A new wrinkle on pain relief: re-engineering clostridial neurotoxins for analgesics

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Botulinum neurotoxins are used to treat of a range of chronic neuromuscular conditions and, increasingly, conditions involving non-neuromuscular transmission, both cholinergic and non-cholinergic, including chronic pain. However, their clinical use is limited by the potential for adverse effects related to the neuromuscular activity, which results from the selectivity of the toxin for the neuromuscular junction. The elucidation of the structure of the botulinum toxin molecule and its relationship to neurotoxin function has enabled the design of novel molecules incorporating selected aspects of toxin function. This review considers the suitability of engineered neurotoxins as the basis for novel therapeutic proteins and the opportunity of developing analgesics based on these neurotoxins.

Botulinum neurotoxins (BoNTs), the causative agents of botulism, are the most potent acute lethal toxins known to man, with lethal doses of 10^{-9} g kg^{-1} of body weight [1]. There are seven immunologically distinct serotypes of botulinum neurotoxin, named A–G (BoNT/A–BoNT/G). BoNTs exert their effect at the neuromuscular junction where, by inhibiting acetylcholine release, they cause a flaccid paralysis. They inhibit neurosecretion by proteolytic cleavage of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins: (i) syntaxin, (ii) synaptosomal protein of 25 kDa (SNAP-25) and (iii) synaptobrevin [2,3]. Each of the BoNTs, with the exception of BoNT/C, cleaves just one of the SNARE proteins at a single peptide bond, which is specific to the particular neurotoxin. BoNT/C is unique among the neurotoxins in having two substrate proteins, SNAP-25 and syntaxin, each of which it cleaves at a single peptide bond. The SNARE proteins are essential for synaptic vesicle fusion at the presynaptic membrane and their cleavage prevents neurosecretion.

The clinical use of BoNT to effect a chemical denervation in a neuromuscular disorder was first described by Alan Scott, who in 1980 reported that injection of small quantities of toxin – far lower than those causing systemic toxicity – into the extraocular muscles produced a correction in strabismus (squint) [4]. Following this pioneering report, BoNT/A was used throughout the 1980s to treat a range of dystonias (involuntary muscle spasms), including blepharospasm (closure of the eyelids), and was shown to be a safe and effective therapy for a large number of neurological diseases. FDA approval of BoNT/A as an orphan drug for the treatment of strabismus, blepharospasm and hemifacial spasm was given in December 1989. It has subsequently been approved for the treatment of cervical dystonia, glabellar wrinkles and hyperhydrosis. The FDA-approved BoNT/A product is manufactured under the trade name BOTOX® by Allergan. An alternative BoNT/A preparation, DYSPORT™, is manufactured in Europe by Ipsen, but is not currently FDA-approved. Although BOTOX® and DYSPORT™ are both

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formulations of BoNT/A, they are not interchangeable products and have different units. More recently, a preparation of BoNT/B received regulatory approval for use in cervical dystonia, and is available as MYOBLOC® in the USA and as NEUROBLOC™ in Europe (Solstice Neurosciences). BoNT/A use has been reported to be effective in >100 different clinical conditions (for reviews, see Refs [5–8]). In addition to effects on muscle contraction and autonomic conditions, recent therapeutic benefits reported for BoNT preparations, particularly BoNT/A, have included pain relief. A major advantage of BoNTs in clinical use, relevant to their use to relieve chronic pain, is their prolonged duration of effect.

Pain and its management

Pain is a protective mechanism signalling present or impending tissue damage by harmful stimuli, but when triggered inappropriately it can cause severe and debilitating medical conditions. There are two types of clinical pain, acute and chronic. Acute pain is defined as short term pain or pain with an easily identifiable cause, and responds well to current medication. Chronic pain is defined as pain that has lasted six months or longer. This constant or intermittent pain will normally have outlived its purpose because it does not help protect against injury. The primary symptoms of chronic pain are allodynia (innocuous stimuli perceived as painful), hyperalgesia (increased intensity of pain sensation) and spontaneous pain. Chronic pain is more difficult to treat than acute pain; current pharmacological treatments are poorly tolerated and ineffective against some types of pain. The pharmacotherapy for pain is dominated by conventional analgesics, such as opioids and non-steroidal anti-inflammatory drugs. With better understanding of the pathophysiology of pain, there is an opportunity to develop new drugs that interact specifically with pain pathways, providing drugs that are more efficacious with fewer side effects.

