Handbook of Clinical Neurology, Vol. 91 (3rd series)
Neuromuscular junction disorders
A.G. Engel, Editor

Chapter 11

Botulism

ERIC A. JOHNSON[1]* AND CESARE MONTECUCCO[2]

[1]*Department of Bacteriology, Food Research Institute, University of Wisconsin, Madison, WI, USA*
[2]*Dipartimento di Scienze Biomediche Sperimentali, Università di Padova, Padova, Italy*

11.1. Introduction

Botulism is an acute neuroparalytic disease of humans and animals caused through the action of botulinum neurotoxins (BoNTs) primarily acting at the neuromuscular junction (NMJ) of somatic nerves that innervate cranial and skeletal muscle. This results in the blockade of release of acetylcholine (ACh) with ensuing denervation and accompanying muscle paralysis and atrophy. BoNT also blocks neurotransmission at cholinergic parasympathetic and postganglionic sympathetic nerves, affecting smooth muscle activity and glandular and secretory functions and impairing certain autonomic activities. Botulism generally presents with symptoms of fatigability affecting bulbar and ocular musculature and, in severe cases, weakness of the neck, limbs, torso and ensuing generalized paralysis. Botulism can be life-threatening, generally due to respiratory paralysis and failure and occasionally due to secondary infections or cardiac arrest. Although botulism is considered an acute intoxication, the duration of paralysis can last for weeks to months and complete recovery requires restoration of neurotransmission and muscle function. During the past century, death caused by botulism has decreased from ca. 70% to ca. 10% worldwide due primarily to clinical recognition of the disease, prompt administration of antitoxin, intensive nursing care, mechanical ventilation, parenteral feeding and control of secondary infections. Botulism outbreaks have had dramatic and devastating impacts on human and animal populations in which they occur (Meyer, 1956; Dolman, 1964).

Botulism is a true toxemia, caused solely through the action of BoNT at cholinergic nerve terminals. BoNTs are protein toxins of 150 kDa produced by neurotoxigenic bacteria of the genus Clostridium. Seven serotypes (A, B, C, D, E, F and G) are currently distinguished (Sugiyama, 1980; Sakaguchi, 1983; DasGupta, 1989; Schiavo et al., 2000). BoNTs are the most poisonous substances known and, currently, there is no antidote to botulism other than passive administration of antitoxin within hours after toxin exposure or immunization of at-risk individuals prior to exposure (Arnon et al., 2005). Since botulism is an extremely rare disease, general immunization of human populations is not practical and would prevent the pharmaceutical use of BoNT for treatment of human disease (Johnson, 1999).

Six clinical forms of botulism are recognized (Hatheway, 1995; Centers for Disease Control, 1998; Cherington, 2004): 1. classic foodborne botulism; 2. wound botulism; 3. intestinal botulism including infant botulism; 4. inhalational botulism; 5. botulism of unknown source; and 6. inappropriate administration of botulinum toxin during its use as a pharmaceutical agent (iatrogenic botulism). Intentional botulism poisoning by oral or inhalation exposure such as in a bioterrorist event could be considered as a seventh class with potentially severe consequences (Hatheway and Dang, 1994; Caya et al., 2004). Foodborne botulism through ingestion of BoNT by the oral route is the most prevalent natural form of botulism that occurs worldwide. However, currently the most common route of exposure of humans to BoNTs is by injection for medicinal treatment of a variety of neurological disorders and therapeutic uses, a remarkable development of this toxin (Scott, 1989; Schantz and Johnson, 1992; Jankovic and Hallett, 1994; Johnson, 1999; Moore and Naumann, 2003). A very large number of injections are performed each year in humans

*Correspondence to: Dr Eric A. Johnson, Department of Bacteriology, Food Research Institute, University of Wisconsin, 1550, Linden Drive, Madison, WI 53711, USA. E-mail: eajohnson@wise.edu, Tel: 1-608-263-7944, Fax: 1-608-262-9865.

and the disease syndromes being treated continue to expand at a rapid rate.

The primary objective of this chapter is to address the pathophysiology of botulism with emphasis on the basic science governing the clinical effects, diagnosis, treatment and recovery. Recent aspects regarding epidemiology, pathophysiology and molecular mechanisms of BoNTs are briefly described. Several excellent and in-depth reviews on the biochemistry, structure and pharmacology of BoNT are available (Schiavo et al., 2000; Brin et al., 2002; Moore and Naumann, 2003; Jahn and Scheller, 2006) and the reader is referred to these treatises for in-depth descriptions of these subjects.

