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16 Mechanism of Action of Botulinum Neurotoxin and Overview of Medical Countermeasures for Intoxication

Michael Adler, George Oyler, James P. Apland, Sharad S. Deshpande, James D. Nicholson, Jaime Anderson, Charles B. Millard, and Frank J. Lebeda

CONTENTS

The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the view of the Department of the Army or the Department of Defense.

I. INTRODUCTION

The botulinum neurotoxins (BoNTs)* comprise a family of seven distinct neurotoxic proteins (A–G) produced by immunologically discrete strains of the anaerobic bacterium *Clostridium botulinum* and in rare cases by *Clostridium baratii* and *Clostridium butyricum* (Habermann and Dreyer, 1986; Harvey et al., 2002; Simpson, 2004). These toxins act on peripheral cholinergic synapses to inhibit spontaneous and impulse-dependent release of acetylcholine (ACh) (Brooks, 1956; Kao et al., 1976). Intoxication by BoNT results in muscle weakness, which can be fatal when the diaphragm and intercostal muscles become sufficiently compromised to impair ventilation (Dickson and Shevky, 1923). The BoNTs are the most potent substances in nature, and exposure to as little as 1–3 ng/kg may be sufficient to cause human lethality (Gill, 1982; Middlebrook and Franz, 1997; Arnon et al., 2001).

The purpose of this chapter is to use the insights gained in our understanding of the mechanism of BoNT action to establish a conceptual framework within which to develop effective treatment strategies for intoxication. The chapter is organized into three major topics: (1) an overview of BoNT action, (2) a description of the manifestations of botulism, and (3) an evaluation of conventional and emergent treatment options. From the first description of botulism in 1793 until the mid-1950s, BoNT was primarily viewed as a public health problem because of its association with food poisoning (Gill, 1982; Hatheway, 1988; Shapiro et al., 1998). Although implicated in only a small fraction of foodborne illnesses (<0.1%), the severity of the clinical syndrome produced by BoNT and the potential for numerous casualties led each outbreak to be considered as a potential health crisis (Hatheway, 1988; Smith and Sugiyama, 1988; Snydman, 1989; Shapiro et al., 1998). Due to its selective targeting of peripheral cholinergic synapses, BoNT has also been used as a tool to study cholinergic pathways, especially to explore the influence of synaptic inactivity and ACh deprivation on muscle function (Drachman and Johnston, 1975; Kao et al., 1976; Thesleff, 1989; Thesleff et al., 1990).

During World War II, BoNT was developed as a biological weapon, because of its potential to create mass casualties on the battlefield (Franz, 1997; Grace, 2003). The battlefield use of BoNT is now viewed as less likely following adoption of the 1972 Biological and Toxin Weapons Convention and the dissolution of the Soviet Union. However, the Iraqi stockpiling of BoNT before the Persian Gulf War of 1991 reveals the ability of a determined nation to acquire biological weapons in relative secrecy (Shoham, 2000; Arnon et al., 2001).

With the rise of global terrorism, exemplified by organizations such as the Japanese Aum Shinrikyo cult and al Qaeda, the potential use of BoNT as a bioterrorist weapon has become a more immediate threat (Franz et al., 1997; Middlebrook and Franz, 1997; Arnon et al., 2001; Grace, 2003). BoNT is well suited for this role because of its extraordinary lethality, capacity to elicit panic, and potential to disrupt the public health system (Atlas, 1998). Additional attributes of BoNT include widespread availability, low cost, and ease of production, transport, and concealment (Arnon et al., 2001). These considerations have led BoNT to be classified as a Category A biothreat agent by the Centers for Disease Control and Prevention (CDC) (Lohenry and Foulke, 2006). Bioterrorist attacks are generally thought to involve dispersal of BoNT as an aerosol, but the toxin can also be used to contaminate the food supply (Wein and Liu, 2005).

Systematic research on the mechanism of action of BoNT began with Emile Pierre van Ermengem's historic isolation and characterization of *C. botulinum* following a large outbreak in Ellezelles, Belgium, in 1895 and has continued with increasing interest and enthusiasm to the present time (Simpson, 2004; Grumelli et al., 2005; Rossetto et al., 2006; Singh, 2006). Early work on BoNT intoxication revealed the existence of multiple serotypes, localized the site of action to peripheral cholinergic synapses, proposed the mechanism of impaired ACh release, and ruled out noncholinergic, sensory, and central nervous system (CNS) involvement (Dickson and Shevky,

* In this chapter, BoNT is used to designate both pure botulinum neurotoxin as well as the neurotoxin complex. Some authors prefer the designation of BoTx for the latter.

1923; Guyton and MacDonald, 1947; Burgen et al., 1949; Ambache, 1951; Brooks, 1956). Most of these findings were established by the mid-1950s and refined during the next three decades (Lundh et al., 1977; Simpson, 1981; Thesleff, 1989).

The remarkable specificity for peripheral cholinergic synapses and long duration of action led to the use of BoNT/A for a growing number of focal dystonias and movement disorders following its approval in 1989 as an "orphan drug" by the U.S. Food and Drug Administration (FDA) for the treatment of strabismus, blepharospasm, and hemifacial spasm (Jankovic and Brin, 1997; Schantz and Johnson, 1997). The attributes that render BoNT a deadly poison also make the neurotoxin an ideal therapeutic agent to treat diseases of muscle hyperactivity. In addition to its original indications, BoNTs are also used for treatment of spasticity following brain and spinal cord injuries, stroke, multiple sclerosis, cerebral palsy, and numerous other disorders. Expansion and refinement in its clinical use constitute the most active focus of current BoNT research, and a number of excellent reviews have been published (Jankovic and Brin, 1997; Schantz and Johnson, 1997; Tugnoli et al., 1997; Johnson, 1999; Aoki, 2002; Charles, 2004; Chaddock and Marks, 2006; Eleopra et al., 2006; Dutton and Fowler, 2007).

In addition to discovering additional indications for the native neurotoxin, a promising new approach has been to alter the BoNT-binding domain to retarget the modified toxins to noncholinergic sites. Notable examples include a novel conjugate of BoNT/A, whose binding domain was replaced by *Erythrina cristagalli* lectin for targeting to pain fibers (Chaddock et al., 2004), and a modified BoNT/C1 in which the binding domain was replaced by epidermal growth factor for targeting to epithelial cells to inhibit excess mucus secretion (Foster et al., 2006). The former has potential for relief of chronic pain, whereas the latter may be useful for treatment of asthma and chronic obstructive pulmonary disease.

During the last two decades, enormous progress has been made in understanding the action of BoNT at the molecular level. This was spurred by a number of crucial developments: (1) elucidation of the amino acid sequence leading to recognition of the zinc-binding motif (Jongeneel et al., 1989), (2) demonstration of zinc metalloprotease activity with identification of substrates and cleavage sites (Schiavo et al., 1992a, 1992b, 1993, 1994; Blasi et al., 1993a, 1993b; Montecucco et al., 1994; Yamasaki et al., 1994), (3) solution of the crystal structure for BoNT beginning with serotypes A and B (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000), and (4) elucidation of the protein receptor for BoNT/B, BoNT/G, and BoNT/A (Nishiki et al., 1994; Dong et al., 2003, 2006; Rummel et al., 2004, 2007; Chai et al., 2006; Jin et al., 2006). These developments provided a detailed understanding of the mechanisms of actions of BoNT and opened the possibility for rational studies of pharmacological antagonists for BoNT toxicity.

A. Characteristics of BoNT Intoxication

The typical manifestation of botulism is a flaccid paralysis that is bilateral and descending, involving skeletal muscle and structures innervated by autonomic ganglia (Habermann and Dreyer, 1986; Smith and Sugiyama, 1988; Merz et al., 2003). Human intoxication is caused by serotypes A, B, E, and, to a much lesser extent, F, and is generally manifested as foodborne, wound, and intestinal (infant) botulism (Simpson, 1981). Wound and infant botulism are usually mediated by serotypes A and B (Pickett et al., 1976; Arnon, 1995). Two additional forms of botulism have been observed that do not occur in nature: inhalation botulism and iatrogenic botulism. The former is so rare in humans that only one occurrence, a laboratory accident, has ever been reported (Holzer, 1962). An outbreak of inhalation botulism would be suspected as a terrorist incident unless other causes were found (Arnon et al., 2001; Park and Simpson, 2003; Adler, 2006). Iatrogenic botulism stems from overdose of clinically or cosmetically used BoNT (Klein, 2004). A recent case in which four individuals were injected with multiple lethal doses of a nonapproved preparation of BoNT/A during a cosmetic procedure illustrates the potential hazard of this otherwise safe use of BoNT (Chertow et al., 2006; Souayah et al., 2006).

C. botulinum spores are widely distributed in soils, sea sediments, decaying vegetation, animal carcasses, and sewage (Smith, 1978). The intestinal tracts of birds, mammals, and fish may also acquire *C. botulinum* as a transient member of their intestinal flora. The hosts do not exhibit botulism since growth of these anaerobic bacteria is suppressed when there is competition from other organisms and a functional immune system (Smith, 1978; Smith and Sugiyama, 1988; Snydman, 1989). The resistance of clostridial spores to harsh environmental conditions enables their dissemination by air currents and dust particles, leading to surface contamination of exposed food products (Stinger et al., 2005). Botulism is not contagious, however, and contact with spores does not usually lead to disease except in young infants under 1 year in age (infant botulism), in adults with altered gastrointestinal (GI) anatomy and microflora (adult intestinal botulism), or following germination in wounds (wound botulism) (Mershon and Dowell, 1973; MacKenzie et al., 1982; Arnon, 1995; Shapiro et al., 1998).

B. Symptomology

The clinical syndrome of botulism reflects toxin-induced blockade of ACh release from neuromuscular and neuroeffector junctions (Burgen et al., 1949; Ambache, 1951). The basic syndrome of BoNT intoxication is similar for foodborne, intestinal, and wound botulism and does not vary appreciably among toxin serotypes (Sobel, 2005). The earliest symptoms generally include visual disturbances (diplopia, blurred vision) and xerostomia (Hughes and Tacket, 1983). With low-level exposure, these symptoms may gradually resolve, even in the absence of medical intervention. In more severe cases, the initial symptoms are followed by dysphasia, dysphonia, and dysarthria, reflecting an especially high susceptibility of cranial efferent terminals to BoNT action (Shapiro et al., 1998). A descending generalized skeletal muscle weakness may then develop, progressing from the upper to the lower extremities. Involvement of the diaphragm and intercostal muscles can lead to ventilatory failure and death, unless appropriate supportive care is provided (Cherington, 1998; Robinson and Nahata, 2003; Sobel, 2005). Although motor function is severely impaired, there is little or no sensory alteration or CNS involvement in botulinum intoxication (Simpson, 1981).

Symptoms are usually observed 12–36 h after exposure, although onset times as short as 4 h or as long as 8 days have been reported (Robinson and Nahata, 2003; Sobel, 2005). The preponderance of symptoms, including the potentially lethal respiratory collapse, stems from inhibition of neuromuscular transmission (Burgen et al., 1949; Brooks, 1956; Kao et al., 1976; Simpson, 1981). Parasympathetic dysfunction is responsible for blurred vision, xerostomia, constipation, and urinary retention (Ambache, 1951; MacKenzie et al., 1982; Merz et al., 2003).

