Published in final edited form as:
Curr Top Microbiol Immunol. 2013 ; 364: 179–196. doi:10.1007/978-3-642-33570-9_9.

Persistence of *Botulinum* neurotoxin inactivation of nerve function

Charles B. Shoemaker[1] and **George A. Oyler**[2]

[1]Department of Biomedical Sciences, Tufts Cummings School of Veterinary Medicine, North Grafton, MA, United States of America

[2]Synaptic Research LLC, Baltimore, MD United States of America

Abstract

The extraordinary persistence of intoxication occurring after exposure to some Botulinum neurotoxin (BoNT) serotypes is both a therapeutic marvel and a biodefense nightmare. Understanding the mechanisms underlying BoNT persistence will offer new strategies for improving the efficacy and extending the applications of BoNT therapeutic agents as well as for treating the symptoms of botulism. Research indicates that the persistence of BoNT intoxication can be influenced both by the ability of the toxin protease or its cleaved SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein substrate to resist turnover. Protease turnover seems to be mediated in part by the ubiquitin-proteasome system (UPS) and efforts to manipulate the UPS may prove to be an effective strategy for improving therapeutic utility of BoNT products and in the development of botulism antidotes.

1. Introduction

Cellular proteins turn over with half-lives that vary from seconds to several days in a highly regulated manner, influencing critical processes such as the cell cycle, apoptosis, stress responses and immunity (Varshavsky, 1996). Neurons, like all cells, possess elaborate systems dedicated to the maintenance of a healthy proteome in which improperly folded proteins are either refolded or shunted for degradation, most commonly via the ubiquitin/proteasome system (UPS) or lysosome/autophagy system (LAS) [1]. Botulinum neurotoxins (BoNTs) entering a neuron will surely encounter these same systems and be degraded at some rate. Rapid turnover of intraneuronal BoNTs would reduce the timeframe of toxin action and mitigate the morbidity and mortality associated with intoxication. Because it is assumed that death of the intoxicated animal is an outcome that benefits *Clostridium botulinum* in nature, evolutionary pressures result in BoNTs having greater persistence of action. This pressure has apparently led to BoNT proteases that resist degradation within the cytosol of their targeted neuronal cells. This chapter describes the remarkable persistence of some BoNT proteases and considers the mechanisms whereby the proteases promote their own stability. Strategies for perturbing BoNT protease persistence, both positively and negatively, to generate more effective therapeutic agents or to develop strategies to accelerate patient recovery from pathogenic intoxication are described.

C. botulinum strains produce a number of different neurotoxin serotypes and subtypes (see Chapter 1) which show variable potency and persistence in different animals, presumably

Corresponding author: Dr. Charles B. Shoemaker, charles.shoemaker@tufts.edu, Tel. 508-887-4324; Fax 508-839-7911.
Current address: Department of Biomedical Sciences, Tufts Cummings School of Veterinary Medicine, 200 Westboro Rd, North Grafton, MA 01536

reflecting evolutionary selection for strains that are lethal to a broader range of target species. The mechanisms responsible for BoNT toxicity and persistence may vary widely between the seven known BoNT serotypes and within the huge range of susceptible animal species, yet have been studied in detail for only a small subset. As a result, the current understanding of BoNT persistence mechanisms is primarily based on human and rodent studies employing BoNT/A and BoNT/E serotypes and may not prove broadly applicable in other species and BoNT serotypes. Nevertheless, substantial progress has been made in our understanding of persistence in humans and this may translate into improved therapeutic BoNT agents and in the development of antidotes for reversal of unwanted intoxication.

The persistence of muscle paralysis in humans following treatment with different BoNT serotypes varies dramatically from 4 to 6 months with BoNT/A and BoNT/C1 to a 1–4 weeks with BoNT/E in the few examples studied [2–4]. BoNT/B paralysis also ranges between 2 and 4 months in humans although persistence for these serotypes appears to be somewhat less than for BoNT/A [3–6]. In rodent models, the time to recovery from muscle paralysis is consistently much less than in humans although the relative order of persistence of the different BoNT serotypes remains similar [7–13]. BoNT/F has been tested in rats and found to have much reduced persistence compared to BoNT/A [14]. Though recovery from BoNT intoxication is faster in rodents, even in mice neurons intoxicated by BoNT/A remain unable to recycle vesicles for a month [15] and observed recovery of muscle function prior to this time is hypothesized to be a consequence of nerve sprouts at the nerve endings that begin forming within days of intoxication [16,17]. The function of the original termini fully recovers during the second and third month post-intoxication and the sprouts are largely eliminated [15,18]. The role of sprouting in the recovery from intoxication by other BoNT serotypes has not been carefully evaluated. A careful analysis of the persistence of neurotransmitter release blockage following treatment of cultured primary neurons with each of five different BoNT serotypes demonstrated a ranking in persistence that strongly correlated to the *in vivo* results in humans and rodents [19]. In summary, the available data to date in humans and rodents consistently ranks the persistence of intoxication for the various BoNT serotypes as $A \geq C1 > B \gg F > E$.

2. Mechanisms of BoNT persistence

All BoNT serotypes intoxicate neurons by delivering the LC protease to the cytosol which specifically cleaves one or more SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins. The cleaved SNARE proteins either are unable to form SNARE complexes or form inactive SNARE complexes. In either case, it is widely accepted that the presence of the BoNT-cleaved SNARE proteins inactivates exocytosis and release of neurotransmitters to cause the flaccid paralysis observed following exposure to BoNT (see Chapter 7 and 8). It follows then that the persistence of BoNT-induced intoxication must depend on: 1) how long the cleaved SNARE proteins remain in the cytosol and the ability of these cleaved SNARE proteins to maintain a block to exocytosis; 2) how long the BoNT protease remains in place to cleave newly synthesized SNARE proteins and/or; 3) the ability of the presynaptic terminal to remodel in a manner to overcome dysfunction. A summary of the mechanisms proposed to explain the variable persistence of the different BoNT serotypes is provided in Figure 1. Evidence exists that all these processes may contribute to BoNT persistence in rodent models.

