych A. B. and Simpson L. L. (1997). Induction of an immune response by oral administration of recombinant botulinum toxin. Infect. Immun. **65:** 4586–4591
47 Agarwal R., Eswaramoorthy S., Kumaran D., Dunn J. J. and Swaminathan S. (2004). Cloning, high level expression, purification and crystallization of the full length Clostridium botulinum neurotoxin type E light chain. Protein Expr. Purif. **34:** 95–102
48 Blanes-Mira C., Ibanez C., Fernandez-Ballester G., Planells-Cases R., Perez-Paya E. and Ferrer-Montiel A. (2001). Thermal stabilization of the catalytic domain of botulinum neurotoxin E by phosphorylation of a single tyrosine residue. Biochemistry **40:** 2234–2242
49 Arndt J. W., Yu W., Bi F. and Stevens R. C. (2005). Crystal structure of botulinum neurotoxin type g light chain: serotype divergence in substrate recognition. Biochemistry **44:** 9574–9580
50 Huang X., Wheeler M. B., Kang Y. H., Sheu L., Lukacs G. L., Trimble W. S. et al. (1998). Truncated SNAP-25 (1–197), like botulinum neurotoxin A, can inhibit insulin secretion from HIT-T15 insulinoma cells. Mol. Endocrinol. **12:** 1060–1070
51 Aguado F., Gombau L., Majo G., Marsal J., Blanco J. and Blasi J. (1997). Regulated secretion is impaired in AtT-20 endocrine cells stably transfected with botulinum neurotoxin type A light chain. J. Biol. Chem. **272:** 26005–26008
52 Lacy D. B. and Stevens R. C. (1997). Recombinant expression and purification of the botulinum neurotoxin type A translocation domain. Protein Expr. Purif. **11:** 195–200
53 Sutton J. M., Wayne J., Scott-Tucker A., O'Brien s M., Marks P. M., Alexander F. C. et al. (2005). Preparation of specifically activatable endopeptidase derivatives of Clostridium botulinum toxins type A, B and C and their applications. Protein Expr. Purif. **40:** 31–41
54 Clayton M. A., Clayton J. M., Brown D. R. and Middlebrook J. L. (1995). Protective vaccination with a recombinant fragment of Clostridium botulinum neurotoxin serotype A expressed from a synthetic gene in Escherichia coli. Infect. Immun. **63:** 2738–2742
55 LaPenotiere H. F., Clayton M. A. and Middlebrook J. L. (1995). Expression of a large, nontoxic fragment of botulinum neurotoxin serotype A and its use as an immunogen. Toxicon **33:** 1383–1386
56 Smith L. A. (1998). Development of recombinant vaccines for botulinum neurotoxin. Toxicon **36:** 1539–1548
57 Potter K. J., Zhang W., Smith L. A. and Meagher M. M. (2000). Production and purification of the heavy chain fragment C of botulinum neurotoxin, serotype A, expressed in the methylotrophic yeast Pichia pastoris. Protein Expr. Purif. **19:** 393–402
58 Byrne M. P., Smith T. J., Montgomery V. A. and Smith L. A. (1998). Purification, potency and efficacy of the botulinum neurotoxin type A binding domain from Pichia pastoris as a recombinant vaccine candidate. Infect. Immun. **66:** 4817–4822
59 Potter K. J., Bevins M. A., Vassilieva E. V., Chiruvolu V. R., Smith T., Smith L. A. (1998). Production and purification of the heavy-chain fragment C of botulinum neurotoxin, serotype B, expressed in the methylotrophic yeast Pichia pastoris. Protein Expr. Purif. **13:** 357–365
60 Woodward L. A., Arimitsu H., Hirst R. and Oguma K. (2003). Expression of HC subunits from Clostridium botulinum types C and D and their evaluation as candidate vaccine antigens in mice. Infect. Immun. **71:** 2941–2944
61 Arimitsu H., Lee J. C., Sakaguchi Y., Hayakawa Y., Hayashi M., Nakaura M. et al. (2004). Vaccination with recombinant whole heavy chain fragments of Clostridium botulinum Type C and D neurotoxins. Clin. Diagn. Lab. Immunol. **11:** 496–502
62 Dux M. P., Barent R., Sinha J., Gouthro M., Swanson T., Barthuli A. et al. (2005). Purification and scale-up of a recombinant heavy chain fragment C of botulinum neurotoxin serotype E in *Pichia pastoris* GS115. Protein Expr. Purif. Sep. 20 [Epub ahead of print]
63 Holley J. L., Elmore M., Mauchline M., Minton N. and Titball R. W. (2000). Cloning, expression and evaluation of a recombinant sub-unit vaccine against Clostridium botulinum type F toxin. Vaccine **19:** 288–297