C. Functional Domains of BoNT

The BoNTs are synthesized as ~150 kDa single-chain protoxins (range, 140–167 kDa). They are proteolytically activated (nicked) to form dichain molecules consisting of a ~50 kDa light chain (LC) and a ~100 kDa heavy chain (HC) (DasGupta and Sugiyama, 1972; Bandyopadhyay et al., 1987). The two chains are coupled by a single disulfide bond and by noncovalent forces. In their natural state, BoNTs exist as complexes consisting of ~150 kDa neurotoxin associated with a group of nontoxic proteins. The latter are designated as neurotoxin-associated proteins (NAPs), some of which possess hemagglutinin activity (Sakaguchi, 1982; Sharma et al., 2006). NAPs associate with BoNT in the bacterial culture medium by noncovalent interactions and protect the neurotoxin from proteolytic and low pH-mediated inactivation. They have also been suggested to facilitate absorption of BoNT from the GI tract into the bloodstream (Sharma and Singh, 1998). The ability of BoNT to manifest oral toxicity has generally been attributed to the presence of these proteins; conversely, the inability of the related tetanus neurotoxin (TeNT) to produce foodborne intoxication has been ascribed to the absence of such NAPs (Singh et al., 1995). Maksymowych et al. (1999) have raised some questions on the importance of NAPs in BoNT toxicity, especially

with regard to their role in transcytosis of the neurotoxin. These investigators demonstrated that pure BoNT/A lacking NAPs was still toxic to mice following intragastric administration, although to a lesser extent than the toxin complex. When examined at elevated concentrations, the differences in efficacy between pure and NAP-containing neurotoxin were progressively reduced. These results indicate that pure neurotoxin does not require accessory proteins for absorption from the GI tract. Moreover, even though the NAPs are clearly protective, sufficient pure neurotoxin can survive the inhospitable environment of the GI tract to produce lethality.

In conformity with the sequential processing of bacterial protein toxins such as diphtheria or cholera toxin, the action of BoNT involves multiple discrete steps: binding to surface receptors, internalization via receptor-mediated endocytosis, and translocation from endosome to cytosol. For BONT, the final step is cleavage of soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) in the cytosol (Simpson, 1981, 2004; Montecucco et al., 1994). Binding and translocation are mediated by the C- and N-terminal domains of the BoNT HC, respectively (Daniels-Holgate and Dolly, 1996; Koriazova and Montal, 2003; Simpson, 2004; Fisher and Montal, 2006). The LC has zinc metalloprotease activity, targeted to one of the three SNARE proteins (SNAP-25, synaptobrevin, or syntaxin) that are required for the docking and fusion of synaptic vesicles with active zones at the cytoplasmic surface of the nerve terminal (Schiavo et al., 1992a; Montecucco and Schiavo, 1993; Montecucco et al., 1994; Schiavo et al., 2000).

Serotypes B, D, F, and G cleave different sites on the synaptic vesicle protein, synaptobrevin (VAMP), whereas serotypes A and E cleave the presynaptic membrane-associated protein SNAP-25 (Schiavo et al., 2000; Simpson, 2004). Serotype C1 is unique in that it cleaves two cytoplasmic proteins, syntaxin and SNAP-25 (Williamson et al., 1996). Interaction of these SNAREs on the surface of synaptic vesicles and active zone membranes is required for voltage- and Ca^{2+}-dependent release of neurotransmitter; cleavage by BoNT inhibits this process, leading to muscle weakness and paralysis (Sutton et al., 1998; Schiavo et al., 2000). Cleavage of SNARE proteins appears to be sufficient to account for all actions of the BoNTs, and the SNARE hypothesis has received near universal acceptance since its introduction in the early 1990s.

For each BoNT serotype, the dichain form constitutes the active configuration of the neurotoxin; the isolated LC and HC are devoid of systemic toxicity. The absence of toxicity is consistent with findings that the LC cannot gain access to the cytosol unless it is coupled to the HC and that the HC lacks the ability to inhibit neurotransmitter release (Stecher et al., 1989; Goodnough et al., 2002). The isolated LC does, however, remain enzymatically active as evidenced by its ability to inhibit exocytosis from permeabilized chromaffin cells (Stecher et al., 1989), by its ability to cleave SNARE proteins in cell-free assays (Adler et al., 1998), and by its capacity to inhibit ACh release in skeletal muscle when delivered by liposomes (de Paiva and Dolly, 1990). It is not clear whether any portion of the HC is translocated along with the LC, and if so, whether it exerts a role in enhancing the catalytic activity or stability of the LC.

All BoNT serotypes suppress ACh release, show high specificity for cholinergic synapses, and share the same overall mode of action; they differ, however, in potency and in duration of action. Type A neurotoxin exhibits the highest potency (Gill, 1982), and types A and C1 produce the longest intoxication times (Eleopra et al., 1998; Keller et al., 1999; Adler et al., 2001; Keller and Neale, 2001; Foran et al., 2003; Keller, 2006). Other differences include targeting of different functional surface receptors on the motor nerve terminal (Black and Dolly, 1986; Montecucco, 1986; Daniels-Holgate and Dolly, 1996; Rummel et al., 2007), and cleaving unique peptide bonds in the appropriate SNARE proteins (Schiavo et al., 2000; Simpson, 2004; Rossetto et al., 2006).

II. MANIFESTATIONS OF BOTULISM

Botulinum intoxication generally results from ingestion of preformed toxin elaborated in contaminated foods (foodborne) or from colonization by *C. botulinum* of deep wounds with subsequent production of toxin (wound botulism) (Mershon and Dowell, 1973; Snydman, 1989). A third form,

termed intestinal botulism, is observed in young infants (infant botulism), or less commonly in adults with altered GI anatomy or microflora (adult intestinal botulism), and originates from colonization of the large intestine by *C. botulinum* with subsequent production and absorption of toxin (Pickett et al., 1976; Arnon, 1995). Two additional forms of botulism are iatrogenic botulism, from accidental overdoses following clinical or cosmetic procedures (Klein, 2004), and inhalation botulism (Franz et al., 1993).

A. Foodborne Botulism

Elaboration of BoNT in foods requires contact with *C. botulinum* spores under conditions that allow bacterial cell proliferation and toxin production. These consist of an anaerobic environment, temperatures between 4°C and 40°C, pH above 4.6, water activity greater than 0.94 (<10% NaCl), and lack of adequate preservatives (Baird-Parker and Freame, 1967; Stinger et al., 2005). The requirements for growth of *C. botulinum* are stringent, especially anaerobiosis, making outbreaks relatively rare; nevertheless, episodes of foodborne botulism constitute a persistent public health threat (Sobel et al., 2004). In fact, food-related botulism outbreaks in the United States have shown no significant reduction during the past century, with an average of approximately 24 cases/year (Shapiro et al., 1998).

The primary vehicle for foodborne botulism presently and during most of the twentieth century has been improperly prepared home-preserved food products, often involving vegetables with a low acid content (Smith and Sugiyama, 1988; Snydman, 1989). Other sources are food consumed in restaurants that use unsafe procedures and contaminated commercially canned food products; the latter has become rare since the introduction of modern methods (O'Mahoney et al., 1990). Data compiled for foodborne botulism during the decade 1990–2000 in the United States indicate that serotype A was responsible for 50% of all cases, whereas serotypes B and E accounted for 10% and 37%, respectively, of intoxications in which serotype involvement was established (Sobel et al., 2004). Human foodborne intoxication by BoNT/F is exceedingly rare; between 1981 and 2002, only a single case was reported to the CDC (Gupta et al., 2005).

Although the number of outbreaks has been relatively constant, the case to fatality ratio has improved markedly. From 1899 to 1950, foodborne botulism was associated with 60% mortality; from 1950 to 1996, the average annual mortality fell to 15.5% (Shapiro et al., 1998), and decreased to 4% during the last decade (Sobel et al., 2004). These advances in survival have come primarily from improvements in critical care (Tacket et al., 1984; Sobel et al., 2004). Further reductions in morbidity and mortality from botulinum intoxication will require better methods for detection and diagnosis of BoNT outbreaks and availability of specific pharmacological treatments (Franz et al., 1997; Dickerson and Janda, 2006).

Perhaps the largest outbreak of foodborne botulism recorded to date occurred in Nan Province, Thailand during 14–18 March 2006 (Ungchusak et al., 2007). The outbreak was traced to consumption of contaminated home-canned bamboo shoots served at a religious festival. The successful handling of this outbreak by the Thai Ministry of Public Health has implications for the appropriate management of a small-scale bioterrorism attack involving deliberate contamination of the food supply or an aerosol attack. A total of 209 people exhibited signs and symptoms of botulism, with abdominal pain, dry mouth, and nausea being the most frequently reported; 134 villagers required hospitalization and 42 required mechanical ventilation. Botulism was suspected as soon as more specific signs such as bulbar muscle paralysis were observed, especially when coupled with respiratory depression. Due to familiarity with botulism in Thailand, the correct diagnosis was reached, and emergency procedures were implemented promptly, which allowed all patients to survive the outbreak. After initial triage, patients were flown to hospitals that had adequate emergency care facilities, including ventilators. In countries where botulism outbreaks are less frequent, health care personnel are often unfamiliar with its clinical presentation (Ruthman et al., 1985). As a result, botulism may not be diagnosed in a timely fashion, leading to delays in

treatment, and in a much poorer prognosis. The lessons learned from this large outbreak suggest that successful management of a bioterrorist attack involving botulism would require early recognition and accurate diagnosis of signs and symptoms, a realistic plan for allocating resources, adequacy of those resources, and coordination among the health care facilities, responsible government agencies, and international partners (Ungchusak et al., 2007). For this outbreak, antitoxin was obtained through informal government channels, and although responses were expeditious, antitoxin from the United States and the United Kingdom did not reach Thailand until 5–9 days after the outbreak. This delay would have resulted in potential deaths in the more severe group were it not for access to ventilators and emergency care. Formal international arrangement for antitoxin delivery is preferable to informal mechanisms, but local strategic stockpiles of critical medicines and supplies are essential to avoid delays in treatment.

B. Wound Botulism

Wound botulism is relatively rare, accounting for only 5% of all outbreaks. The majority of these are caused by serotype A, and the remainder by serotype B (Shapiro et al., 1998). The neurological symptoms of wound botulism differ little from those of foodborne botulism except for the general absence of GI symptoms. Historically, this form of botulism was so uncommon that it was not even recognized until the last half of the twentieth century. From its discovery in 1943 until 1990, only 47 incidences of wound botulism were documented (Weber et al., 1993). An examination of these cases indicated that wounds susceptible to *C. botulinum* are generally deep with avascular areas but need not appear obviously infected or necrotic. Additional risk factors include compound fractures and extensive crush injuries (Mershon and Dowell, 1973). Contamination of wounds with *C. botulinum* spores leads to germination and colonization at the site of infection. Localized weakness results from production of toxin at the wound, and systemic botulism can occur from toxin transmitted via the bloodstream to distant targets (Weber et al., 1993).

From 1980 to the present time, wound botulism has been observed predominantly in illicit drug users following repeated subcutaneous administration of black tar heroin, or in individuals with nasal or sinus lesions from chronic cocaine abuse (Anderson et al., 1997). During the last decade alone, wound botulism from black tar heroin has exceeded the total reported wound botulism cases during the preceding 40 years by a factor of almost three (Sandrock and Murin, 2001).