Evidence for neurotoxin analgesic activity

Treatment of neuromuscular disorders with neurotoxin is associated with a marked analgesic activity [8]. Chronic painful musculoskeletal conditions that have been treated with the BoNT include: temporomandibular joint dysfunction, cervicothoracic pain, lower back pain and myofascial pain. A common cause of patients presenting at pain clinics is myofascial pain syndrome (MPS), a chronic regional pain syndrome. Both BoNT/A and BoNT/B have proved useful in the treatment of refractory MPS [9,10]. Data from several studies suggest a role for BoNT in the treatment of several chronic headache conditions, including migraine, tension-type headache, cervicogenic headache and cluster headache [11]. Reports are also beginning to appear on the use of the neurotoxins in the treatment of neuropathic pain (postherpetic neuralgia, complex regional pain syndrome and spinal cord injury pain) [12].

Scientific basis for effect on pain

The majority of situations where BoNT therapy has been reported to produce pain relief are in the context of inhibition of muscle spasm, and mechanisms have been proposed to explain the pain relief based upon the muscle-relaxant properties of BoNT [13,14]. One such mechanism involves the relief of local ischemia in the muscle caused by compression to blood vessels. Similarly, it has been suggested that activation of nociceptive afferents caused by compression of the nerve could be relieved by the muscle-relaxant activity of the neurotoxins. Another possible mechanism of pain relief by BoNT in neuromuscular conditions involves effects on muscle spindle afferents. Afferent nerves from the muscle spindle – Ia afferents – feedback to the spinal cord and are involved in stretch reflexes. By reducing muscle contractile activity, BoNTs would reduce the Ia afferent signal from muscle spindles to the spinal cord. This could influence pain both through direct reduction of sensory input and via central reorganization resulting from prolonged reduced feedback to the CNS by the Ia afferents [12].

Muscle relaxation cannot, however, entirely explain pain relief by BoNT. It has long been recognized that patients receiving neurotoxin for the treatment of cervical dystonia often display pain relief that exceeds the motor benefit. It has also been reported that pain relief is experienced by patients receiving neurotoxin for the treatment of dystonia before there is any observable muscle relaxation, and that the pain relief often outlasts the reduction in muscle spasm [8]. These observations have led to the suggestion that BoNTs have a direct analgesic activity, which would also explain their beneficial activity in pain syndromes not associated with muscle spasm.

Inhibition of neuropeptide release from dorsal root ganglion (DRG) neurons *in vitro* by BoNTs has been reported [15,16]. Ishikawa *et al.* reported inhibition of both acetylcholine and substance P (SP)-mediated responses by BoNT/A in neurons innervating the iris sphincter and dilator muscles in rabbits [17]. Release of neuropeptides, including SP and calcitonin gene-related peptide (CGRP), from peripheral nerve endings following antidromic activation of nociceptive C-fibres can induce neurogenic inflammation. SP and CGRP have marked effects on blood vessels, causing vasodilation and increased permeability and SP is also known to sensitize nociceptors. BoNTs, by inhibiting the release of neuropeptides, would reduce neurogenic inflammation and relieve pain. Reduction of neuropeptide release from primary afferent nociceptors by BoNTs would also abolish the tenderness and pain in conditions where altered tissue nociceptor sensitivity underlies the pathology [18]. The alleviation of pain in various headache syndromes, including migraine, by neurotoxin could involve such mechanisms. The reduction of pain by BoNTs in inflammatory conditions has been suggested to involve blockade of inflammatory mediator release [19].