2.1 Role of SNARE proteins in the persistence of BoNT intoxication

Studies on the mechanism of BoNT persistence have largely focused on BoNT/A and BoNT/E because these two serotypes have dramatically different persistence in mammals yet they cleave the same SNARE protein, SNAP-25 (synaptosomal associated protein of 25 kDa), at positions separated by only 17 amino acids. LC/E cleavage of SNAP-25 removes a

carboxyl-terminal peptide of 26 amino acids and there is evidence that this small carboxyl-terminal product is capable, on its own, of inhibiting nerve function. Specifically, several peptides corresponding to different portions of the SNAP-25 carboxyl-terminus have been shown to inhibit neurosecretion in different neuronal model systems [20–23]. Although the carboxyl-end peptides have the ability to interfere with neurosecretion, their role in natural BoNT intoxication of motor neurons remains uncertain. For example, the carboxyl-terminal nine amino acids appear unnecessary for BoNT/A intoxication as expressing the truncated SNAP-25 corresponding only to the amino-terminal cleavage product of LC/A caused an intoxication equivalent to that from BoNT/A in insulinoma HIT-T17 cells [24]. Furthermore, there is no evidence that the carboxyl-terminal peptides cleaved from SNAP-25 following LC/A or LC/E exposure play any role in the relative persistence of intoxication of these two toxin. In fact, the 9-mer peptide from cleavage by the most persistent serotype BoNT/A has an amino-terminal arginine that is predicted by the N-end rule [25] to be highly unstable while the isoleucine at the amino-terminus of the less persistent LC/E carboxyl-terminal cleavage product should be more stable. A recent study validates this prediction in neuroblastoma cells [26] and it now seems highly unlikely that the carboxyl-terminal peptides cleaved from SNAP-25 play any role in promoting the extraordinary persistence of BoNT/A function in neurons.

The large amino-terminal SNAP-25 products resulting from the proteolytic activities of BoNT/A (SNAP-25^{A}) or BoNT/E (SNAP-25^{E}) have been proposed by several groups to play an important role in the persistence of these two toxins. If the SNAP-25 cleavage products are the primary determinant of persistence, this leads to the testable prediction that persistence of BoNT/A intoxication would be reduced to that of BoNT/E by ‘super-intoxication’ with BoNT/E which would truncate the SNAP-25^{A} to SNAP-25^{E}. Note that this prediction assumes sufficient endosome recycling will remain following BoNT intoxication such that a different BoNT serotype can enter the cell. Despite the caveat, the prediction has reportedly been validated in several studies. A study by Eleopra et al.[5] in human volunteers found that BoNT/A paralysis of the EDB foot muscle (3 IU) was much more persistent than BoNT/E paralysis of this muscle (3 IU) in the contralateral limb. When another group of volunteers were paralyzed in both EDB foot muscles with a mixture of 2 IU BoNT/A and 4 IU BoNT/E, or 4 IU BoNT/A and 2 IU BoNT/E, all muscles recovered at the same faster rate previously observed for 3 IU BoNT/E alone. Another similar study by Meunier et al.[7] showed that intoxication by BoNT/E accelerated recovery from BoNT/A intoxication in mice while intoxication by short-lived BoNT/F, which does not cleave SNAP-25, did not affect recovery time from BoNT/A. Raciborska and Charlton [27] employed a frog model and reported that SNAP-25^{A} was more persistent at the nerve termini than SNAP-25^{E}.

One possible explanation for BoNT/E treatment accelerating recovery from BoNT/A intoxication could be that the SNAP-25^{A} product of LC/A is much more stable than SNAP-25^{E} so cleavage of SNAP-25^{A} to SNAP-25^{E} by super-intoxication accelerates turnover of the inactive SNAP-25 and subsequent formation of functional SNARE complexes from newly synthesized proteins. Foran et al.[19] tested this hypothesis directly by performing pulse-chase studies to compare the half-lives of the SNAP-25 forms in rat cerebellar primary neurons treated with BoNT/A or BoNT/E. The study showed that the intact or cleaved SNAP-25 proteins all have half-lives of 20–24 hours and they concluded that, in their model system, the half-life of cleaved SNAP-25 “does not account for the longevity of BoNT/A-induced inhibition”.

Another explanation for the accelerated recovery from BoNT/A by super-intoxication with BoNT/E has been proposed by several groups [5,12,27,28]. These groups hypothesize that further cleavage of SNAP-25^{A} to SNAP-25^{E} allows replacement of the damaged SNAP-25

with newly synthesized, uncleaved, SNAP-25 to restore nerve function. Bajohrs et al. [28] directly demonstrated that SNAP-25^{A} remains bound to syntaxin and localized to the plasma membrane, while cleavage to SNAP-25^{E} results in the loss of association with syntaxin and release to the cytosol. The hypothesis that cleavage to SNAP-25^{A}, but not SNAP-25^{E}, results in stable, unproductive SNARE complexes is consistent with earlier work of Otto et al. [29] showing that cleavage of SNAP-25^{A} does not affect the formation or stability of SNARE complexes and by Hayashi et al. [30] which showed that SNARE complexes with SNAP-25^{E} are less stable to denaturants than those with SNAP-25^{A}.

2.2 Role of BoNT proteases in the persistence of BoNT intoxication

A number of studies report data strongly in support of the argument that the remarkable persistence BoNT/A intoxication results from retention of active BoNT/A protease within the nerve termini. An early study by Bartels et al. [31] showed that exocytosis in bovine chromaffin cells previously intoxicated by BoNT/A could be rescued using electroporation to deliver antibodies to the cell cytosol that bind to the BoNT/A protease domain (LC, light chain). Electroporation of antibodies that bind only to the BoNT/A heavy chain domain did not restore exocytosis. The fact that exocytosis was restored to normal within a few days after antibody treatment indicates that, if the BoNT/A protease is inhibited or removed, exocytosis function is rapidly restored by endogenous synthesis of full-size SNAP-25. This implies, at least for BoNT/A, that recovery from intoxication is dependent on the loss of BoNT/A protease activity.

More recent studies, designed similar to those in the previous section, monitored recovery of neurons intoxicated by both BoNT/A and BoNT/E. A study by Keller et al. [32] showed that intoxication of fetal mouse spinal nerve cells with BoNT/A lasted more than eleven weeks based on SNAP-25 cleavage data, while BoNT/E intoxication persisted less than three weeks. When these nerve cells were first intoxicated by BoNT/A and then super-intoxicated by BoNT/E, all of the SNAP-25 became SNAP-25^{E} showing that all neurons had become intoxicated by BoNT/E. By monitoring the SNAP-25 with time post-intoxication, these authors found that SNAP-25^{E} was slowly replaced by SNAP-25^{A}. This demonstrated that the BoNT/A protease persisted and continued to cleave newly synthesized SNAP-25 even after the BoNT/E protease was no longer active. In a similar study, Adler et al. [8] found that persistence of intoxication by BoNT/A in a rat EDL model was not reduced by subsequent or previous intoxication with BoNT/E and the authors conclude that BoNT/A persistence in this model is a consequence of the half-life of the BoNT protease rather than that of the cleaved SNAP-25.