For reasons that are not completely understood, wounds are much more likely to be contaminated by *Clostridium tetani* than with *C. botulinum*. Although an aggressive vaccination program has nearly eliminated tetanus in developed nations, the absence of universal tetanus vaccination in many developing countries results in substantial mortality (Vandelaer et al., 2003). A large number of TeNT intoxications occur in neonates, often by infection of the umbilical stump. The mortality rates in developing countries were reported to be 85% for neonatal tetanus and 50% for nonneonatal tetanus during the mid-1980s (Schofield, 1986). For the year 2002, deaths from TeNT were estimated by the World Heath Organization (WHO) at 213,000 worldwide, of which 198,000 occurred in children under 5 years of age (Vandelaer et al., 2003).

C. Intestinal Botulism

1. Infant Botulism

Infant botulism is a consequence of intoxication by BoNT following ingestion or inhalation of clostridial spores that colonize the large intestine; young infants, especially those between 2 and 4 months of age, are susceptible to this form of botulism (Pickett et al., 1976; Arnon, 1995). Germination of spores and growth of vegetative cells lead to production of BoNT; the neurotoxin thus elaborated crosses the intestinal wall and reaches susceptible targets such as skeletal muscle

via the bloodstream (Arnon, 1995). The characteristic symptoms are poor sucking, constipation, generalized weakness, and respiratory insufficiency. The risk factors are not completely understood, but the incidence drops off sharply after 28 weeks of age, which is likely to be related to development of a more diversified intestinal flora. The latter has been shown to suppress germination and growth of *C. botulinum* spores in mice (Sugiyama and Mills, 1978). Of all food products that may be contaminated with *C. botulinum* spores, honey has been the one most often implicated in infant botulism; it is therefore recommended that honey not be given to young infants (Arnon et al., 1979).

Although infant botulism was not recognized until a large outbreak occurred in California in 1976 (Pickett et al., 1976), it is currently the most prevalent form of botulism in the United States, accounting for approximately 70% of all cases (Shapiro et al., 1998). Because infant botulism results from a continual production of BoNT, it appears to be more effectively treated by antitoxin than is foodborne botulism. In a recently concluded 5 year randomized clinical trial carried out with a human botulinum immune globulin (BIG-IV), it was found that administration of BIG-IV within 3 days of hospitalization resulted in a 3 week reduction in the mean hospital stay, as well as substantial reductions in the time needed for intensive care and mechanical ventilation (Arnon et al., 2006). In a nationwide open label study, BIG-IV was found to be effective even when administered 4–7 days after hospital admission, although to a somewhat lesser extent than when infusion was initiated at 3 days (Arnon et al., 2006).

2. Adult Intestinal Botulism

Under rare conditions, adults may manifest a syndrome similar to that of infant botulism. Such cases generally occur in hospitalized patients treated with a long course of multiple antibiotics that eliminate the normally suppressive intestinal flora; other predisposing factors include inflammatory bowel disease and surgical alterations of the bowel (Fenicia et al., 1999).

D. Inhalation Botulism

Inhalation botulism is so rare that only one human outbreak has ever been reported (Holzer, 1962). Three laboratory investigators became intoxicated by BoNT while performing necropsies on animals exposed earlier to an aerosol of BoNT/A that was stabilized by addition of colloids. It is assumed that the BoNT/A was reaerosolized from the animals' fur during the course of performing the necropsies. Signs and symptoms of intoxication, consisting of dysphagia, dysphonia, dizziness, headache, and blurred vision, were observed 3 days later. The patients were hospitalized for approximately 1 week but did not require artificial ventilation. Although BoNT gained entry via the lungs, no alteration of pulmonary function was reported in any of the individuals. In animal experiments (guinea pigs and nonhuman primates), it was demonstrated that no specific pulmonary histopathology resulted from inhalation of BoNT, even at lethal doses (Franz et al., 1993; Gelzleichter et al., 1999). This is consistent with findings that (with the possible exception of serotype C1), exposure to BoNT does not result in morphological damage (Duchen, 1971). However, in a more recent study, histopathological alterations were reported in mice that survived intranasally administered BoNT/A when examined 14 days after toxin challenge. Moreover, immunization with the pentavalent toxoid protected mice from lethality but not from lung damage (Taysse et al., 2005). The discrepancy with earlier results may stem from differences in toxin administration.

Since aerosol dispersal of BoNT can create a toxic cloud over large areas, it is considered to be a likely route for use by terrorists. Consequently, a critical question for effective medical management of potential bioterrorist attacks is whether the conventional vaccine and antitoxin would be effective in treating patients following an inhalation exposure of BoNT. In experiments where guinea pigs were immunized with the pentavalent toxoid, the vaccine was found to be as protective against an

inhalation challenge of BoNT as against challenge by other routes of administration (Cardella et al., 1963). More recently, a vaccine derived from recombinant C fragment of BoNT/B (binding domain of the HC) was shown to protect rhesus monkeys from an inhalation challenge of BoNT/B (Boles et al., 2006).

Both human- and equine-derived antitoxins have been tested for protection against an inhalation challenge of BoNT/A, and both were found to be highly effective. Rhesus monkeys were injected with human hyperimmune globulin or equine $F(ab')_2$ antitoxin, yielding plasma titers of <0.02 to 0.6 IU, and challenged with an ~6 LD_{50} dose of aerosolized liquid BoNT/A 48 h later (Franz et al., 1993). Animals not only survived this challenge but were also protected from any signs of BoNT intoxication. Rhesus monkeys even survived challenge by BoNT/A 6 weeks after a single administration of 16 IU/kg of human hyperimmune globulin. Control animals, on the other hand, died 2–4 days after BoNT/A challenge and exhibited clinical signs of intoxication 12–18 h before death. These signs were similar to those observed with other forms of botulism in nonhuman primates and consisted of, in order of onset, muscle weakness, intermittent ptosis, poor head control, dysphasia, and lateral recumbency (Franz et al., 1993).

Since inhalation botulism does not occur in nature, all outbreaks must be considered as suspicious. Prudence would dictate that each should be treated as a criminal or terrorist attack, unless other causes are found (Arnon et al., 2001). From the limited human and animal data currently available, inhalation botulism does not have a unique presentation; rather, the signs and symptoms resemble those of other forms of botulism. The latent period is comparable with that of foodborne botulism without the early GI signs (Adler, 2006).

Detection of inhalation botulism presents a unique challenge for confirming the outbreak and identifying the source of toxin. There would be no obvious wounds, contaminated food, or history of clinical or cosmetic use, where overdoses, although rare, are possible (Klein, 2004; Chertow et al., 2006). Confirmation of inhalation botulism would have to be based on finding toxin in the blood and perhaps in the nasal mucosa of intoxicated patients, on detecting toxin residues in the environment, or on observing an unusual clustering or distribution of cases. Unfortunately, BoNT cannot always be identified in laboratory samples (Shapiro et al., 1998), and the threshold for detecting toxin or residues in the nasal mucosa or in the environment is currently unknown.

III. PROPHYLAXIS AND TREATMENT OPTIONS

BoNTs are the most potent toxins known to mankind, and exposure to as little as 1 ng/kg by injection or 3 ng/kg by inhalation can result in human fatality (Gill, 1982; Arnon et al., 2001; Adler, 2006). Recovery, especially from type A intoxication, is slow (Keller et al., 1999), and residual physical and psychological signs and symptoms may persist for years after exposure (Mann, 1983; Cohen et al., 1988). Some of the residual problems may be a consequence of the prolonged period of inactivity during intoxication (Wilcox et al., 1990). Treatment consists of intensive care, ventilatory support, if required, and infusion of trivalent equine antitoxin (Tacket et al., 1984). The role of antibiotics in the treatment of botulism is controversial: they may be of benefit in eradicating *C. botulinum* in wound botulism (Sandrock and Murin, 2001), but they are considered to be ineffective in the treatment of intestinal or foodborne botulism (Santos et al., 1981). In addition, aminoglycoside antibiotics and tetracycline may exacerbate the BoNT-induced inhibition of neuromuscular transmission (Santos et al., 1981). There is also concern that if antibiotics are used to treat secondary bacterial infections in intestinal botulism, the subsequent lysis of *C. botulinum* vegetative cells may result in increased toxin absorption. This is considered to be less of a problem currently due to the availability of human-derived botulism antitoxin (BIG-IV), with a long residence time ($t_{1/2}$ ~ 28 days), which can neutralize the additional toxin load (Arnon et al., 2006).

A. Antitoxin

1. Equine Botulinum Antitoxin

Trivalent equine antitoxin is administered by intravenous infusion and contains antibodies to BoNT/A (7500 IU), BoNT/B (5500 IU), and BoNT/E (8500 IU) (Shapiro et al., 1998). These potencies are 100-fold greater than those required to neutralize the highest toxin levels detected by the CDC in BoNT-intoxicated patients (Hatheway et al., 1984). The trivalent equine antitoxin is currently the only FDA-approved product for botulism, and is effective in limiting the severity of the intoxication if administered early during the course of illness (Shapiro et al., 1998). The temporal limitation of antitoxin treatment has long been appreciated (Hewlett, 1929), and is related to the fact that clostridial neurotoxins exert their actions inside the nerve terminal, where they are not susceptible to antitoxin action (Simpson, 1981). Accordingly, at the time when signs and symptoms of botulism become apparent, a substantial quantity of toxin has already become internalized, and only the fraction that is still in the circulation is available to be neutralized. In a retrospective study of 134 patients, those who received antitoxin within 24 h after onset of signs and symptoms had a lower fatality rate (10%) than those who received antitoxin after 24 h of onset (15%), or those who did not receive antitoxin (46% fatality). In addition, patients who received antitoxin within 24 h had shorter hospital stays and spent fewer days on a ventilator than those who received antitoxin after 24 h (Tacket et al., 1984).

Although it is commonly believed that circulating BoNT is rapidly cleared from the bloodstream this is not always the case. In the recent Oakland Park, Florida outbreak, detectable levels of toxin were observed in one patient 8 days after receiving a massive overdose of nonapproved BoNT/A during a cosmetic procedure (Chertow et al., 2006). For such severely intoxicated patients, antitoxin administration, even if delayed, may still be effective in limiting the duration of illness, since it would neutralize circulating BoNT and prevent further toxin internalization.

2. Recombinant Monoclonal Antibodies

Although the equine antitoxin is effective in reducing the progression and severity of BoNT intoxication, there are significant limitations to its use. First, it is difficult to generate adequate supplies for the potential population at risk in a mass casualty event. In addition, since it is an equine product, the antitoxin has a brief plasma half-life in humans (5–8 days), and there is substantial risk of adverse reactions ranging from mild hypersensitivity to serum sickness (Black and Gunn, 1980; Hatheway et al., 1984). To overcome these limitations, new formulations of antitoxin are currently under development, including production of recombinant single-chain human BoNT antibodies (scFvs) by phage display technology (Marks, 2004). Initial surface plasmon resonance measurements identified four nonoverlapping epitopes on the BoNT/A C-terminal half of HC (C fragment, binding domain) for a set of scFvs (Amersdorfer et al., 1997).

The measured equilibrium-binding constants were encouraging ($K_d = 7.3 \times 10^{-8}$ to 1.1×10^{-9} M), which indicate a potential for neutralizing capability. To elicit protection against BoNT/A, a combination of three human-compatible monoclonal antibodies proved to be necessary (Marks, 2004). Interestingly, no single monoclonal antibody was found to be protective. These findings suggest that large-scale production of high-potency botulinum antitoxins for human use is technically feasible.