Aoki and colleagues provided direct evidence for inhibition of inflammatory pain by BoNT/A using a rat model of formalin-induced inflammatory pain [20,21]. Subcutaneous injection of formalin into the hindpaw produces a two phase pain response. Phase I is an acute pain immediately following injection due to chemical stimulation of the nociceptor neurons by formalin; this phase resolves within 10 min and is followed by a quiescent phase. Phase II starts after ~15 min, when peripheral neurons become sensitized by the local inflammatory response, and is maintained for over an hour. Subcutaneous administration of BoNT/A into the hindpaw 5 h or 12 days before the formalin challenge dose-dependently inhibited the second phase of formalin-induced nociceptive behaviour [21]. At doses of BoNT/A up to 15 U Kg^{-1}, there was no effect on the first phase of the formalin response, and no local muscle weakness was observed. However, at 30 U Kg^{-1}, in addition to almost total blockade of the second phase of the formalin response, BoNT/A also caused complete ablation of the first phase. At this highest dose of BoNT/A, there was also evidence of lethargy, and effects on muscle function were observed as assessed by the ability of the animals to stay on a rotating rod (rotarod test) [21]. The highest level of blockade of inflammatory pain that could be achieved with BoNT/A in the formalin model without the appearance of the neuromuscular effects was a ~40% reduction in phase II. The antinociceptive effect was presumed to result from inhibition of release from primary afferent terminals, as evidenced by inhibition of formalin-induced release of glutamate in the injected paw [20,21]. It is proposed that this would block peripheral sensitization and, indirectly, reduce central sensitization. This reduction in central sensitization was supported by both electrophysiological recording from the spinal cord and by measurement of neuronal activation in the dorsal horn [20].

The regulation, by inflammatory mediators, of the surface expression of receptors and channels in the peripheral terminals of nociceptive afferent neurons could be an important mechanism for both the development and maintenance of inflammatory hyperalgesia. 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 [22]. Such a blockade might represent another mechanism by which the toxin could reduce inflammatory pain, particularly the development of hyperalgesia.

Although BoNTs cannot be considered 'first-line' therapy for pain, in refractory cases they could offer a chance for pain relief [10]. A major restriction to the use of BoNTs in the treatment of chronic pain, however, is their highly potent and selective activity at the neuromuscular junction [9]. In the treatment of chronic pain, therefore, the clinical use of BoNT will be limited to conditions amenable to localized, peripheral administration of the toxin. Use will also be restricted to pain syndromes where the underlying pathophysiology involves mechanisms susceptible to toxin inhibition, namely reduction of neuromuscular activity or inhibition of neurogenic inflammation.

BoNTs inhibit neurotransmitter release from both motor nerves and sensory nerves by the same biochemical mechanism: proteolytic cleavage of SNARE proteins. The selectivity of BoNTs for motor neurons is due to their potent binding to motor nerve terminals. Recent advances in our understanding of neurotoxin structure and function have presented the opportunity to engineer the toxin molecule to produce new molecules that are able to inhibit nociceptive afferent function without effects on other neurons, including motor neurons.

Structure–function and re-engineering neurotoxins

BoNTs share a common structure, being di-chain proteins of ~150 kDa, consisting of a heavy chain (HC) of ~100 kDa covalently joined by a single disulfide bond to a light chain (LC) of ~50 kDa [23]. The HC consists of two domains, each of ~50 kDa. The C-terminal domain (H_C) is required for the high-affinity neuronal binding [24,25], whereas the N-terminal domain (H_N) is proposed to be involved in membrane translocation [26]. The LC is a zinc-dependent metalloprotease responsible for the cleavage of the substrate SNARE protein [3]. Intoxication of the neuromuscular junction by BoNT is believed to occur in a three-stage process: (i) an initial binding stage leading to internalization via endocytosis; (ii) a membrane translocation stage; and (iii) a secretion blockade stage (Figure 1).