64 Byrne M. P., Titball R. W., Holley J. and Smith L. A. (2000). Fermentation, purification and efficacy of a recombinant vaccine candidate against botulinum neurotoxin type F from Pichia pastoris. Protein Expr. Purif. **18:** 327–337
65 Johnson S. K., Zhang W., Smith L. A., Hywood-Potter K. J., Todd Swanson S., Schlegel V. L. et al. (2003). Scale-up of the fermentation and purification of the recombinant heavy chain fragment C of botulinum neurotoxin serotype F, expressed in Pichia pastoris. Protein Expr. Purif. **32:** 1–9
66 Zhou Y. and Singh B. R. (2004). Cloning, high-level expression, single-step purification and binding activity of His6-tagged recombinant type B botulinum neurotoxin heavy chain transmembrane and binding domain. Protein Expr. Purif. **34:** 8–16
67 Helting T. B. and Zwisler O. (1977). Structure of tetanus toxin. I. Breakdown of the toxin molecule and discrimination between polypeptide fragments. J. Biol. Chem. **252:** 187–193
68 Pastan I. and FitzGerald D. (1991). Recombinant toxins for cancer treatment. Science **254:** 1173–1177
69 Bizzini B. (1984). Investigation of the mode of action of tetanus toxin with the aid of hybrid molecules consisting in part of tetanus toxin-derived fragments. In: Bacterial Protein Toxins, pp. 427–434, Alouf J., (ed.), Academic Press, London
70 Chaddock J. A., Purkiss J. R., Friis L. M., Broadbridge J. D., Duggan M. J., Fooks S. J. et al. (2000). Inhibition of vesicular

secretion in both neuronal and nonneuronal cells by a retargeted endopeptidase derivative of Clostridium botulinum neurotoxin type A. Infect. Immun. **68:** 2587–2593
71 Streit W. J., Schulte B. A., Balentine D. J. and Spicer S. S. (1985). Histochemical localization of galactose-containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of the rat. J. Histochem. Cytochem. **33:** 1042–1052
72 Streit W. J., Schulte B. A., Balentine J. D. and Spicer S. S. (1986). Evidence for glycoconjugate in nociceptive primary sensory neurons and its origin from the Golgi complex. Brain Res. **377:** 1–17
73 Duggan M. J., Quinn C. P., Chaddock J. A., Purkiss J. R., Alexander F. C., Doward S. et al. (2002). Inhibition of release of neurotransmitters from rat dorsal root ganglia by a novel conjugate of a Clostridium botulinum toxin A endopeptidase fragment and Erythrina cristagalli lectin. J. Biol. Chem. **277:** 34846–34852
74 Chaddock J. A., Purkiss J. R., Alexander F. C., Doward S., Fooks S. J., Friis L. M. et al. (2004). Retargeted clostridial endopeptidases: inhibition of nociceptive neurotransmitter release in vitro and antinociceptive activity in in vivo models of pain. Mov. Disord. **19 Suppl 8:** S42–S47
75 Stancombe P. R., Alexander F. C. G., Ling R., Matheson M. A., Shone C. C. and Chaddock J. A. (2003). Isolation of the gene and large-scale expression and purification of recombinant Erythrina cristagalli lectin. Protein Expr. Purif. **30:** 283–292
76 Lappi D. A., Matsunami R., Martineau D. and Baird A. (1993). Reducing the heterogeneity of chemically conjugated targeted toxins: homogeneous basic FGF-saporin. Anal. Biochem. **212:** 446–451
77 Kreitman R. J. (2003). Recombinant toxins for the treatment of cancer. Curr. Opin. Mol. Ther. **5:** 44–51
78 Ford E., Cruttwell C., Nute E., Chaddock J., Barnes P., Sutton J. et al. (2005). Inhibition of mucin secretion from A549 cells using a re-targeted clostridial endopeptidase. Proc. Am. Thor. Soc. **2:** A219
79 Zdanovskaia M. V., Los G. and Zdanovsky A. G. (2000). Recombinant derivatives of clostridial neurotoxins as delivery vehicles for proteins and small organic molecules. J. Protein Chem. **19:** 699–707
80 Maksymowych A. B. and Simpson L. L. (2004). Structural features of the botulinum neurotoxin molecule that govern binding and transcytosis across polarized human intestinal epithelial cells. J. Pharmacol. Exp. Ther. **310:** 633–641