As a further refinement, a yeast display system was used as a technology platform for performing molecular evolution by increasing the affinity of those scFv antibodies that bind to BoNT/A. Yeast-displayed scFv libraries have been constructed by selecting scFvs that bind with increased association rates. A single cycle of error-prone mutagenesis increased the affinity for BoNT/A by 45-fold (from $K_d = 9.43 \times 10^{-10}$ to 2.1×10^{-11} M) (Razai et al., 2005). Thus, it is possible that these and other biotechnology approaches could be harnessed to develop neutralizing monoclonal antibodies directed at all seven serotypes of BoNT. Such technological advances would be expected to result in antitoxin

with relatively long plasma circulation times, a reduced incidence of hypersensitive reactions, and the availability of a sufficient quantity to protect the population at potential risk.

B. Prophylaxis

Prophylaxis generally involves vaccination with a pentavalent (A–E) toxoid. Vaccination provides a high degree of protection and is commonly administered to laboratory investigators who are at risk of exposure. The current vaccine has been available from the CDC as an Investigational New Drug (IND) for the past 45 years (Fiock et al., 1963). The vaccine is administered intramuscularly at 0, 2, and 12 weeks and requires a booster at 1 year to generate long-term protection. A heptavalent vaccine (A–G) originally developed by the U.S. Army (Franz, 1997; Middlebrook and Franz, 1997), will be produced by DynPort Vaccine Co. LLC under the aegis of the National Institute of Allergy and Infectious Diseases (NIAID), and is expected to be available by the end of this decade.

Recently, a vaccine made from the recombinant BoNT C fragment has been reported to protect nonhuman primates from an aerosol exposure of BoNT, and neutralizing antibody titers were detected for up to 2 years following vaccination (Boles et al., 2006). In addition to needle delivery, there is a considerable interest in developing a mucosal vaccine for BoNT. This is based on the premise that the mucosal immune system would be the first line of defense for inhaled BoNT, and administration of mucosal vaccines may be easier to carry out in the general population (Park and Simpson, 2004; Fujihashi et al., 2007).

Although these vaccines are effective, they require multiple inoculations and require up to a year from onset to generate adequate protection. In addition, since the BoNT antibodies remain elevated for a prolonged period, vaccinated individuals may be precluded from use of BoNT for treatment of spasticity or movement disorders that might develop during their lifetime (Jankovic and Brin, 1997). These limitations argue strongly in favor of a supplementary pharmacological approach for the management of botulism.

C. Pharmacological Intervention

From the time that inhibition of ACh release was established as the mechanism of BoNT action, attempts were made to antagonize the neurotoxin by measures that enhance ACh release. Until recently, however, development of a treatment for BoNT intoxication had low priority, in part because early efforts were generally unsuccessful, and in part because effective vaccines and antitoxins were already available. Currently, there is an increased impetus to develop pharmacological treatments following recognition of the potential for overdose with the expanding clinical use of BoNT (Klein, 2004; Chertow et al., 2006; Souayah et al., 2006). In addition, the experience gained in preparation for a potential BoNT threat during the Persian Gulf War made it clear that delays in generating adequate protection by the BoNT vaccine were not consistent with the requirement for rapid deployment of military personnel (Atlas, 1998). In addition, marked sequence variability has been found within BoNT serotypes, where subtypes exhibit differences in susceptibility to antibody neutralization (Smith et al., 2005). Such variability will need to be incorporated in the development of future vaccines and antitoxins to ensure adequate protection.

Some of the earliest putative BoNT antagonists were cholinesterase inhibitors, selected for their ability to prolong the actions of ACh. Carbamate anticholinesterase agents such as neostigmine and physostigmine were investigated in animals (Edmunds and Keiper, 1924), and in nerve-muscle preparations (Guyton and MacDonald, 1947), but they were unable to antagonize the effect of BoNT. More recent studies have tended to confirm earlier findings (Adler et al., 1995), although there have been occasional reports of human botulism responding to the short-acting cholinesterase inhibitor, edrophonium (Cherington, 1998).

Other potential antagonists of BoNT action such as elevated calcium, calcium ionophores, lanthanum, black widow spider venom, 2,4-dinitrophenol, and agents that raise cyclic AMP levels

were examined for their ability to reverse BoNT toxicity. Addition of the above compounds to BoNT-intoxicated nerve-muscle preparations led to increases in the frequency of spontaneous miniature endplate potentials (MEPPs) but resulted in little or no enhancement in the amplitude of evoked endplate potentials (EPPs) (Simpson, 1988; Thesleff, 1989). Since these compounds generally increased spontaneous but not evoked ACh release, they were not considered to be of practical value for treatment of BoNT intoxication.

1. Potassium Channel Blockers

Potassium channel blockers were found to be more effective in antagonizing the paralytic action of BoNT than were the former group of compounds. Their higher efficacy comes from their ability to prolong the duration of the presynaptic action potential (Penner and Dreyer, 1986), leading to a greater influx of calcium during nerve stimulation. Coupling of increased calcium influx to nerve impulses enables the potassium blockers to produce striking increases in the amplitude of EPPs and of nerve-evoked twitch tensions (Adler et al., 1979).

A number of potassium channel blockers have been evaluated for their ability to antagonize the actions of BoNT, including guanidine, 4-aminopyridine, 3,4-diaminopyridine (3,4-DAP), and tetraethylammonium (Lundh et al., 1977; Molgo et al., 1980; Simpson, 1986). Of these, the most promising candidate was 3,4-DAP; 4-aminopyridine exhibited undesirable CNS side effects, and tetraethylammonium caused a marked postsynaptic depression of endplate potentials and muscle contractions that actually exacerbated BoNT-mediated inhibition (Adler et al., 1979, 1995; Simpson, 1988; Thesleff, 1989; Cherington, 1998). When added to nerve-muscle preparations before BoNT, 3,4-DAP produced a marked delay in the time-to-block of nerve-evoked muscle contractions (Simpson, 1986). When applied after BoNT paralysis, 3,4-DAP was able to restore tensions to near-control values (Lundh et al., 1977; Molgo et al., 1980; Simpson, 1986; Adler et al., 1995). Unlike many candidate antagonists, 3,4-DAP could restore tension even several days after total paralysis was established (Adler et al., 1996). In spite of these successes with 3,4-DAP, two fundamental limitations were noted: its efficacy was largely limited to serotype A (Simpson, 1986), and it had a brief in vivo lifetime relative to that of BoNT (Adler et al., 1996). Of the two, the latter is less critical since the short lifetime can be compensated by use of an infusion delivery as shown by Adler et al. (2000).

The basis for the lack of response to 3,4-DAP by the other serotypes is not well understood. At a functional level, serotype A-intoxicated neuromuscular junctions undergo an attenuated but synchronous release of ACh following stimulation; preparations intoxicated by serotypes B, D, and F produce asynchronous release where the ACh quanta are dispersed and cannot summate to produce suprathreshold EPPs (Lundh et al., 1977; Molgo et al., 1980; Thesleff, 1989). It is readily apparent that the lack of synchrony would prevent 3,4-DAP from restoring transmitter release; however, the factors that lead to asynchronous release are not currently understood.

An additional concern with potassium blockers comes from human case reports. These indicate that although the potassium blockers guanidine and 3,4-DAP produced a moderate increase in muscle strength, their use did not lead to the return of spontaneous ventilation in BoNT-intoxicated individuals (Cherington and Schultz, 1977; Davis et al., 1992). It is not clear if human diaphragm or intercostal muscles are less responsive to potassium blockers than are limb muscles, or whether the doses used clinically were insufficient to reverse muscle paralysis (Davis et al., 1992). The latter may be the case, since BoNT/A-paralyzed rat or mouse diaphragm muscles respond vigorously to the actions of 3,4-DAP (Simpson, 1986; Adler et al., 1995). Higher doses of 3,4-DAP were not attempted in these patients to avoid the risk of seizures and other potential side effects. At the present time, the potassium blockers hold promise as potential therapeutic agents, but development of more selective compounds or targeting of the inhibitors to neuromuscular and neuroeffector synapses will be required to exploit their full potential.

2. Inhibitors for Specific Stages of Intoxication

The examples given earlier of treatment strategies are based on antagonizing the actions of BoNT after the neurotoxin has undergone internalization and subsequent cleavage of its target protein. In addition, since these approaches were developed before the intracellular targets were identified, they do not specifically antagonize the action of toxin at the molecular level. Rather, these compounds act by elevating intracellular calcium levels in an attempt to compensate for the toxin-mediated inhibition of ACh release. The discrete stages of clostridial neurotoxin action of binding, internalization, translocation, and catalysis suggest that there are multiple sites for direct pharmacological intervention. These stages are mediated by different domains of BoNT, and in principle, each can be specifically inhibited (Simpson, 1988, 2004). Three areas where significant progress has been made will be discussed in the following sections.

a. *Inhibitors of Binding*

A reasonable starting point for developing pharmacological countermeasures for BoNT intoxication is the use of receptor antagonists to reduce or prevent the binding of toxin to the nerve terminal. Complications with this approach are that many BoNTs bind to dual polysialoganglioside–protein receptors on the surface of nerve terminals, and that different BoNT serotypes recognize different protein–ganglioside combinations (Dolly et al., 1984; Montecucco, 1986; Yowler et al., 2002; Kohda et al., 2007). This implies that multiple receptor antagonists would need to be developed to protect against the BoNT serotypes responsible for human intoxications.

1. Ganglioside Component of BoNT Receptor. Evidence for involvement of gangliosides in botulinum intoxication is extensive (Van Heyningen and Miller, 1961; Montecucco, 1986; Shapiro et al., 1997; Kitamura et al., 1999). The most direct demonstration, however, comes from the study of Bullens et al. (2002). These authors reported that in diaphragm muscles obtained from a line of knockout mice that lack polysialogangliosides (GalNac-$T^{-/-}$), a paralytic concentration of BoNT/A (~2 ng/mL BOTOX) had no effect on spontaneous or evoked release of ACh. In agreement with this finding, Kitamura et al. (1999) showed that the LD50 of BoNT/A is 40-fold greater in GalNac-$T^{-/-}$ mice than in wild-type littermates. The relative resistance to BoNT/A, but absence of complete protection, suggested that GD3 or GM3 gangliosides, which were upregulated in the knockout mice, may serve as potential receptors for BoNT in these mice. Alternatively, it is possible that the protein receptor may be sufficient for binding of toxin at these higher concentrations.

Pronounced antagonism of neurotoxin binding has been achieved with lectins from *Triticum vulgaris* (TVL) and *Limax flavus* (Bakry et al., 1991). Pretreatment by these lectins led to a concentration-dependent inhibition in the binding of BoNT/B and TeNT to preparations of rat brain membranes, approaching total inhibition at the highest concentration. The most effective lectins were those that had an affinity for *N*-acetyl-α-sialic acid; six lectins with specificities for other carbohydrates were ineffective (Bakry et al., 1991). In complementary experiments on mouse phrenic nerve-hemidiaphragm preparations, TVL delayed the time-to-block of nerve-elicited muscle contractions with all BoNT serotypes examined (A–F). If one defines the time-to-block in the presence and absence of BoNT antagonist as a protective index, the values for the different serotypes ranged from 1.3 to 1.9. Although the physiological actions of TVL appear less striking than its antagonism of binding, it must be borne in mind that a 10-fold decrease in bound neurotoxin can only be expected to produce a 2-fold slowing in the time-to-block (Bakry et al., 1991).