The crystal structures of BoNT/A [27] and BoNT/B [28] have been solved and show three distinct structural domains, each one corresponding to one of the three steps of the mechanism of toxification: binding, translocation and catalytic activity [29,30]. The three domains are arranged sequentially in a linear fashion with the translocation domain in the middle; therefore, there are no interactions between the binding and catalytic domains. The catalytic domain, the LC, has a compact globular structure, and contains a zinc-binding HExxH motif in the middle of the chain. The active-site zinc is located inside a groove into which the substrate has to insert to enable catalytic cleavage of the peptide bond. The translocation domain, H_N, is mainly α-helical, with a pair of α-helices 105 Å in length and an unusual 'belt' region that wraps around the catalytic domain. It is believed that, following internalization into an endosomal compartment, acidification of the endosome leads to a conformational change in the toxin and formation by the H_N domain of a channel in the membrane through which the LC can gain access to the cytosol [31]. The binding domain, H_C, consists of two subdomains, H_{CC} and H_{CN}. The H_{CN} subdomain contains two seven-stranded antiparallel β-sheets sandwiched together, resembling a jelly roll motif, and the H_{CC} subdomain contains a six-stranded β-barrel and a β-trefoil motif. The whole binding domain is tilted away from the central translocation domain and has minimal interaction with it. BoNTs bind