Another approach to understanding the factors responsible for the unusual persistence of BoNT/A intoxication was to attempt rescue of nerve function at different times post-intoxication with BoNT/A by transient expression of full-size SNAP-25 [33]. In this study, cultured bovine chromaffin cells were intoxicated with BoNT/A and then transfected by plasmids promoting expression of wild-type SNAP-25 or a mutated SNAP-25 that was not cleaved by BoNT/A yet retained the ability to form functional SNARE complexes. Their results showed that BoNT/A cleaved SNAP-25^{A} is not a dominant block to exocytosis because function was restored by expression of the BoNT/A insensitive mutant SNAP-25 when the chromaffin cells were transfected just a day post-intoxication. The authors then used this strategy to test whether the BoNT/A protease remained persistent in the chromaffin cells. Chromaffin cells that had been intoxicated for 16 days were transfected with plasmids to over-express either wild-type or BoNT/A insensitive mutant SNAP-25 and measured 5 days later for functional exocytosis. The results showed that expression of wild-type SNAP-25 remained reproducibly unable to rescue exocytosis while mutant SNAP-25 did rescue function, thus demonstrating that active BoNT/A protease persists in these cells for at

least three weeks. The authors conclude that the “continued action of BoNT/A-LC is a major contributory factor in the prolonged inhibition of release, at least in this model system”.

Ideally, studies on the role of the BoNT proteases in the persistence of intoxication would monitor the cytosolic levels of the proteases in neuronal cells at various times following toxin exposure and attempt to correlate protease levels with the cells’ ability to perform exocytosis. This is not practically possible for a number of reasons. Most significant is the inability to quantify cytosolic BoNT proteases within intoxicated cells using current detection methods. Although the number of cytosolic protease molecules necessary to inactivate exocytosis is not known, it has been estimated to be no more than about a thousand [34] for BoNT proteases, and less than ten molecules for the closely related tetanus toxin protease [35]. Even these remarkably low numbers may be overestimations as for other toxins such as ricin or diphtheria toxin, a single molecule has been shown sufficient to elicit a measurable effect (cell death) [36,37]. It is also problematic to differentiate cytosolic BoNT proteases from proteases that remain in the endosomal fraction using imprecise available immunolocalization or subcellular fractionation methods, especially if only a small percentage of BoNT proteases escape the endosomal fraction to the cytosol [38].

2.2.1 Contribution of intraneuronal BoNT protease subcellular localization to persistence—Because of the difficulties in detecting cytosolic BoNT protease following natural intoxication, several labs have employed DNA transfection methods to promote expression of the proteases within cells to study persistence [26,39–41]. Fernandez-Salas et al. over-expressed either recombinant BoNT/A or BoNT/E proteases within rat PC12 neuroblastoma cells as fusions to green fluorescent protein (GFP) and monitored protease localization by fluorescence microscopy. They found that BoNT/A protease (LC/A) co-localized with SNAP-25^{A} at the plasma membrane where intact SNAP-25 localizes. Surprisingly they found that the BoNT/E light chain protease (LC/E) was primarily found in the cytosol rather than localized at the plasma membrane. These authors suggest that the cytosolic localization of LC/E may be the reason that this protease is turned over more rapidly than the LC/A protease which they speculate remains in “a slow cycling compartment at the plasma membrane”. In a recent study in a different neuroblastoma cell line (N18), Tsai et al.[26] found that both recombinant LC/A and LC/E co-localized at the plasma membrane and argued that differential localization is not likely to be responsible for the differential persistence of these proteins. It is not possible to know whether cytosolic synthesis and over-expression of recombinant LC/A and LC/E through recombinant expression as employed in these studies produced localizations that accurately reflect the protease localization achieved following natural intoxication events in which these proteases are delivered from endosomes to the cytosol in vanishingly small amounts by a highly evolved and sophisticated translocation process [42].

In an effort to understand the mechanistic basis for the differing subcellular localizations of LC/A and LC/E observed in their model system, some groups have studied the effects of specific protease mutations on their localization within cells. Fernandez-Salas et al.[39] produced a variety of mutant forms of recombinant LC/A to identify signals that lead to its plasma membrane localization. They found that removal of eight amino acids at the amino terminal end of the protease, or 22 amino acids from the carboxyl end, reduced plasma membrane association by LC/A and this indicated that signals may exist at both ends of the protease to promote plasma membrane association. These results were extended by Chen and Barbieri [43] who used LC/A mutants to demonstrate that LC/A localization results from its binding to the SNAP-25 substrate. To reach this conclusion, the authors showed that LC/A mutants lacking the amino terminal eight amino acids, or containing lysine to alanine mutations near the amino terminus at positions 6 and 11, significantly reduced plasma membrane localization. They then showed that SNAP-25 mutants which lack their putative

palmitoylation sites no longer localized to the plasma membrane and when cells were engineered to express these SNAP-25 mutants, the co-expressed LC/A also became localized to the cytosol. Since it was previously demonstrated that SNAP-25 is released to the cytosol following cleavage by LC/E but not following cleavage with LC/A [28], Chen and Barbieri [43] tested the localization of recombinant LC/A when it was co-expressed with LC/E. As expected, they found that LC/A now localized to the cytosol, presumably because the SNAP-25 to which it associates becomes localized to the cytosol following its conversion to SNAP-25^{E}. These results imply that LC/A does not naturally localize to the plasma membrane in the absence of SNAP-25, yet other studies have found plasma membrane localization for LC/A within MDCK cells which lack SNAP-25 (G. Oyler, unpublished observation). As such, other mechanisms responsible for alterations in LC localization cannot be ruled out.

Fernandez-Salas et al.[39] hypothesized that a dileucine motif [44] present near the carboxyl end (aa 427/428) played an important role in the plasma membrane localization of LC/A. To test this concept, they generated an LC/A mutant in which only this dileucine was altered to a dialanine (LC/A^{AA}) and compared its localization to wild type LC/A within transfected cells. They found that this mutant displayed reduced plasma membrane localization and suggested that the dileucine in LC/A helps promote the membrane localization of LC/A- and its characteristic persistence. The LC/A^{AA}, though, was expressed to different levels than wild type LC/A and had reduced protease activity, and these factors may also have contributed to the altered localization.

The SNAP-25 mechanism for LC/A localization is somewhat more complicated since LC/A has been seen to associate with membranes when expressed in cell lines such as cells lacking SNAP-25 (unpublished observations, GAO). Thus, there may be additional mechanism involved in targeting LC/A to membranes.