81 Byrne M. P. and Smith L. A. (2000). Development of vaccines for prevention of botulism. Biochimie **82:** 955–966
82 DePaz R. A., Henderson I. and Advant S. J. (2005). Formulation of botulinum neurotoxin heavy chain fragments for vaccine development: mechanisms of adsorption to an aluminum-containing adjuvant. Vaccine **23:** 4029–4035
83 Park J. B. and Simpson L. L. (2004). Progress toward development of an inhalation vaccine against botulinum toxin. Expert Rev. Vaccines **3:** 477–487
84 Francis J. W., Hosler B. A., Brown R. H. Jr and Fishman P. S. (1995). CuZn superoxide dismutase (SOD-1):tetanus toxin fragment C hybrid protein for targeted delivery of SOD-1 to neuronal cells. J. Biol. Chem. **270:** 15434–15442
85 Francis J. W., Figueiredo D., vanderSpek J. C., Ayala L. M., Kim Y. S., Remington M. P. et al. (2004). A survival motor neuron:tetanus toxin fragment C fusion protein for the targeted delivery of SMN protein to neurons. Brain Res. **995:** 84–96
86 Goodnough M. C., Oyler G., Fishman P. S., Johnson E. A., Neale E. A., Keller J. E. et al. (2002). Development of a delivery vehicle for intracellular transport of botulinum neurotoxin antagonists. FEBS Lett. **513:** 163–168
87 Morenilla-Palao C., Planells-Cases R., Garcia-Sanz N. and Ferrer-Montiel A. (2004). Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. J. Biol. Chem. **279:** 25665–25672
88 Chen F., Foran P., Shone C. C., Foster K. A., Melling J. and Dolly J. O. (1997). Botulinum neurotoxin B inhibits insulin-stimulated glucose uptake into 3T3-L1 adipocytes and cleaves cellubrevin unlike type A toxin which failed to proteolyze the SNAP-23 present. Biochemistry **36:** 5719–5728
89 Stanley E. F. (2003). Syntaxin I modulation of presynaptic calcium channel inactivation revealed by botulinum toxin C1. Eur. J. Neurosci. **17:** 1303–1305
90 Ji J., Tsuk S., Salapatek A. M., Huang X., Chikvashvili D., Pasyk E. A. et al. (2002). The 25-kDa synaptosome-associated protein (SNAP-25) binds and inhibits delayed rectifier potassium channels in secretory cells. J. Biol. Chem. **277:** 20195–20204
91 Segelke B., Knapp M., Kadkhodayan S., Balhorn R. and Rupp B. (2004). Crystal structure of Clostridium botulinum neurotoxin protease in a product-bound state: Evidence for noncanonical zinc protease activity. Proc. Natl. Acad. Sci. USA **101:** 6888–6893
92 Hanson M. A. and Stevens R. C. (2000). Cocrystal structure of synaptobrevin-II bound to botulinum neurotoxin type B at 2.0 A resolution. Nat. Struct. Biol. **7:** 687–692
93 Jayaraman S., Eswaramoorthy S., Ahmed S. A., Smith L. A. and Swaminathan S. (2005). N-terminal helix reorients in recombinant C-fragment of Clostridium botulinum type B. Biochem. Biophys. Res. Commun. **330:** 97–103
94 Agarwal R., Eswaramoorthy S., Kumaran D., Binz T. and Swaminathan S. (2004). Structural analysis of botulinum neurotoxin type E catalytic domain and its mutant Glu212→Gln reveals the pivotal role of the Glu212 carboxylate in the catalytic pathway. Biochemistry **43:** 6637–6644
95 Agarwal R., Binz T. and Swaminathan S. (2005). Structural analysis of botulinum neurotoxin serotype f light chain: implications on substrate binding and inhibitor design. Biochemistry **44:** 11758–11765
96 Rao K. N., Kumaran D., Binz T. and Swaminathan S. (2005). Structural analysis of the catalytic domain of tetanus neurotoxin. Toxicon **45:** 929–939
97 Umland T. C., Wingert L., Swaminathan S., Schmidt J. J. and Sax M. (1998). Crystallization and preliminary X-ray analysis of tetanus neurotoxin C fragment. Acta Crystallogr. D Biol.. Crystallogr. **54 (Pt 2):** 273–275
98 Emsley P., Fotinou C., Black I., Fairweather N. F., Charles I. G., Watts C. et al. (2000). The structures of the H(C) fragment of tetanus toxin with carbohydrate subunit complexes provide insight into ganglioside binding. J. Biol. Chem. **275:** 8889–8894
99 Esnouf R. M. (1999). Further additions to MolScript version 1.4, including reading and contouring of electron-density maps. Acta Crystallogr. D Biol. Crystallogr. **55 (Pt 4):** 938–940