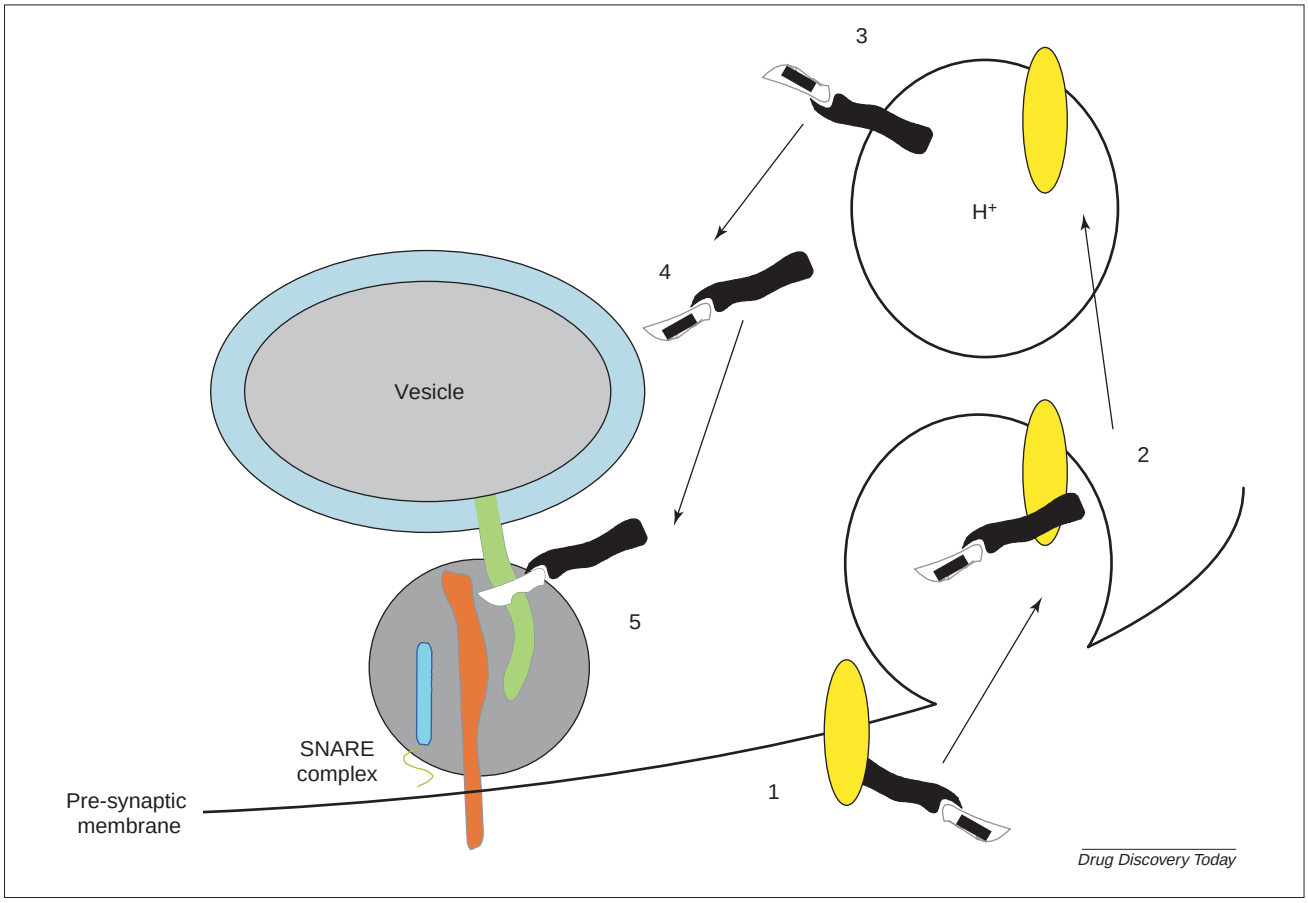

FIGURE 1

Schematic of neurotoxin action at pre-synaptic nerve terminal. (1) Botulinum neurotoxin binds to receptor(s) at the pre-synaptic membrane. (2) Receptor with bound neurotoxin undergoes endocytosis. (3) Endosome acidifies leading to insertion of neurotoxin heavy chain (H_N) into the membrane and translocation of the neurotoxin into the neuronal cytosol. (4) Neurotoxin, or light chain, in the cytosol. (5) Neurotoxin light chain cleaves specific SNARE protein at unique peptide bond.

specifically to neuronal cells with subnanomolar binding affinities. It is believed that this high-affinity binding involves both protein and carbohydrate moieties [32]. The protein receptor has only been characterized for BoNT/B and BoNT/G, and is synaptotagmin I and II [33,34]. It has been suggested recently that binding is a two stage process involving a capture step via oligosaccaharides, producing a concentration effect at the membrane, followed by interaction with receptor protein arrays in membrane microdomains [35]. The second stage is believed to render the binding effectively irreversible and trigger endocytosis. Several studies have shown that key residues involved in neurotoxin binding activity are located in the H_{CC} subdomain [36] (Figure 2).

Limited proteolytic cleavage of BoNT/A by trypsin produces a 100 kDa species termed LH_N/A (representative of the LC and H_N domains of BoNT/A coupled by a single disulfide bond) [25]. Recently, expression and purification of an equivalent species from a heterologous expression host has been reported [37]. This opens up the possibility of producing LH_N species of BoNTs other than BoNT/A, which is not possible from the native neurotoxins because they lack the endogenous protease site found at the H_N–H_C interface in BoNT/A.

The LH_N/A fragment lacks the H_C domain with which to bind to the neuronal surface and is therefore effectively non-toxic. It has proved possible to retarget the LH_N/A fragment into a range of neuronal and non-neuronal cells by replacing the H_C domain with alternative ligands [38,39]: this results in cleavage of the substrate SNARE protein, SNAP-25, and consequent inhibition of secretion from the cells targeted. The ability to inhibit secretion from a variety of neuronal and non-neuronal cells using re-targeted LH_N/A is because the SNARE proteins that are the substrates for the BoNT endopeptidases represent a universal mechanism of vesicle fusion and secretion in eukaryotic cells [40]. The neuronal selectivity of the BoNTs is due to the binding characteristics of the binding domain, H_C. Therefore, by replacing this domain with alternative binding ligands, the possibility of targeting the neurotoxin endopeptidases to new target cells of choice is opened up. This offers the potential to produce therapeutic molecules based upon the neurotoxins but with new cellular targets. One opportunity for this strategy

is to develop analgesics specifically targeted at primary nociceptive afferents.