Since the isolated diaphragm muscle has a limited viability in vitro ($\leq$8 h; Adler et al., 1995), it is generally tested with high concentrations of BoNT where protection may be difficult to demonstrate. It was therefore of interest to determine the efficacy of TVL in a physiological preparation where lower toxin doses can be used, and where protection can be assessed over a longer time interval. In a recent study from our laboratory, TVL was injected locally in the rat extensor digitorum longus (EDL) muscle 30 min before a local injection of 0.6 units (U) of BoNT/A. Muscle tension was recorded in situ 7 days after BoNT administration. As shown in Table 16.1, pretreatment by TVL led to

TABLE 16.1
Pretreatment by *Triticum vulgaris* Lectin (TVL) Protects Rat EDL Muscle from Paralysis Following Local Injection of BoNT/A

BoNT/A Dose	Pretreatment	TVL Dose (mg)	Number of Muscles	Twitch Tension (g) Mean ± SEM
None	None		5	69.4 ± 3.4
0.6 U	None		4	2.0 ± 0.9[a]
None	TVL	0.375	3	61.2 ± 10.0
0.6 U	TVL	0.375	3	9.7 ± 3.1[a]
0.6 U	TVL	0.75	6	23.8 ± 6.4[a,b]

Note: The dose of BoNT/A was selected to produce ≥95% inhibition of muscle tension 48 h after injection. TVL was injected 30 min before BoNT/A. Tensions were tested 7 days after BoNT/A administration; doses are expressed as mouse i.p. LD_{50} units (U).

[a] Tensions differ significantly from pre-BoNT/A values ($p < 0.05$).

[b] Tensions differ significantly from muscles injected with BoNT/A alone ($p < 0.05$).

protection, albeit incomplete, of muscle tension following injection of a paralytic dose of BoNT/A. Moreover, protection was sustained for at least 1 week, at which time muscles that were not pretreated with TVL still showed nearly complete paralysis (2.9% of control tension).

The major advantage of the lectins is that they are effective against all clostridial toxin serotypes (Bakry et al., 1991). The disadvantage of using lectins to protect against BoNT is that they must be administered as pretreatments. This limitation is inherent in the basic mechanism of BoNT action; thus no antagonist of surface receptor binding would be expected to be protective once BoNT is internalized and symptoms are manifested.

2. Protein Component of BoNT Receptor. Based on the high-affinity, cholinergic selectivity, and sensitivity to proteolytic enzymes (Black and Dolly, 1986), the binding of BoNT to the nerve terminal was suggested to involve a protein component in addition to polysialogangliosides (Montecucco, 1986). Nishiki et al. (1994, 1996a, 1996b) were the first to identify a protein receptor for BoNT. Using rat brain synaptosomal membranes, these authors demonstrated that BoNT/B bound to synaptotagmin (Syt), a synaptic vesicle protein that also serves as the calcium sensor for evoked transmitter release (Nagy et al., 2006). Binding to Syt was found to be saturable and to be enhanced by the inclusion of the ganglioside G_{D1a} or G_{T1b} (Nishiki et al., 1994). However, since the work was performed in tissue that is not the physiological target for the clostridial neurotoxins, and the authors did not demonstrate that the binding of BoNT to Syt led to toxin internalization, the results were not universally accepted (Middlebrook and Franz, 1997).

Compelling evidence that Syt was indeed the protein receptor for BoNT/B was provided by the seminal work of Dong et al. (2003). These authors demonstrated that Syt isoforms I and II were responsible for the productive binding of BoNT/B to PC12 cells and to motor nerve terminals of diaphragm muscle; Syt-I had lower affinity for BoNT/B and required gangliosides for binding, whereas Syt-II produced higher-affinity binding that did not require gangliosides (Dong et al., 2003). The authors also demonstrated that fragments of Syt-II that contained the toxin-binding domain (luminal region) delayed the time to death in mice challenged by intravenous BoNT/B. Syt was subsequently demonstrated to be the protein receptor for BoNT/G but not for any of the remaining five BoNT serotypes (Rummel et al., 2004).

In addition to serotypes B and G, the protein receptor for serotype A has now been identified. Dong et al. (2006) demonstrated that the synaptic vesicle protein SV2 (isoforms A, B, and C) is the protein receptor for BoNT/A. SV2 is an integral membrane protein with 12 putative transmembrane domains and is normally associated with secretory vesicle membranes (Janz and Sudhof, 1999).

Several lines of evidence implicated SV2 as the protein receptor for BoNT/A: (1) binding of BoNT/A but not BoNT/B was reduced in hippocampal neurons cultured from SV2 knockout mice, (2) fragments of SV2 that contain the toxin interaction domain (luminal loop 4) inhibited binding of BoNT/A to neurons, and (3) SV2B knockout mice were found to be less sensitive to an intravenous challenge of BoNT/A and, in addition, diaphragm muscles from these mice showed a significantly reduced binding of BoNT/A.

SV2 and Syt are thought to become incorporated in the plasma membrane of the nerve terminal during the process of transmitter release, where the secretory vesicle membrane is transiently fused with the plasma membrane (Bonanomi et al., 2006). This explains the presence of synaptic vesicle proteins on the surface of nerve terminal and accounts for the finding that the rate of BoNT intoxication increases with synaptic activity (Hughes and Whaler, 1962).

Recent insight into the binding of BoNT/B has come from X-ray crystallographic studies in which the luminal domain of Syt-II was crystallized with BoNT/B (Chai et al., 2006) or with the C fragment of BoNT/B (Jin et al., 2006). These studies revealed that a helix is induced in the luminal domain of Syt-II, which binds to a hydrophobic groove or saddle-shaped crevice near the C terminus of the BoNT/B HC. Gangliosides bind to nonoverlapping adjacent sites that are separated from the Syt-binding site by 15Å (Chai et al., 2006; Jin et al., 2006). This binding orients the translocation domain of the HC for optimal interaction with the plasma membrane. Mutations in the luminal domain of Syt-II as well as fragments of this peptide were found to inhibit the binding of BoNT/B (Chai et al., 2006). In addition, single-site mutations in recombinant BoNT/B neurotoxin in the region of the Syt-II-binding domain resulted in marked loss of potency when tested in the mouse phrenic nerve-hemidiaphragm preparation, especially mutations A1196K and K1192E (Jin et al., 2006). These findings suggest that peptide fragments of the protein-binding domains and inactive mutant BoNT HC variants have the potential to be developed into inhibitors. More extensive work will be required to determine if these inhibitors have practical application and if they provide benefit over immunological approaches.

Since the details of toxin binding have been reported only for a limited number of serotypes, it is not clear whether the remaining serotypes will also be found to bind to adjacently located protein–ganglioside receptors, or whether the proteins will be derived from transient synaptic vesicle fusions with the plasma membrane. Assuming the former, it is tempting to speculate on the cholinergic selectivity of these neurotoxins. Possibilities include: (1) a high density of synaptic vesicle proteins on cholinergic nerve terminals from the generally rapid transmitter release rates of cholinergic motor neurons (Brooks, 1956; Thesleff, 1989), (2) a high density of the appropriate polysialogangliosides on cholinergic terminals, and (3) favorable localization (geometric arrangement) of the appropriate protein- and ganglioside-binding components.

It must be pointed out, however, that the cholinergic selectivity of the BoNTs is not absolute, and a variety of cell types can be intoxicated in vitro with sufficiently high toxin concentrations or prolonged incubation times (MacKenzie et al., 1982; Schiavo et al., 2000). In addition, direct injection of BoNT into the CNS can give rise to central actions that are not observed during exposure by conventional routes (Bozzi et al., 2006). The finding that noncholinergic cell types and central neurons are not ordinarily affected by BoNT suggests that they may have a limited access to toxin, a lower density of productive receptors, or unfavorable protein–ganglioside geometry.

b. *Inhibitors of Internalization**

Following binding of the clostridial neurotoxins to receptors on cholinergic nerve terminals, the toxins undergo internalization before reaching their ultimate intracellular targets (Schiavo et al., 2000; Simpson, 2004). Internalization is thought to involve endocytosis of the BoNT–receptor complex, acidification of the resulting endocytotic vesicle, dissociation of the LC and HC, and

* Internalization, in this section, is used broadly to describe the entry of toxin or LC into the nerve terminal cytosol and includes both trafficing from the cell surface to the endosoma and translocation across the endosomal membrane.

release of the LC into the cytosol (Koriazova and Montal, 2003; Simpson, 2004). The most direct evidence for internalization comes from experiments in which colloidal gold-BoNT conjugates have been visualized inside cholinergic motor axon terminals (Dolly et al., 1984) and torpedo electric organ synaptosomes (Blasi et al., 1992).

Internalization affords the next opportunity to ameliorate the toxic actions of BoNT. A number of pharmacological agents have been examined for inhibition of this process with various degrees of success. Simpson (1983) demonstrated that pretreatment of phrenic nerve-hemidiaphragm preparations with the lysosomotropic agents ammonium chloride or methylamine hydrochloride delayed the time-to-block of nerve-evoked muscle contractions after exposure to BoNT serotypes A, B, C1, and TeNT. Incubation of nerve-muscle preparations with ammonium chloride and methylamine hydrochloride was effective if applied before, concurrently, or up to 20 min after toxin exposure. The efficacy of the lysosomotropic agents was reduced rapidly with further delays, such that no effect was observed if they were administered 30–35 min after toxin exposure. At optimal concentrations, these compounds produced a twofold delay in the time-to-block (Simpson, 1983).

Other candidates examined for inhibiting BoNT-mediated internalization were the antimalarial agents chloroquine and hydroxychloroquine (Simpson, 1982). These drugs were selected on the basis of interfering with the actions of a large group of peptide hormones and protein toxins that exert their actions following internalization (Goldstein et al., 1979). The maximal efficacies of the above 4-aminoquinolines were similar to those of ammonium chloride and methylamine hydrochloride, and both groups exhibited a comparable therapeutic window. They differed in that effective concentrations of the 4-aminoquinolines also produced a reversible depression of neuromuscular transmission.

Work on antimalarial agents was extended by Deshpande et al. (1997) to identify candidates that did not block neuromuscular transmission, had a longer therapeutic window, and could delay the time-to-block to a greater degree. These investigators examined a large group of 4- and 8-aminoquinoline compounds as well as analogous acridines for their efficacy against BoNT in mouse diaphragm preparations. The most effective compounds were quinacrine, amodiaquine, and chloroquine; 8-aminoquinolines such as primaquine were ineffective. The highest protective index, 3.9, was obtained with 20 μM amodiaquine. This was achieved with no deleterious effects on neuromuscular transmission, and thus defines the present limit for inhibitors of internalization. Unfortunately, the therapeutic window could not be extended; no protection was observed if the antimalarial agents were added $\geq$40 min after exposure to BoNT/A or BoNT/B.