Wang et al.[45] attempted to directly determine whether the amino terminal amino acids of BoNT/A, or the dileucine present at the carboxyl end, contributed to the unusual persistence of this toxin. In their study, the authors engineered recombinant holotoxins in which the BoNT/A protease either lacked the seven amino terminal amino acids (residues 2–8) or had the dileucine 427/428 converted to a dialanine. They then compared the persistence of intoxication elicited by the mutant toxins compared to wild type recombinant BoNT/A or BoNT/E holotoxins. Strikingly, the BoNT/A mutation converting the dileucine to dialanine was clearly shown to significantly reduce the persistence of intoxication from this mutant holotoxin in primary rat neuronal cells or in vivo in the mouse hind limb. To further demonstrate the role of dileucines in persistence, these authors also produced a catalytically inactive form of BoNT/A in which the LC/A protease was inactivated by mutation, with or without the dileucine mutations, and the complete LC/E protease was fused to the amino terminus. The chimeric protein that lacked the dileucine mutation, called LC_E-$BoTIM_A$, was toxic to neurons and produced intoxication with the persistence of wild type BoNT/A. As previously shown by Bade et al. [38] this demonstrated that a fusion of two complete BoNT proteases could be effectively delivered to the cytosol by the heavy chain translocation domain. It also demonstrated that, in this system, the mechanism responsible for the prolonged persistence of LC/A was not overcome when it was fused to LC/E, despite the intrinsic rapid turnover of LC/E alone within neurons. When the dileucine within the catalytically inactive LC/A in LC_E-$BoTIM_A$ was mutated to dialanine, the persistence of intoxication from this chimeric toxin was substantially reduced near to that of native BoNT/E. Thus, by removing the dileucine signal within the inactive LC/A fusion partner, the functional LC/E protease was no longer being stabilized within the neurons by the LC/A. Surprisingly, when these authors tested the BoNT/A holotoxin with the mutation that removed amino acids 2–7 of LC/A, this toxin elicited intoxication that had the persistence of

wild type BoNT/A. Since a similar mutation of LC/A was earlier reported to eliminate the plasma membrane localization of LC/A [39], Wang et al. [45] postulated that subcellular localization probably did not play a role in BoNT/A persistence, but rather that the dileucine somehow protected the protease from proteolytic degradation.

The subcellular localizations of two additional proteases, LC/B and LC/C, have been studied following their expression by plasmid transfection. Both of these proteases appeared to be dispersed throughout the cell, not localized to the plasma membrane [41,46]. These results suggest that localization to the plasma membrane is not required for protease persistence in these other long-lasting BoNT serotypes and their persistence depends on other mechanisms.

2.2.2 Contribution of the ubiquitin-proteasome system to BoNT protease persistence—Protein turnover in cells is most commonly mediated by the lysosome/autophagy system (LAS) and/or the ubiquitin-proteasome systems (UPS) [1,47,48]. The LAS is considered to be a more general system responsible for turnover of macromolecules and the UPS is utilized for more targeted and regulated protein turnover processes. While no evidence yet exists that LAS degradation plays a role in the relative persistence of the different BoNT serotypes, there is a growing body of evidence that the UPS may be an important factor. Shi et al. [49] showed that modulation of LC/B polyubiquitination altered its protease activity. When LC/B was heavily polyubiquitinated *in vitro*, these authors found that the enzymatic activity was reduced by 34%. In the same study, LC/B polyubiquitination in SHSY-5Y cells was increased about 2.5 fold treated with phorbol 12-myristate 13-acetate (PMA), and about 3.5 fold when treated with both PMA and a proteasome inhibitor, following exposure to a very high dose of BoNT/B (10 μg/ml). The increased intracellular polyubiquitination of LC/B led to a significant reduction in intracellular VAMP (vesicle associated membrane protein) cleavage in BoNT/B treated cells although it was not possible to distinguish the contribution of reduced protease activity or accelerated LC/B turnover to this reduction.

The role of the UPS in BoNT persistence was investigated by Tsai et al, 2010 [26] through a comparison of the polyubiquitination of the persistent LC/A vs the short-lived LC/E. These authors first showed that, in N18 cells, LC/E is much more rapidly turned over than is LC/A and that this is associated with a much heavier polyubiquitination of LC/E than of LC/A. The half-life of the LC/E was significantly increased in cells treated with proteasome inhibitors suggesting a role of the UPS in LC/E turnover. Tsai et al. [26] then demonstrated that LC/A turnover could be accelerated by developing a specific biomolecule that promoted LC/A polyubiquitination. The biomolecule they designed was a fusion protein containing both an LC/A targeting protein and an E3-ligase domain (HECT domain from E6AP or RING domain from XIAP). As a targeting domain, the authors employed the SNAP-25 substrate for LC/A in which the protein was mutated to a form that could not be cleaved by LC/A (SNAP-25nc; R198T). These ‘designer ubiquitin ligases’ were co-expressed in cells with LC/A and the LC/A was found to be more heavily ubiquitinated and to have a much shorter half-life than LC/A from control cells. Kuo et al. [40] also produced biomolecules designed to promote polyubiquitination of LC/A and LC/B, and demonstrated that these agents increased intra-neuronal cell turnover of the targeted proteases and, importantly, the agents also accelerated the recovery of cells from natural BoNT/A intoxication (see Section 3). These results imply that LC/A is more resistant than LC/E to polyubiquitination in intoxicated neurons and that the polyubiquitination step may be rate limiting in the regulation of LC/A turnover- and likely the protease turnover for other persistent BoNT serotypes.

Tsai et al. performed additional studies in an effort to explain why the BoNT/A protease is unusually resistant to polyubiquitination within cells in comparison to BoNT/E protease.

The authors immunoprecipitated LC/A or LC/E from transfected N18 neuroblastoma cells and used proteomic tools to identify proteins that bound preferably to one or the other protease. One protein, TRAF2, was found to be specifically bound to LC/E. TRAF2 is a RING finger protein that is involved in TNF receptor signaling that has been implicated in ubiquitination [50]. To test for a possible role of TRAF2 in LC/E turnover, expression of this protein was either increased in cells by expression plasmid transfection or knocked down by siRNA. The half-life of LC/E was shown to be reduced by overexpression of TRAF2 and increased by knockdown of TRAF2. The outcome of these studies was that TRAF2 appears to interact specifically with LC/E and promote its turnover by the UPS, and this may explain, in part, the reduced persistence of BoNT/E intoxication. In unpublished work, a number of studies have found that LC/A binds to several de-ubiquitinases (DUBs) and that knockdown of these DUBs reduces the half-life of LC/A (G. Oyler, personal communication), suggesting the possibility that recruitment of DUBs by LC/A may play a role in stabilizing the protease and promoting persistence of BoNT/A intoxication.