Botulinum neurotoxin derived proteins with analgesic potential

Given the evidence for the analgesic properties of the BoNTs, it is reasonable to conclude that if BoNT endopeptidases could be targeted in a selective manner to peripheral nociceptive afferents, the transmission of pain responses could be prevented specifically. A key requirement to enable achievement of this goal is the identification of a ligand that can target nociceptive afferents specifically and, following binding, internalize these afferents. Galactose-containing carbohydrates have been reported to be present selectively on nociceptive afferents relative to other neurons in the central and peripheral nervous system [41,42]. Lectins from the leguminous *Erythrina* species have been identified to bind such galactose-containing carbohydrates and the lectin from *Erythrina cristagalli* (ECL) was selected to test the hypothesis that BoNT endopeptidases could be retargeted selectively to nociceptive afferents [43,44].

A chemical conjugate of ECL and LH_N/A (ECL–LH_N/A) inhibited both SP and glutamate release from embryonic DRG neurons in culture [43]. This inhibition required ECL-mediated delivery of the conjugate and involved endopeptidase-mediated cleavage of SNAP-25. ECL–LH_N/A was relatively ineffective at inhibiting glycine release from cultured embryonic spinal cord neurons (SCN) from anatomically adjacent regions of the spinal cord to the DRG. By contrast, BoNT/A was a potent inhibitor of glycine release from SCN. An indication of the different selectivities of ECL–LH_N/A and BoNT/A for inhibition of neurotransmitter release from DRG neurons relative to SCN, can be gained by comparing the ratio of IC_{50} values for inhibition of neurotransmitter release from the two types of neuronal culture (see Ref [43]). BoNT/A showed higher potency for inhibition of glycine release from SCN compared with inhibition of SP release from DRG neurons, with the ratio of IC_{50} values being 0.005:1. By contrast, ECL–LH_N/A was relatively ineffective at inhibiting glycine release with no IC_{50} being observed at the concentrations tested, giving an equivalent ratio of >6.9:1. The selectivity of ECL–LH_N/A for DRG neurons relative to SCN based on Ref [43] is, therefore, at least 1400-fold greater than that of BoNT/A.

The prolonged duration of action of BoNTs is key to their clinical utility. If neurotoxin derivatives of the type represented by ECL–LH_N/A are to be of value in the clinic, they will need to retain this property. ECL–LH_N/A inhibits SP release from cultured DRG neurons for at least 25 days following treatment of the cells [43]. The conjugate has therefore retained the duration of action that is characteristic of the native neurotoxin, which is consistent with this being a characteristic of the proteolytic domain. The differences in the duration of inhibition of neurotransmitter release by different botulinum neurotoxin serotypes has been proposed to be a feature of the LC, either because of survival of the LC in the neuronal terminal [45–47] and/or because of properties of the substrate cleavage product [46,48]. Therefore, the production of ligand–LH_N hybrid proteins opens up the potential to engineer therapeutic proteins where the ligand determines cellular selectivity and specificity, while the LC serotype determines duration of action.

Following intrathecal administration (delivery to the intrathecal space surrounding the spinal cord) of the ECL–LH_N/A conjugate into the lumbar region of the spinal cord of rats, sensory inputs by primary nociceptive afferents were significantly attenuated [43,44]. This effect was observed 24 h after administration of conjugate, suggesting a prolonged effect *in vivo*. The ability of ECL–LH_N/A to block neurotransmitter release from nociceptive afferent neurons for prolonged periods *in vitro* is therefore retained *in vivo*. Intrathecal administration of ECL–LH_N/A into the lumbar region of the spinal cord of mice resulted in a prolonged withdrawal latency in a model of thermal pain, thus demonstrating the analgesic activity of the conjugate [46]. This effect was sustained for more than 30 days post-administration of the conjugate, whereas morphine in the same model ceased to demonstrate analgesic activity within less than a day. The ECL–LH_N/A conjugate, intrathecal or subcutaneous, has also been shown to inhibit phase II inflammatory pain in the rat formalin model [49].