A somewhat different approach for attempting to prevent or reduce the internalization of BoNT was to treat nerve-muscle preparations with the proton ionophores monensin and nigericin (Adler et al., 1994; Sheridan, 1996). These ionophores act by depleting vesicular pH gradients, thereby interfering with several stages in the delivery of active LC in the cytosol. These ionophores were found to be approximately as effective as the other inhibitors of internalization. They were more toxic, however, and high concentrations led to a depression of neuromuscular transmission (Adler et al., 1994; Sheridan, 1996). Toxicity is difficult to avoid with this group of agents since proton gradients are required for a number of cellular reactions such as the synthesis of ATP and filling of synaptic vesicles.

c. *Metalloprotease Inhibitors*

The third area for therapeutic intervention is inhibition of the metalloprotease activity of the BoNT LCs. This field is potentially the most promising, especially since the crystal structures of the LCs for all serotypes have been solved (Agarwal et al., 2005; Arndt et al., 2005, 2006). The presence of a zinc-binding motif in the LC of clostridial neurotoxins, and the finding that zinc is required for neurotoxin-mediated proteolysis of SNARE proteins (Schiavo et al., 1992a; Montecucco and Schiavo, 1993), suggest that three classes of potential inhibitors may be effective in antagonizing the toxic actions of BoNT LC: metal chelators, zinc metalloprotease inhibitors, and exosite inhibitors. Simpson et al. (1993) demonstrated that the zinc chelator *N,N,N′,N′*-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) caused a marked slowing in the time-to-block of nerve-evoked muscle

contractions when administered before BoNT in phrenic nerve-hemidiaphragm preparations. The maximum efficacy was equivalent to that achieved with TVL, ammonium chloride, methylamine hydrochloride, or the most potent antimalarial drugs.

In common with the above inhibitors, TPEN was effective against all BoNT serotypes examined. In addition, when coapplied with TVL or the lysosomotropic agents, the protection observed with TPEN was approximately additive with that of the former compounds. These results are encouraging because they demonstrate that, in principle, concerted inhibition of the different stages in the production of toxicity is a viable strategy for managing BoNT intoxication. Sheridan and Deshpande (1995) examined a number of additional chelators on nerve-evoked twitch tensions and concluded that both a high affinity for zinc and membrane permeability are required for antagonism of BoNT.

The results with TPEN in the isolated nerve-muscle preparations were sufficiently encouraging to test this chelator for protection against challenge by BoNT; unfortunately, these results were less encouraging. First, TPEN was found to be highly toxic in vivo, producing rapid lethality at doses above 20 mg/kg in mice (Adler et al., 1997). Second, at the highest tolerated dose, TPEN only increased survival by 2–3 h following a 20 LD_{50} challenge of BoNT/A or BoNT/B. Toxicity of TPEN was also observed with primary and clonal cells. TPEN concentrations $\geq$10 μM produced morphological damage with characteristics of apoptosis (Adler et al., 1999b).

Studies with ion replacement indicated that chelation of zinc was the proximal cause of cytotoxicity, and examination of a variety of chelators suggested that those with high membrane permeability were especially apt to produce cell death (Sheridan and Deshpande, 1998). Based on these findings, metal chelators may have a limited use in the therapy of botulinum intoxication since the requirements for efficacy against BoNT are the same ones that promote cellular toxicity.

A more promising approach is the development of metalloprotease inhibitors to target the catalytic activity of BoNT LC. This endeavor was made possible by the discovery of the zinc metalloprotease activity of the clostridial toxins that began almost 20 years ago when the HEXXH signature sequence of zinc-binding proteins was noted in the TeNT LC by Jongeneel et al. (1989). Their finding suggested that clostridial neurotoxins possessed zinc-dependent protease activity. During the next 4 years, the SNARE protein substrates and serotype-specific cleavage sites were identified and correlated with intoxication in a systematic series of studies (Montecucco et al., 1994; Rossetto et al., 1995). It is noteworthy that an enzymatic activity for BoNT/A was suspected as far back as 1947 by Guyton and Marshall in their pioneering study on botulinum intoxication where it is stated that:

> ...this minute quantity of toxin necessary to produce poisoning, the duration of poisoning and the physical properties of the toxin all tend to characterize the toxin as a destructive enzyme...

Metalloprotease inhibitors have the advantage of being potentially effective after onset of BoNT intoxication. Of the pharmacological agents discussed thus far, metalloprotease inhibitors (and metal chelators) are the only ones not constrained by the brief time window that limits antitoxin efficacy, as well as the efficacy of inhibitors of BoNT binding, internalization, and translocation. To be viable candidates, metalloprotease inhibitors would need to be highly potent, be able to gain access to the internalized BoNT LC in the cytosol, and have unusually high stability to match that of the BoNT LC (Keller et al., 1999; Adler et al., 2001; Keller and Neale, 2001; Foran et al., 2003; Meunier et al., 2003).

Early work with zinc metalloprotease inhibitors focused on the well-characterized agents captopril ((2S)-1-[(2S)-2-methyl-3-sulfanyl-propanoyl] pyrrolidine-2-carboxylic acid) and phosphoramidon (*N*-alpha-L-rhamnopyranosyloxy[hydroxyphosphinyl]-L-leucyl-L-tryptophan). These compounds, however, were found to have little inhibitory activity against BoNT (Adler et al., 1994, 1999a). The poor efficacy of captopril was suggested to stem from unfavorable steric constraints in the binding of proline at the active site of BoNT (Schmidt and Stafford, 2002).

Phosphoramidon analogs in which Leu–Trp was replaced by Phe–Glu to resemble the cleavage site of synaptobrevin exhibited little increase in inhibitory activity; one analog was marginally more potent and two were significantly less potent than the parent compound (Adler et al., 1999a).

Several classes of organic compounds were examined for their ability to inhibit the catalytic activity of BoNT/B, chiefly isocoumarins and phosphonates. Adler et al. (1998) tested a series of isocoumarin compounds that were originally designed as elastase inhibitors. Molecular modeling studies suggested that these compounds may interact favorably with the BoNT/B-active site, and several candidates were able to inhibit BoNT/B LC activity. The most effective compound in this series was 7-*N*-phenylcarbamoylamino-4-chloro-3-propyloxyisocoumarin, which had an IC_{50} of 28 μM when tested in a cleavage assay using a 50-mer synaptobrevin peptide.

Since existing metalloprotease inhibitors showed little promise for development, the focus for drug discovery shifted to peptide inhibitors by systematically modifying key residues in the substrate and examining the consequences of these alterations on enzymatic activity. Schmidt et al. (1998) made single residue changes near the cleavage site of a 17-mer SNAP-25 peptide that was a minimal substrate for BoNT/A. Substitution of Cys in the P1 or P2 position transformed the peptide from a substrate to a competitive inhibitor. The best inhibitors in this series had K_i values of ~2 μM in cell-free assays. Efficacy was attributed to the favorable location of the sulfhydryl group of Cys, which was postulated to interact with the catalytically important zinc in the BoNT-active site. With further elaboration of this approach, more potent inhibitors were developed, as exemplified by the pseudo peptide, 2-mercapto-3-phenylpropionyl-RATKML ($K_i = 0.33$ μM; Schmidt and Stafford, 2002). The most potent inhibitor of BoNT/F ($K_i = 1$–2 nM) was a peptidomimetic in which d-Cys was substituted for Gln-58 in the minimum synaptobrevin fragment (Schmidt and Stafford, 2005).

Sukonpan et al. (2004) also developed substrate-based inhibitors for BoNT/A using a similar 17-mer SNAP-25 peptide (aa 187–203). The authors incorporated α-thiol amide in place of Gln-197 (P1 residue) in the 17-mer SNAP-25 peptide to generate submicromolar inhibitors of BoNT/A catalytic activity. A similar approach with a 35-mer synaptobrevin peptide resulted in inhibitors of BoNT/B LC protease with K_i values in the low micromolar range (Oost et al., 2003). A combinatorial approach using a hinged peptide for BoNT/A and BoNT/B has also been reported (Moore et al., 2006). In spite of their generally high potencies, peptide inhibitors are considered to be unsuitable as therapeutic agents since they are unstable in vivo and would have difficulty gaining access to the nerve-terminal cytosol to inhibit internalized BoNT LC. They have, however, served as templates for synthesis of organic drug candidates and have been used successfully by Burnett et al. (2003, 2007) to select effective compounds from natural products libraries.

Anne et al. (2005) demonstrate that a pseudotripeptide inhibitor and its disulfide-coupled dimer could prevent BoNT/B-mediated inhibition of norepinephrine release from cortical synaptosomes. This action was observed in the presence of inhibitor concentrations >15 μM. The relatively high concentrations required for inhibition of BoNT/B on norepinephrine release contrasts with the 2 nM IC_{50} observed for inhibition of the BoNT/B LC-mediated cleavage of a synaptobrevin peptide in a cell-free enzymatic assay (Anne et al., 2005). The lower potency in cell-based assays appears to reflect the difficulty of these charged peptidomimetic inhibitors to cross plasma membranes, which represents a major limitation of this class of compounds.

Small organic inhibitors with moderately high potency against BoNT protease activity are beginning to emerge. In general, the organic inhibitors described to date are less potent than the most effective peptide-based inhibitors; however, continued work on structure optimization should lead to improvements in their potency, selectivity, and cellular efficacy. Park et al. (2006) performed in silico screens of 2.5 million compounds that were capable of coordinating with the active site zinc of BoNT/A LC and determined a structure commonality for inhibition. Using multiple molecular dynamics simulations, the authors examined the interaction of the initial set of effective compounds with the BoNT/A LC-active site. Modeling studies suggested that inclusion of a hydroxamate would improve interactions with the active site zinc, whereas an additional aromatic ring would

increase interactions with the serotype-specific Phe-193 in the active site. From these insights, the authors were able to synthesize an organic inhibitor with a K_i of 12 μM, which is among the most potent and serotype-selective small molecule inhibitor of BoNT/A thus far reported in a cell-free enzymatic assay.

Boldt et al. (2006b) also screened organic inhibitors of BoNT/A metalloprotease activity on the basis of their interactions with the active site zinc. Initial efforts with arginine hydroxamate led to an inhibitor with a K_i of 60 μM. Subsequently, a hydroxamate library with carboxylic acids was tested. The best agent in this screen was 4-chlorocinnamic hydroxamate (with an IC_{50} of 15 μM). Further chloro substitution led to 2,4 dichlorocinnamic hydroxamic acid, a competitive inhibitor with a K_i of 0.30 μM. The potency of this compound is comparable with that of the best peptide inhibitors for BoNT/A (Schmidt and Stafford, 2002). However, Burnett et al. (2007) reported that this compound was less potent, and had an IC_{50} of 29 μM under their assay conditions. A novel compound, Fmoc-d-Cys(Trt)-OH, was recently tested by Boldt et al. (2006a). This compound was found to be a moderately potent inhibitor of BoNT/A LC protease activity ($K_i = 18$ μM) and was nearly equipotent in its ability to antagonize BoNT/A in neuro-2 cells. The latter is encouraging since most LC protease inhibitors are much less effective in cellular assays than in cell-free assays (Anne et al., 2005).

In addition to the BoNT-active site, other regions of the LC have received attention as potential areas for inhibition. Lebeda and Olson (1994) first suggested that the high substrate selectivity of the family of BoNT proteins depended on "the critical positioning of the substrate by... non-perfectly conserved residues" and by "the residues flanking the active-site region." This suggestion is consistent with the observation that two exosites are crucial for SNAP-25 binding to the LC of BoNT/A (Breidenbach and Brunger, 2004). The α- and β-exosites were recognized as being potentially important in the development of specific inhibitory drugs, and targeting the peripheral sites of substrate binding has focused on a family of bis-imidazole inhibitors for BoNT/A (Merino et al., 2006).