2.2.3 Contribution of the BoNT heavy chain to BoNT protease persistence—

Another factor that could contribute to BoNT persistence is the uptake and protease translocation process itself. Serotype differences in the cell entry or endosomal release processes that are mediated by the BoNT translocation domains could lead to differences in the protease trafficking to its site of action or exposure of the protease to cytosolic degradation pathways. For example, differential trafficking might steer the BoNT proteases of more persistent serotypes through protected environments that result in less damage and/or more rapid access to SNARE protein substrates. Nevertheless, there is evidence, at least for BoNT/A and BoNT/E, that the receptor binding and translocation domains do not play a role in persistence. Wang et al. [51] produced chimeras in which the 50 kDa receptor binding domain of BoNT/A was replaced by the corresponding domain from BoNT/E (AE) or in which the receptor binding domain of BoNT/E was replaced by the corresponding domain from BoNT/A (EA). For these chimeras, the persistence of intoxication appeared to be unrelated to which receptor binding domain was present on the toxin. Cells treated with the AE chimera remained intoxicated about as long as cells treated with wild type BoNT/A while the EA chimera persisted about as long as intoxication with BoNT/E. Similar results were obtained in a subsequent study [52] in which this laboratory produced chimeras exchanging the BoNT/A and BoNT/B receptor binding domains. Again, even though cell susceptibility to intoxication was determined by the receptor binding domain, persistence of the resulting intoxication was determined by the LC and translocation domains. Thus for the BoNT serotypes considered the most dangerous to humans, persistence does not seem to be affected by which receptor mediates entry. However, it remains possible that the HC plays a more significant role in the persistence for other more distantly related BoNT serotypes that bind to different receptors or target different neuronal cell populations.

3. Exploiting BoNT persistence for therapeutic applications

Substantial research is currently directed at enhancing the efficacy of BoNT as a treatment for a variety of conditions or to the development of new therapeutic applications, and this research includes efforts to enhance the persistence of nerve inactivation. On the flip side, there are also a number of research groups that seek to diminish the threat posed by BoNT as an agent of bioterror, and one focus is an effort to diminish the persistence of paralysis so as to promote a more rapid recovery from toxin exposure. This research has benefited greatly from the improved understanding of the mechanisms of BoNT persistence that are outlined above. As a result, research to modify the persistence of BoNT intoxication has focused on the protease domain.

3.1 Increasing BoNT persistence for improved therapeutic benefit

Wang et al. [45] generated a chimeric toxin in an effort to combine the prolonged persistence of BoNT/A with the therapeutically beneficial features of BoNT/E such as more rapid and comprehensive intoxication. As described above (Section 2.2.1), this chimera, called LC_E-$BoTIM_A$, was a full-sized BoNT/A containing mutations in the light chain that eliminate LC/A protease activity in which the LC/E was appended to the amino terminus to create a protease dimer. The chimeric protein was found to intoxicate neurons with the persistence of BoNT/A and to permit the intoxication of sensory neurons not normally sensitive to BoNT/E. The authors claim that their LC_E-$BoTIM_A$ toxin is a long-acting agent with potential for pain therapy and other conditions caused by overactive cholinergic nerves.

3.2 Reversing paralysis by accelerated turnover of BoNT protease

The first report of an agent that was created with the purpose of promoting ubiquitination and accelerated turnover of a BoNT protease was by Tsai et al. [26]. In their study, described above in Section 2.2.2, a fusion protein was produced in which E3-ligases were joined to a mutant form of SNAP-25 (SNAP-25nc) that was not cleaved by the BoNT/A protease, LC/A. Cells expressing these ‘designer ubiquitin ligases’, successfully promoted increased polyubiquitination and accelerated turnover of co-expressed LC/A, presumably because the SNAP-25nc recruited the LC/A to become a substrate for E3-ligase action. This strategy is not particularly practical for therapeutic use because of difficulties commercially producing such a complex protein and delivering it to the cytosol of intoxicated neurons in patients. In addition, different non-cleavable SNARE proteins would need to be developed for each BoNT serotype and there are concerns about the off-target effects of SNARE-based E3-ligases leading to degradation of other cellular proteins.

An alternative strategy for promoting accelerated ubiquitination and turnover of BoNT proteases was proposed by Kuo et al. [40]. Like Tsai et al. [26], the approach was to deliver a fusion protein to intoxicated neurons that would lead to polyubiquination and rapid turnover of the intoxicating BoNT protease. The new concept was to use camelid heavy-chain-only V_H domains (VHHs) as targeting domains that recognized BoNT proteases. These are small (14 kDa) proteins that retain their binding activity and specificity within neurons [53] and can be readily developed for proteases from all known BoNT serotypes. Since VHHs should be highly specific only to their LC target, side-effects following their delivery to cells should be of minimal concern. In place of a large E3-ligase, Kuo et al. proposed to employ a minimal F-box domain derived from β-TrCP, a protein known to be expressed within neurons [54]. The F-box domain is responsible for recruiting an E3-ligase to β-TrCP and promoting polyubiquitination of bound proteins. As a fusion partner with a VHH, this small F-box domain (15 kDa) was expected to be sufficient to recruit neuronal E3-ligases to the BoNT protease bound by the VHH for polyubiquitination. Kuo et al. demonstrated that expression of a VHH/F-box fusion protein, called a targeted F-box or TFB (Figure 2), in which the VHH was specific for LC/A, led to polyubiquitination of LC/A and to a much more rapid turnover of this protease within both N2A and M17 neuroblastoma cells. Replacing the VHH with a different VHH that binds to BoNT/B protease, LC/B, resulted in a new TFB that promoted turnover of LC/B. Using the TFBs targeting LC/A or B resulted only in the accelerated turnover of the targeted protease and had no effect on the non-targeted protease, even within the same cell population. Most importantly, Kuo et al. demonstrated that their TFB agents were effective in promoting the recovery of neuronal cells that were naturally intoxicated by BoNT/A. Using SNAP-25 as an indicator, M17 neuroblastoma cells expressing an LC/A-targeted TFB recovered from BoNT/A intoxication at least 2.5 fold faster than the control cells. Having validated the approach for both BoNT/A and BoNT/B, development of TFB agents targeting other BoNT serotypes should quickly follow as it requires only that VHHs binding the other LCs be

identified using established methods [53]. These results suggest it will be possible to accelerate recovery from botulism caused by any toxic serotype if the appropriate TFB agent can be successfully delivered to the cytosol of BoNT intoxicated neurons. Therapeutic delivery strategies might employ protein vehicles such as atoxic mutants of neurotoxins (e.g. BoNT [55]) or non-specific toxins that have been re-targeted for neuronal specificity (e.g. *C. difficile* toxin [56]), or perhaps genetic vehicles such as viruses modified for motor neuron tropism.