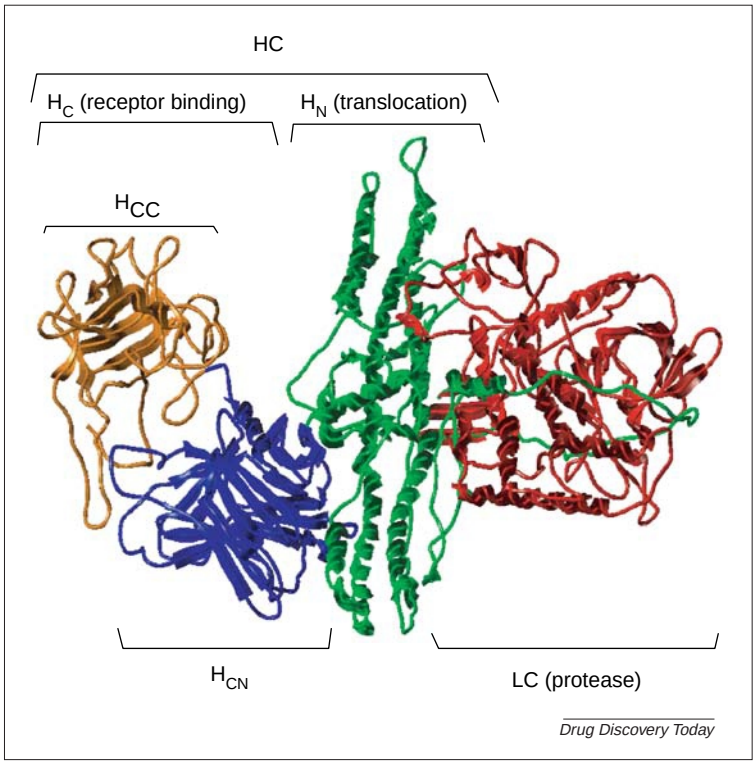

FIGURE 2

Botulinum neurotoxin domain structure. Ribbon representation of botulinum neurotoxin type A structure generated using Rasmol from the tertiary structure, as reported by Lacy *et al.* [27] (Protein Data Bank code 3BTA). Abbreviations: HC, heavy chain; H_C, carboxy-terminal half of HC; H_N, amino-terminal half of HC; H_{CC}, carboxy-end of H_C; H_{CN}, amino-end of H_C; LC, light chain.

Conclusion

As a result of the widespread involvement of SNARE proteins in vesicle fusion events in eukaryotic cells, BoNT endopeptidase activity has the potential to inhibit vesicular fusion and secretion in a wide range of cell types, but is unable to do so because of the highly specific and selective targeting of the endopeptidase by the neurotoxin-binding domain. Recent understanding of the structural basis of neurotoxin activity has enabled this barrier to the use of BoNT endopeptidases to be overcome, and has opened up the opportunity to produce novel proteins that target the endopeptidase activity selectively to cells of choice. The ECL–LH_N/A conjugate described in this review has shown how, by suitable selection of targeting ligand, a molecule can be engineered that selectively targets a specific neuronal population. Importantly, this molecule retains the duration of action of the parent neurotoxin and demonstrates analgesic activity *in vivo* for weeks post application. ECL–LH_N/A thus provides proof-of-principle for the ability to retarget clostridial endopeptidase and obtain therapeutically relevant effects. However, the inherent heterogeneity of chemical conjugates, and the difficulty of developing a regulatory compliant process based upon them, makes them unsuitable for the basis of a potential pharmaceutical product. The requirement is to develop a fully recombinant chimera protein incorporating the targeting and endopeptidase domains; given the size and complexity of such a fusion protein this is a challenging task. Once available, such a recombinant protein would have the potential to be developed for the relief of chronic pain. This technology has also been exemplified using other targeting ligands and in a range of cell types, and targets a universal mechanism of cell biology, bringing about a long-lasting inhibition of secretion without cytotoxicity. Pain is therefore likely to be the first of many clinical applications of this technology, producing a family of therapeutic proteins that is able to treat a variety of chronic diseases.

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