11.2. Brief history of botulism as a neuromuscular disorder

Botulism is a presynaptic myasthenic neuromuscular syndrome exhibiting muscle weakness as its primary clinical sign. As such, it shares similarities with other myasthenic syndromes such as myasthenia gravis and Lambert–Eaton syndrome as well as a number of other congenital and acquired diseases and chemical and biological intoxications (Kaminski, 2003; Meriggioli et al., 2005; Holmes et al., 2006). Botulism likely occurred as a dreaded food poisoning in ancient cultures, including the 10th century edict of Emperor Leo VI of Byzantium (886–911), who forbid the preparation of raw sausages (Wright, 1955; Koenig, 1971; Smith and Sugiyama, 1988). The symptoms of botulism were described as a muscle-weakening syndrome by Justinus Kerner (1786–1862) in persons who had consumed uncooked blood sausages (Kerner, 1820; Devriese and Devriese, 2001; Ergguth, 2004). Despite the recognition of the clinical aspects of the disease, the responsible toxic agent remained elusive until 1895, when the toxigenic bacterium (Bacillus botulinus) and the causative toxin (botulinum neurotoxin, BoNT) were described by Emile Pierrre van Ermengem in a remarkable series of experiments from a food poisoning outbreak in Belgium (van Ermengem, 1897a, b, 1979; Devriese and Devriese, 2001). In the mid-to-late 1900s it was established that botulism also could result from wound and intestinal infections in humans and that botulism affected a variety of animals (Johnson and Goodnough, 1988; Smith and Sugiyama, 1988; Hatheway, 1995). Transmission of BoNT in stable aerosols in bioterrorism events has been evaluated and even attempted in modern times and botulism occurrence from bioterrorist events is considered to be a serious concern (Hatheway and Dang, 1994; Caya et al., 2004; Bigalke and Rummel, 2005).

Botulism is a rare disease, and it has been studied less extensively than many other neuromuscular disorders. It is often considered of low priority in the differential diagnoses of myasthenic disorders except under special circumstances such as in foodborne botulism outbreaks that affect several individuals from a single food source. Being rare, many physicians and neurologists have not encountered botulism in their practices. As such, less information is available regarding certain clinical aspects and pathophysiology of botulism compared to more common myasthenic syndromes.

11.3. Sources of botulinum neurotoxins

Botulinum neurotoxins are produced by a heterogeneous group of clostridial bacteria that differ widely in genetic and metabolic characteristics (Popoff, 1995; Hatheway and Johnson, 1998; Franciosa et al., 2003; Johnson, 2007). The exceptional feature of neurotoxigenic clostridia is their formation of a characteristic neurotoxin of extraordinary potency for humans and certain animals (botulinum and tetanus neurotoxins) (Sugiyama, 1980; Sakaguchi, 1983; Schiavo et al., 2000). Other key features of the neurotoxigenic clostridia are their anaerobic metabolism and the production of spores, which have high resistance to chemicals and physical agents (Hatheway and Johnson, 1998; Setlow and Johnson, 2007). The resistant spores are widely distributed in the biosphere and are disseminated in dust, waters, vapors, sewage and various fomites such as insects from which they readily contaminate most environments including soils, waters, humans and animals, households and buildings, as well as commodities such as foods (Hatheway and Johnson, 1998; Johnson, 2007). Under permissive nutritional and physical conditions (anaerobiosis, low acid, pH $>$ 4.5, relatively low concentrations of salt and sugar and nutrient sufficiency) the spores germinate to form vegetative cell populations and elicit BoNT (Smith and Sugiyama, 1988; Franciosa et al., 2003; Johnson, 2007). The natural habitats of neurotoxigenic clostridia are soils and sediments and the intestinal contents of certain insects and animals (but not healthy humans) (Popoff, 1995; Hatheway and Johnson, 1998; Johnson, 2005c). Unlike many other pathogenic bacteria, neurotoxigenic clostridia are saprophytic and do not have an obligatory relationship with an animal host (Hatheway and Johnson, 1998; Johnson, 2005c).