4. Conclusions

There are seven known serotypes of Botulinum neurotoxins (BoNTs) and these elicit variable levels of toxicity and persistence when different animal species become exposed. The preponderance of research on these toxins has sensibly been focused on humans and the common rodent models- so little is known regarding toxicity and persistence in most other animal species. In comparing human and rodent BoNT intoxication, there is substantial consistency with regard to the relative persistence of the different serotypes which suggests that the mechanisms of persistence that are identified in rodent models will likely predict those in humans. The persistence of paralysis occurring with BoNT intoxication is clearly dependent on the maintenance of truncated SNARE proteins following their cleavage by the BoNT proteases within motor neurons. The truncated SNARE proteins may themselves persist in the nerve endings, and/or the BoNT protease may persist and continue to cleave newly synthesized SNARE proteins. Recent literature would suggest that, for the longest lived BoNT serotype, BoNT/A, the persistence of intoxication primarily depends on the ability of the BoNT protease to resist natural turnover via the ubiquitin-proteasome system (UPS). Quite possibly, with other much shorter lived BoNT proteases such as from BoNT/E, it is the turnover of the truncated SNARE protein that determines the rate of recovery from intoxication. It does appear clear that efforts to develop BoNT-based therapeutics with improved persistence properties will benefit from alterations to the native toxins which increases the intracellular half-life of the protease, for example, by reducing its recognition by the UPS. Similarly, new treatments for reversing unwanted BoNT intoxication, such as following a bioterror incident, are likely to be possible through the delivery of biomolecules that accelerate the turnover rate of the BoNT protease. Such new therapeutic approaches are becoming increasingly practical as we gain new understanding of the mechanisms by which these remarkable toxin biomolecules enter and persist within their motor neuron targets.

Abbreviations

BoNT	Botulinum neurotoxin
UPS	Ubiquitin-proteasome system
LAS	Lysosome/autophagy system
GFP	Green fluorescent protein
LC/X	Light chain of BoNT/A-G
PMA	Phorbol 12-myristate 13-acetate
SNAP-25	Synaptosomal associated protein of 25 kDa
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TFB	Targeted F-box
TNF	Tumor necrosis factor
VAMP	Vesicle-associated membrane protein

VHH Camelid heavy-chain-only V_H domains

References

1. Wong E, Cuervo AM. Integration of clearance mechanisms: the proteasome and autophagy. Cold Spring Harbor perspectives in biology. 2010; 2:a006734. [PubMed: 21068151]
2. Whittaker RL, Gilbertson RB, Garrett AS Jr. Botulism, Type E; Report of Eight Simultaneous Cases. Ann Intern Med. 1964; 61:448–454. [PubMed: 14218929]
3. Eleopra R, Tugnoli V, Rossetto O, Montecucco C, De Grandis D. Botulinum neurotoxin serotype C: a novel effective botulinum toxin therapy in human. Neuroscience letters. 1997; 224:91–94. [PubMed: 9086464]
4. Sloop RR, Cole BA, Escutin RO. Human response to botulinum toxin injection: type B compared with type A. Neurology. 1997; 49:189–194. [PubMed: 9222189]
5. Eleopra R, Tugnoli V, Rossetto O, De Grandis D, Montecucco C. Different time courses of recovery after poisoning with botulinum neurotoxin serotypes A and E in humans. Neuroscience letters. 1998; 256:135–138. [PubMed: 9855358]
6. Brin MF, Lew MF, Adler CH, Comella CL, Factor SA, et al. Safety and efficacy of NeuroBloc (botulinum toxin type B) in type A-resistant cervical dystonia. Neurology. 1999; 53:1431–1438. [PubMed: 10534247]
7. Meunier FA, Lisk G, Sesardic D, Dolly JO. Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation. Molecular and cellular neurosciences. 2003; 22:454–466. [PubMed: 12727443]
8. Adler M, Keller JE, Sheridan RE, Deshpande SS. Persistence of botulinum neurotoxin A demonstrated by sequential administration of serotypes A and E in rat EDL muscle. Toxicon : official journal of the International Society on Toxinology. 2001; 39:233–243. [PubMed: 10978741]
9. Sellin LC, Kauffman JA, Dasgupta BR. Comparison of the effects of botulinum neurotoxin types A and E at the rat neuromuscular junction. Medical biology. 1983; 61:120–125. [PubMed: 6308364]
10. Keller JE. Recovery from botulinum neurotoxin poisoning in vivo. Neuroscience. 2006; 139:629–637. [PubMed: 16490322]
11. Adler M, Macdonald DA, Sellin LC, Parker GW. Effect of 3,4-diaminopyridine on rat extensor digitorum longus muscle paralyzed by local injection of botulinum neurotoxin. Toxicon : official journal of the International Society on Toxinology. 1996; 34:237–249. [PubMed: 8711757]
12. Dolly, JO.; Lisk, G.; Foran, PG.; Meunier, F.; Mohammed, N.; O'Sullivan, G.; de Paiva, A. Insights into the extended duration of neuroparalysis by Botulinum Neurotoxin A relative to the other shorter-acting serotypes: differences between motor nerve terminals and cultured neurons. In: Brin, MF.; Hallett, M.; Jankovic, J., editors. Scientific and Therapeutic Aspects of Botulinum Toxin. Vol. 91. Philadelphia: Lippincott Williams & Wilkins; 2002. p. 102
13. Morbiato L, Carli L, Johnson EA, Montecucco C, Molgo J, et al. Neuromuscular paralysis and recovery in mice injected with botulinum neurotoxins A and C. The European journal of neuroscience. 2007; 25:2697–2704. [PubMed: 17561839]
14. Kauffman JA, Way JF Jr, Siegel LS, Sellin LC. Comparison of the action of types A and F botulinum toxin at the rat neuromuscular junction. Toxicology and applied pharmacology. 1985; 79:211–217. [PubMed: 2988154]
15. de Paiva A, Meunier FA, Molgo J, Aoki KR, Dolly JO. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci U S A. 1999; 96:3200–3205. [PubMed: 10077661]
16. Brown MC, Holland RL, Hopkins WG. Motor nerve sprouting. Annual review of neuroscience. 1981; 4:17–42.
17. Angaut-Petit D, Molgo J, Comella JX, Faille L, Tabti N. Terminal sprouting in mouse neuromuscular junctions poisoned with botulinum type A toxin: morphological and electrophysiological features. Neuroscience. 1990; 37:799–808. [PubMed: 1701041]