A recent study by Eubanks et al. (2007) has highlighted limitations of conventional drug development paradigms. The authors found that high-throughput screens did not consistently predict the candidates that would be most effective in their cell-based assays. Some obvious reasons include cytotoxicity and poor membrane permeability of potential inhibitors. However, other reasons are that current drug screens generally use small peptide fragments of substrates, which may have different secondary structures and often lack exosite regions (Chen and Barbieri, 2006). In addition, in cells, the LCs of at least some serotypes (e.g., BoNT/A) are localized to the cytoplasmic surface of the membrane, and are not in free solution (Fernandez-Salas et al., 2004). Eubanks et al. (2007) also observed that efficacy in cell culture did not predict protection in vivo. Complicating factors include absorption, distribution, metabolism, clearance, and systemic toxicity of inhibitor candidates. A prudent approach, therefore, is to combine high-throughput screening with cellular, isolated tissue, and in vivo assays as an iterative strategy, rather than to rely primarily on cell-free assays.

Although metalloprotease inhibitors are not constrained by a brief therapeutic window, recovery from BoNT intoxication may still be delayed because of the time required to replace cleaved SNARE proteins with intact ones. Estimates from pulse-chase experiments suggest that a half-time of ~1 day would be required to replace SNAP-25, 4–5 days to replace synaptobrevin, and ~6 days to replace syntaxin, even if no further cleavage were to occur (Foran et al., 2003). Examination of the fraction of total SNARE proteins cleaved by BoNT reveals that a relatively small fraction is cleaved at the neuromuscular junction at the time of total muscle paralysis. From local injections of BoNT/A in vivo, Jurasinski et al. (2001) estimated that paralysis requires cleavage of less than 35% of the total SNAP-25. Meunier et al. (2003) reported that isolated diaphragm muscles exposed to 2 nM BoNT/A have only 6.5% of their SNAP-25 in the cleaved form, and suggested that the SNAP-25 relevant to transmitter release must exist in a small specialized pool. It is likely that the critical pool of SNAP-25 is even lower than that found by Meunier et al. (2003), since the

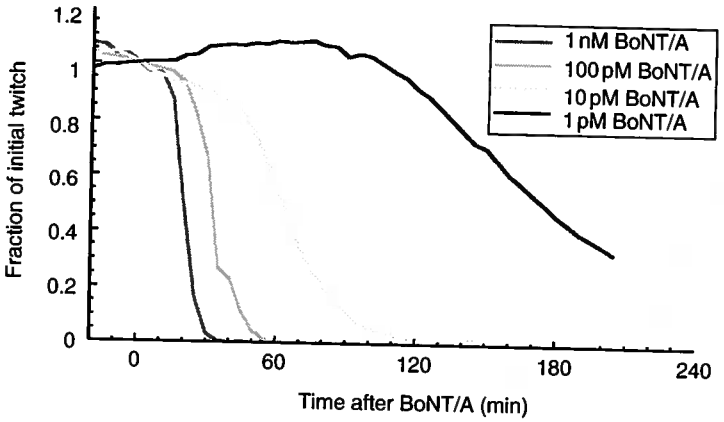

FIGURE 16.1 The effect of BoNT/A on muscle tension in isolated mouse phrenic nerve-hemidiaphragm preparations. Muscles were exposed to BoNT/A at concentrations ranging from 1 pM to 1 nM and the time to paralysis was monitored. Toxins were added to the muscle bath at 0 time from concentrated stock solutions; each concentration was tested on a separate hemidiaphragm muscle. Twitch tensions were elicited by supramaximal stimulation of the phrenic nerve at 30 s intervals. Temperature, 37°C.

concentration of BoNT/A required to paralyze diaphragm muscle is <1 pM (Figure 16.1), considerably lower than the concentration used in the above study. In fact, data from our laboratory on rat EDL muscle indicate that the SNAP-25 cleavage following local injection of a paralytic dose of BoNT/A (1.25 U) is often undetectable (Figure 16.2). The above suggests that after complete inhibition of LC protease activity, recovery of synaptic function will be determined by the turnover rates of the cleaved SNARE proteins, which are in the same range as the turnover rates of the native SNAREs (Foran et al., 2003).

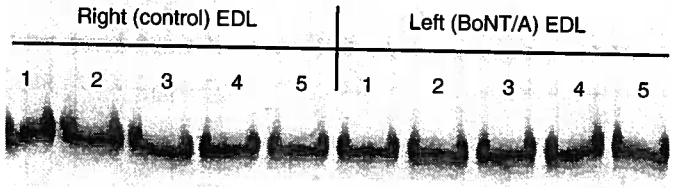

FIGURE 16.2 Western blots of SNAP-25 from rat EDL muscle following local injection of 1.25 U of BoNT/A (15 μL) (left EDL). The right EDL muscles served as uninjected controls. The expected 24 kDa truncated fragment resulting from BoNT/A cleavage of SNAP-25 was not detectable with a paralytic dose of BoNT/A. SMI-81 (Sternberger Monoclonals, Inc) was used for precipitation, whereas rabbit polyclonal antiserum 2777 was used as the primary antibody (BioSynthesis, Inc.). The numbers 1, 2, 3, 4, and 5 correspond to 2, 4, 7, 14, and 30 days after BoNT/A injection, respectively. Neurally elicited twitch tensions were recorded in situ just before excision. Tensions were 2%, 5%, 3%, 32%, and 39% of control, at the above respective time points. Note that the truncated SNAP-25 fragment could not be detected even at day 2, when tensions were 2% of control.

d. *BoNT Inhibitors Derived from Natural Products*

Toosendanin is a triterpenoid compound obtained from the bark of *Melia toosendan* and has been used in traditional Chinese medicine as an antiparasitic agent and agriculture insecticide. Toosendanin was reported to inhibit BoNT intoxication in animal models including nonhuman primates, and to alter the action of a number of ion channels, including the BoNT/A translocation channel (Shi and Wang, 2004; Li and Shi, 2006). Based on reports of its efficacy against BoNT in the Chinese literature, considerable interest has arisen in toosendanin as a potential BoNT antagonist (Sheridan and Parris, 2003; Dickerson and Janda, 2006). In our laboratory, we have found that toosendanin was able to antagonize BoNT/A intoxication in isolated mouse phrenic nerve-hemidiaphragm preparations when administered at the same time or 30 min before BoNT/A, but not when given 30 min after BoNT (Figure 16.3). We have also found that toosendanin is equally effective against BoNT serotypes BoNT/A, BoNT/B, or BoNT/E. The absence of serotype selectivity, coupled with its reported action on the BoNT translocation channel, suggests that toosendanin may be acting on a common step such as a slowing of internalization of the LC into the nerve-terminal cytosol. Current efforts are focused on examining analogs of toosendanin to shed more light on its mechanism of action.

In addition to studies on toosendanin, there are a number of other efforts to develop natural products as BoNT inhibitors, most notably from the laboratory of Dr Bavari. Using the peptide-based inhibitor 2-mercapto-3-phenylpropionyl-RATKML developed by Schmidt and Stafford (2002), Burnett et al. (2007) examined the interaction of this molecule with the active site of BoNT/A LC. The investigators then screened small nonpeptide molecules from the National Cancer Institute's Open Repository and tested these for inhibitory activity using an HPLC assay and in chick spinal motor neurons. Several compounds were effective in the HPLC assay ($K_i = 3$–10 μM), and one, NSC 240898, was found to be effective in producing a concentration-dependent protection of SNAP-25 in cultured spinal cord cells with no evidence of toxicity up to 40 μM.

e. *Removal of BoNT from the Nerve Terminal*

The persistence of BoNT-mediated paralysis is one of the most striking features of intoxication by clostridial neurotoxins. Although persistence is desirable for providing sustained relief of muscle hyperactivity (Jankovic and Brin, 1997), it is a major problem for management of BoNT intoxication (Souayah et al., 2006). In addition to the drug discovery efforts described earlier, a therapy that

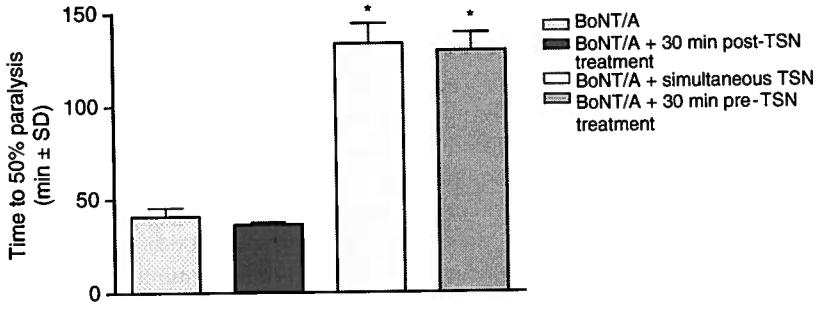

FIGURE 16.3 Toosendanin (TSN) delays the time to 50% paralysis in isolated mouse hemidiaphragm muscle exposed to BoNT/A. TSN (3 μM) was added to tissue baths 30 min before (pre), simultaneously with (simult.), or 30 min following addition of 20 pM BoNT/A (post). Paralysis times were significantly prolonged in muscles when TSN was added before or at the same time as BoNT/A ($p < 0.01$, ANOVA followed by Bonferroni post tests) as indicated by asterisks. Muscles tensions were elicited by supramaximal stimulation of the phrenic nerve at 30 s intervals. Temperature, 37°C.

shortens the period of intoxication would be a major contribution to the development of BoNT treatments.

There are two principal pathways for the elimination of proteins from cells: the ubiquitin proteasome system (UPS) and the lysosomal/autophagy system (Rubinsztein, 2006). These systems are both highly regulated and can be specific for targeting degradation; however, the UPS is generally considered to be the more critical pathway for the specific regulation of protein stability. The essential elements of the UPS consist of ubiquitin (a highly conserved 76 amino acid peptide), a cascade of enzymes to attach ubiquitin to a specific target protein, and the proteasome, which is a large macromolecular complex (Hochstrasser, 1996).

The signature for designating a protein for degradation is attachment of a ubiquitin chain. Ubiquitin serves a number of cellular functions but the most widely investigated is its role in targeting to the proteasome. Ubiquitin is attached to a specific protein through the action of a cascade of enzymes designated E1, E2, and E3, which can sequentially relay ubiquitin to culminate in the covalent ligation of the ubiquitin carboxyl terminus to a lysine side chain of the target protein. The covalent attachment of ubiquitin is exquisitely regulated, and the selection of the protein target for ubiquitination resides primarily in the function of E3 ubiquitin ligases. A specific E3 may have a single or very small number of protein targets that it will recognize to bring about ubiquitination of that target (Figure 16.4). Examples of such specific relationships are the recognition and ubiquitination of the antitumor protein p53 by the E3 ubiquitin ligase MDM2 as well as the regulation of IkK ubiquitination by an SCF ubiquitin ligase complex (Brooks and Gu, 2006).