18. Meunier FA, Schiavo G, Molgo J. Botulinum neurotoxins: from paralysis to recovery of functional neuromuscular transmission. Journal of physiology, Paris. 2002; 96:105–113.
19. Foran PG, Mohammed N, Lisk GO, Nagwaney S, Lawrence GW, et al. Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A. Basis for distinct durations of inhibition of exocytosis in central neurons. J Biol Chem. 2003; 278:1363–1371. [PubMed: 12381720]
20. Ferrer-Montiel AV, Gutierrez LM, Apland JP, Canaves JM, Gil A, et al. The 26-mer peptide released from SNAP-25 cleavage by botulinum neurotoxin E inhibits vesicle docking. FEBS Lett. 1998; 435:84–88. [PubMed: 9755864]
21. Gutierrez LM, Viniegra S, Rueda J, Ferrer-Montiel AV, Canaves JM, et al. A peptide that mimics the C-terminal sequence of SNAP-25 inhibits secretory vesicle docking in chromaffin cells. J Biol Chem. 1997; 272:2634–2639. [PubMed: 9006897]
22. Gutierrez LM, Canaves JM, Ferrer-Montiel AV, Reig JA, Montal M, et al. A peptide that mimics the carboxy-terminal domain of SNAP-25 blocks Ca(2+)-dependent exocytosis in chromaffin cells. FEBS Lett. 1995; 372:39–43. [PubMed: 7556639]
23. Mehta PP, Battenberg E, Wilson MC. SNAP-25 and synaptotagmin involvement in the final Ca(2+)-dependent triggering of neurotransmitter exocytosis. Proc Natl Acad Sci U S A. 1996; 93:10471–10476. [PubMed: 8816825]
24. Huang X, Wheeler MB, Kang YH, Sheu L, Lukacs GL, et al. Truncated SNAP-25 (1-197), like botulinum neurotoxin A, can inhibit insulin secretion from HIT-T15 insulinoma cells. Molecular endocrinology. 1998; 12:1060–1070. [PubMed: 9658409]
25. Varshavsky A. The N-end rule: functions, mysteries, uses. Proc Natl Acad Sci U S A. 1996; 93:12142–12149. [PubMed: 8901547]
26. Tsai YC, Maditz R, Kuo CL, Fishman PS, Shoemaker CB, et al. Targeting botulinum neurotoxin persistence by the ubiquitin-proteasome system. Proc Natl Acad Sci U S A. 2010; 107:16554–16559. [PubMed: 20823219]
27. Raciborska DA, Charlton MP. Retention of cleaved synaptosome-associated protein of 25 kDa (SNAP-25) in neuromuscular junctions: a new hypothesis to explain persistence of botulinum A poisoning. Canadian journal of physiology and pharmacology. 1999; 77:679–688. [PubMed: 10566945]
28. Bajohrs M, Rickman C, Binz T, Davletov B. A molecular basis underlying differences in the toxicity of botulinum serotypes A and E. EMBO reports. 2004; 5:1090–1095. [PubMed: 15486565]
29. Otto H, Hanson PI, Chapman ER, Blasi J, Jahn R. Poisoning by botulinum neurotoxin A does not inhibit formation or disassembly of the synaptosomal fusion complex. Biochemical and biophysical research communications. 1995; 212:945–952. [PubMed: 7626135]
30. Hayashi T, McMahon H, Yamasaki S, Binz T, Hata Y, et al. Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. Embo J. 1994; 13:5051–5061. [PubMed: 7957071]
31. Bartels F, Bergel H, Bigalke H, Frevert J, Halpern J, et al. Specific antibodies against the Zn(2+)-binding domain of clostridial neurotoxins restore exocytosis in chromaffin cells treated with tetanus or botulinum A neurotoxin. J Biol Chem. 1994; 269:8122–8127. [PubMed: 8132537]
32. Keller JE, Neale EA, Oyler G, Adler M. Persistence of botulinum neurotoxin action in cultured spinal cord cells. FEBS Lett. 1999; 456:137–142. [PubMed: 10452545]
33. O'Sullivan GA, Mohammed N, Foran PG, Lawrence GW, Oliver Dolly J. Rescue of exocytosis in botulinum toxin A-poisoned chromaffin cells by expression of cleavage-resistant SNAP-25. Identification of the minimal essential C-terminal residues. J Biol Chem. 1999; 274:36897–36904. [PubMed: 10601242]
34. Hanig JP, Lamanna C. Toxicity of botulinum toxin: a stoichiometric model for the locus of its extraordinary potency and persistence at the neuromuscular junction. Journal of Theoretical Biology. 1979; 77:107–113. [PubMed: 221751]
35. Erdal E, Bartels F, Binscheck T, Erdmann G, Frevert J, et al. Processing of tetanus and botulinum A neurotoxins in isolated chromaffin cells. Naunyn-Schmiedeberg's archives of pharmacology. 1995; 351:67–78.

36. Yamaizumi M, Mekada E, Uchida T, Okada Y. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. Cell. 1978; 15:245–250. [PubMed: 699044]
37. Eiklid K, Olsnes S, Pihl A. Entry of lethal doses of abrin, ricin and modeccin into the cytosol of HeLa cells. Experimental cell research. 1980; 126:321–326. [PubMed: 7363949]
38. Bade S, Rummel A, Reisinger C, Karnath T, Ahnert-Hilger G, et al. Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates. J Neurochem. 2004; 91:1461–1472. [PubMed: 15584922]
39. Fernandez-Salas E, Steward LE, Ho H, Garay PE, Sun SW, et al. Plasma membrane localization signals in the light chain of botulinum neurotoxin. Proc Natl Acad Sci U S A. 2004; 101:3208–3213. [PubMed: 14982988]
40. Kuo CL, Oyler GA, Shoemaker CB. Accelerated neuronal cell recovery from *Botulinum* neurotoxin intoxication by targeted ubiquitination. PLos ONE. 2011 In press.
41. Fernandez-Salas E, Ho H, Garay P, Steward LE, Aoki KR. Is the light chain subcellular localization an important factor in botulinum toxin duration of action? Mov Disord. 2004; 19(Suppl 8):S23–34. [PubMed: 15027051]
42. Montal M. Botulinum neurotoxin: a marvel of protein design. Annual review of biochemistry. 2010; 79:591–617.
43. Chen S, Barbieri JT. Association of botulinum neurotoxin serotype A light chain with plasma membrane-bound SNAP-25. J Biol Chem. 2011; 286:15067–15072. [PubMed: 21378164]
44. Aiken C, Konner J, Landau NR, Lenburg ME, Trono D. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell. 1994; 76:853–864. [PubMed: 8124721]
45. Wang J, Zurawski TH, Meng J, Lawrence G, Olango WM, et al. A dileucine in the protease of botulinum toxin A underlies its long-lived neuroparalysis: transfer of longevity to a novel potential therapeutic. J Biol Chem. 2011; 286:6375–6385. [PubMed: 21138836]
46. Land J, Zhang H, Vaidyanathan VV, Sadoul K, Niemann H, et al. Transient expression of botulinum neurotoxin C1 light chain differentially inhibits calcium and glucose induced insulin secretion in clonal beta-cells. FEBS Lett. 1997; 419:13–17. [PubMed: 9426210]
47. Hershko A, Ciechanover A. The ubiquitin system. Annual review of biochemistry. 1998; 67:425–479.
48. Ciechanover A. Proteolysis: from the lysosome to ubiquitin and the proteasome. Nature reviews Molecular cell biology. 2005; 6:79–87.
49. Shi X, Curran JE, Liao Z, Gordon RK. The biological activity of ubiquitinated BoNT/B light chain in vitro and in human SHSY-5Y neuronal cells. Journal of cellular biochemistry. 2009; 108:660–667. [PubMed: 19681043]
50. Chen ZJ. Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol. 2005; 7:758–765. [PubMed: 16056267]
51. Wang J, Meng J, Lawrence GW, Zurawski TH, Sasse A, et al. Novel chimeras of botulinum neurotoxins A and E unveil contributions from the binding, translocation, and protease domains to their functional characteristics. J Biol Chem. 2008; 283:16993–17002. [PubMed: 18400760]
52. Wang J, Zurawski TH, Bodeker MO, Meng J, Boddul S, et al. Longer-acting and highly potent chimaeric inhibitors of excessive exocytosis created with domains from botulinum neurotoxin A and B. Biochem J. 2012; 444:59–67. [PubMed: 22360156]
53. Tremblay JM, Kuo CL, Abeijon C, Sepulveda J, Oyler G, et al. Camelid single domain antibodies (VHHs) as neuronal cell intrabody binding agents and inhibitors of Clostridium botulinum neurotoxin (BoNT) proteases. Toxicon : official journal of the International Society on Toxinology. 2010; 56:990–998. [PubMed: 20637220]
54. Westbrook TF, Hu G, Ang XL, Mulligan P, Pavlova NN, et al. SCFbeta-TRCP controls oncogenic transformation and neural differentiation through REST degradation. Nature. 2008; 452:370–374. [PubMed: 18354483]
55. Webb RP, Smith TJ, Wright P, Brown J, Smith LA. Production of catalytically inactive BoNT/A1 holoprotein and comparison with BoNT/A1 subunit vaccines against toxin subtypes A1, A2, and A3. Vaccine. 2009; 27:4490–4497. [PubMed: 19450643]