In cells, the brief duration of BoNT/E LC appears to be related to its more extensive ubiquitination relative to that of BoNT/A LC (Figure 16.4b). The E3 ubiquitin ligases are generally modular, with one domain of the E3 specifically binding and recognizing the target protein and another domain, E3 ligase catalytic domain, functioning to facilitate the attachment of ubiquitin to the target protein lysine residue (Figure 16.5a). Such modular design allows for the generation of chimeric recombinant proteins, which contain a combination of a recognition domain for novel targets together with an E3 ligase catalytic domain to attach ubiquitin to this novel target (Figure 16.5b). This approach of generating chimeric recombinant or "designer" ubiquitin ligases is applied to a number of fields, including cancer therapy (Zhou et al., 2000; Oyake et al., 2002; Hatakeyama et al., 2005). The possibility of designating a target protein for degradation may be applicable to the development of therapeutics for BoNT intoxication and is being actively pursued.

In addition to the development of designer ubiquitin ligases for accelerating the elimination of BoNT LC from the presynaptic terminal, studies to determine the role of the endogenous neuronal ubiquitin system in regulating differences in persistence between BoNT/A and BoNT/E have been undertaken. Recent efforts have focused on the identification of cellular protein-binding partners for BoNT/A and BoNT/E LCs. The goal is to identify elements of the UPS that may preferentially recognize one of the two serotypes of BoNT LC and account for the greater rate of UPS degradation of BoNT/E. Two possibilities are emerging. In the first, BoNT/A interacts more extensively with enzymes in the deubiquitinating pathway (systems that actively reverse the ubiquitination process); in the second, cellular E3 enzymes interact more extensively with BoNT/E LC to accelerate its UPS degradation (Amerik and Hochstrasser, 2004). Both alternatives have therapeutic implications. Thus, development of a designer E3 ubiquitin ligase directed to the BoNT/A LC and the delivery of this designer E3 to affected neurons may shorten the duration of intoxication. Alternatively, it may be possible to identify deubiquitination enzymes that are responsible for the persistence of BoNT/A LC, and to inhibit these by small molecule pharmaceutical agents. The era of therapeutics directed toward regulation of protein stability by the UPS is emerging and holds promise in the evolution of novel therapeutic agents to address the persistence of BoNT intoxication.

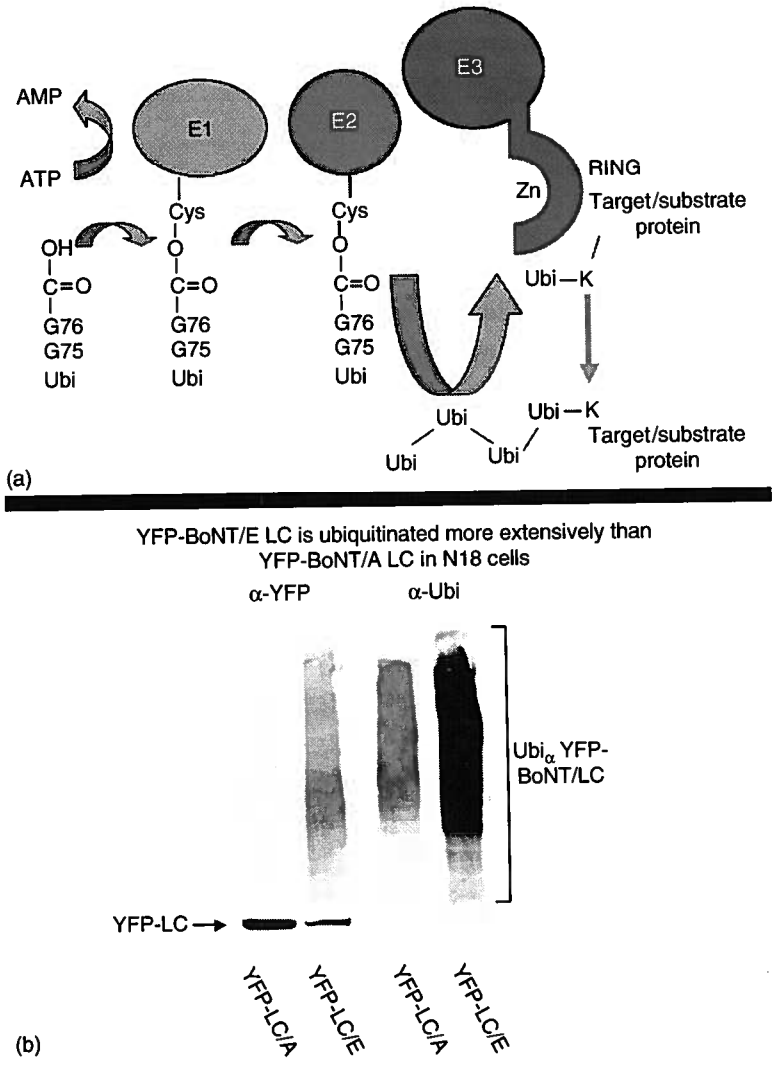

FIGURE 16.4 Cascade of ubiquitination enzymes and the extensive ubiquitination of BoNT/E LC in cells. (a) Ubiquitin activated and attached to lysine (K) residues in target substrate proteins by a cascade of enzymes, designated E1, E2, and E3. The specificity for selecting the target substrate protein for ubiquitin modification resides in the E3 enzyme. (b) Ubiquitination of BoNT/A and BoNT/E LC in N18 neuroblastoma cells. YFP-BoNT/A LC and YFP-BoNT/E LC were transfected into N18 cells, the cells were lysed, and YFP-BoNT LC proteins were immunoprecipitated with anti-YFP antibody. The immunoprecipitated YFP-BoNT LCs were separated and detected by western blotting with antiubiquitin antibody. The YFP-BoNT/E LC is more extensively ubiquitinated than the YFP-BoNT/A LC, as demonstrated by the immunoreactive smearing of the multi-ubiquitinated YFP-BoNT/E LC.

IV. CONCLUSIONS AND FUTURE RESEARCH

Efforts to develop pharmacological inhibitors of BoNT have increased substantially during the last decade. The major focus of the current research is the design and synthesis of specific metalloprotease inhibitors. Early drug discovery efforts were hampered by the lack of information on targets and the absence of the crystal structure for BoNT. Current efforts will be aided enormously by the

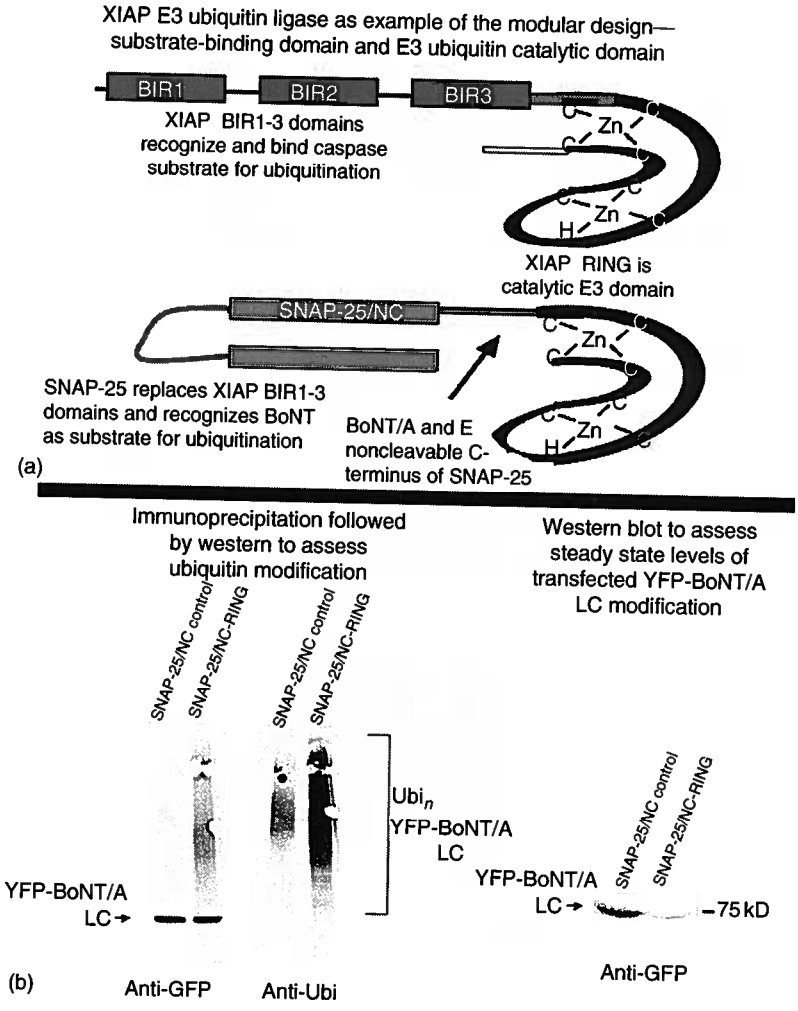

FIGURE 16.5 Modular composition of E3 ubiquitin ligases and increased ubiquitination of BoNT/A LC by a "designer" ubiquitin ligase. (a) The ubiquitin ligase XIAP is an example of the modular composition of E3 ubiquitin ligases generally where the amino-terminal BIR domains bind the target substrate caspases, and the carboxyl-terminal RING domain facilitates the attachment of ubiquitin to the target substrate. A designer E3 ubiquitin ligase for BoNT/A LC was constructed by substituting BoNT noncleavable (NC) SNAP-25 for the BIR domains of XIAP to allow targeting of the RING domain toward BoNT/A LC to induce ubiquitination. (b) Demonstration of the activity of the SNAP-25/NC-RING designer ubiquitin ligase. Cells were transfected with plasmid-expressing YFP-BoNT/A LC along with either SNAP-25/NC control plasmid or SNAP-25/NC-RING-expressing plasmid. The cells were lysed and the YFP-BoNT/A LC was assessed by immunoprecipitation. The cells transfected with SNAP-25/NC-RING displayed increased ubiquitination of the YFP-BoNT/A LC and a lower steady-state level of the YFP-BoNT/A LC.

availability of precise structural information and by knowledge of the mechanism of LC-mediated proteolysis of SNARE proteins (Chen and Barbieri, 2006).

Results to date indicate that a number of low molecular weight inhibitors and small peptides are effective against BoNT in cell-free in vitro systems (Sukonpan et al., 2004; Boldt et al., 2006a, 2006b; Park et al., 2006; Burnett et al., 2007; Eubanks et al., 2007).

Development of safe and effective metalloprotease inhibitors with in vivo efficacy will no doubt be difficult. Some of the challenges involve targeting of drugs to the nerve terminal, ensuring their access to the intracellular compartment and increasing the bioavailability of the drugs to match the duration of the toxin (Goodnough et al., 2002; Eubanks et al., 2007). In addition, different inhibitors may be needed for each serotype, requiring multiple parallel efforts. A more complete characterization of BoNT receptors and a better understanding of the internalization process have recently become available and will aid in accomplishing these objectives by refining the drug delivery methodologies (Chai et al., 2006; Jin et al., 2006).

It may also be necessary to accelerate the removal of cleavage products from the nerve terminal and to introduce noncleavable SNARE analogs for a more rapid recovery (O'Sullivan et al., 1999). The latter is especially relevant for treatment of persistent serotypes such as BoNT/A (Keller et al., 1999; Adler et al., 2001; Keller and Neale, 2001; Foran et al., 2003). The progress in understanding the mechanism of action of the BoNTs and detailed structural information gained during the last decade suggest that pharmacological treatments for BoNT intoxication will soon be a reality.

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