56. Krautz-Peterson G, Zhang Y, Chen K, Oyler GA, Feng H, et al. Retargeting Clostridium difficile Toxin B to Neuronal Cells as a Potential Vehicle for Cytosolic Delivery of Therapeutic Biomolecules to Treat Botulism. Journal of toxicology. 2012; 2012:760142. [PubMed: 21941543]

1. SNARE cleavage products of BoNT persist and maintain block to exocytosis
    a. Cleaved SNARE remains in SNARE complexes inhibiting exocytosis
    b. Cleaved SNARE remains free and inhibits new SNARE complex formation
    c. Small peptide cleaved from SNARE remains and inhibits new SNARE formation

2. BoNT protease persists and continues to cleave new SNARE proteins
    a. Protease localizes to membrane and becomes sequestered from degradation pathways
    b. Protease naturally resists native degradation pathways
    c. Protease actively recruits inhibitors of native degradation pathways
        i. Proteasome
        ii. Lysosome
    d. Protease enters a protected environment within the cytosol following translocation from the endosomes

Figure 1.
Summary of proposed mechanisms of BoNT persistence

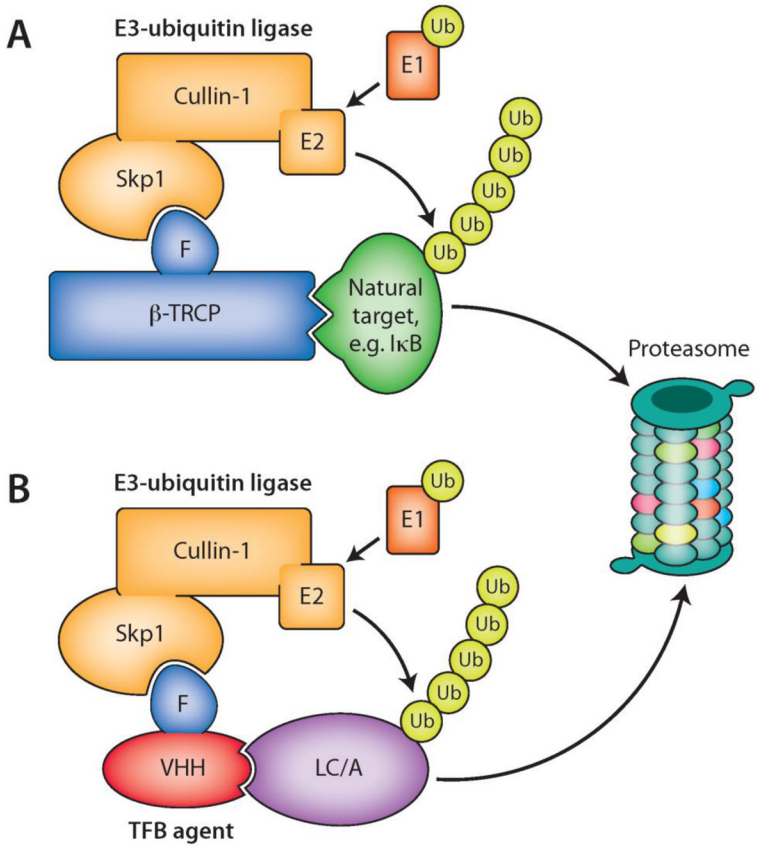

Figure 2. Targeted F-box (TFB) agents exploit the natural process by which F-box proteins promote accelerated turnover of cytosolic proteins

A. F-box proteins participate in the proteasome-mediated turnover of natural cellular proteins. Ubiquitin (Ub) is activated by E1 and transferred to E2 which associates with Cullin-1 and Skp1 to form the E3 ubiquitin ligase. F-box proteins such as β-TrCP bind to natural targets, such as IκB, and recruit the E3 ubiquitin ligases through binding of the small F-box domain on β-TrCP to the Skp1 component of the ligase. The E3 ubiquitin ligase then attaches a chain of Ub molecules to the natural target and this polyubiquitinated protein becomes a substrate for proteasome-mediated degradation. **B.** TFB agents exploit natural pathways to promote accelerated turnover of selected cytosolic proteins such as BoNT proteases. The F-box domain from β-TrCP is expressed as a fusion protein to a VHH with affinity for a BoNT protease such as LC/A. This TFB agent binds to the LC in the cytosol of an intoxicated neuron and the F-box component recruits and E3-ubiquitin ligase which polyubiquitinates the LC and promotes its accelerated turnover by cytosolic proteasomes.