Recognized species of neurotoxigenic clostrida are *Clostridium botulinum* (a large collection of heterogeneous strains that have the common property of producing BoNT) and rare strains of *Clostridium butyricum* and *Clostridium baratii* (Hatheway, 1995; Popoff, 1995; Johnson and Goodnough, 1998; Franciosa et al., 2003; Johnson, 2005a, c). The heterogeneous nature of the neurotoxigenic clostridia suggests that genes for BoNT

have been laterally transferred by plasmid exchange or bacteriophage infection to distinct clostridial species throughout evolution and possibly in contemporary time since most of the new toxigenic species have been isolated from the human intestine (Popoff, 1995; Johnson, 2005b).

Since the primary target of BoNTs is the neuromuscular junction of eukaryotes and particularly vertebrates that evolved billions of years after the initial appearance of the clostridia, the evolution of the BoNT toxin is an intriguing question. Our present view certainly represents only a snapshot in millions of years of evolution. Nucleotide and amino acid sequence studies of the gene as well as structural analyses of the neurotoxins has shown that the BoNT family of toxins comprises a group of highly chimeric molecules of various origins (Niemann, 1991; Lacy and Stevens, 1999; Lacy et al., 1998). Structural and amino acid sequence analyses have shown that BoNT contains segments of genes/proteins from a myriad of organisms (Lacy and Stevens, 1999). Certain properties of BoNTs, especially their synthesis as a polyprotein in a protein complex (Johnson and Bradshaw, 2001; DasGupta, 2006), and similarities in mechanisms of cellular uptake related to certain viruses, suggest that neurotoxigenic clostridia may have acquired portions of the BoNT gene from a neurotropic viral source. Several of the BoNT gene complexes were shown to be associated with mobile genetic elements including bacteriophages, plasmids, transposons and IS (insertion) elements that can be transferred to other bacterial cells and populations (Johnson and Bradshaw, 2001; Johnson, 2005b). Thus, it is likely that the evolutionary diversity of the BoNTs will continue to expand and it is anticipated that additional clostridial species and perhaps other groups of bacteria will be discovered that produce functional BoNTs poisonous to humans and animals.

BoNTs are currently categorized into seven immunologically distinguishable serotypes (serotypes A–G), whereby polyclonal antibodies raised against purified BoNTs neutralize the toxicity of the homologous but not the heterologous serotypes in the mouse bioassay (Hatheway, 1988; Giménez and Giménez, 1993). The mouse bioassay, first introduced in 1939 for detection of botulism in humans (Schneider and Fisk, 1939), is the principal method for assaying and serotyping BoNTs. In the currently used intraperitoneal mouse bioassay (Schantz and Kautter, 1978; Hatheway, 1988; Centers for Disease Control, 1998; Solomon et al., 2001), the LD50 for a 20 g mouse is ca. 7–12 pg for purified BoNT/A and is ca. 20–36 pg for BoNT/A-complex (Schantz and Johnson, 1992; Malizio et al., 2000). All seven serotypes of BoNTs have an intraperitoneal specific toxicity of $\sim 10^8$ LD50 per mg in mice (Sugiyama, 1980), but the toxicity for different animal species including humans varies markedly according to serotype and route of exposure (Morton, 1961; Schantz and Johnson, 1992).

The mouse bioassay has certain drawbacks including the need for large numbers of mice, 2–4 days to obtain definitive results and specimens such as stools and foods may contain lethal substances unrelated to BoNT (Hatheway, 1988). For example, pyridostigmine that had been administered to a patient originally thought to have myasthenia gravis was lethal to mice and presented difficulties in the diagnosis of botulism (Horowitz et al., 1976). Therefore, the assay must be carefully performed with proper controls and the use of serotype-specific antibodies to definitively identify the lethal substance as BoNT (Hatheway, 1988). Several other methods provide biological assays of BoNTs including neuronal cells, tissue preparations such as mouse or rat hemidiaphragm, and others (Habermann and Dreyer, 1986; Habermann, 1989; Johnson, 2005a; Pellett et al., 2007).

Most neurotoxigenic clostridia strains produce a single serotype of BoNT, while some strains produce more than one serotype or have silent unexpressed genes for a different serotype of BoNT (Hatheway, 1995; Franciosa et al., 2003; Johnson, 2005a). Recently, strains of *C. botulinum* have been recognized that produce subtypes of BoNT within a given serotype that differ substantially in amino acid composition, structure and biological activity compared to the primordial toxin (Smith et al., 2005; Arndt et al., 2006b). The serotypes of BoNT that cause human botulism are A, B, E and (rarely) F (Wright, 1955; Koenig, 1971; Woodruff et al., 1992; CDC, 1998; Sobel, 2005). All serotypes of BoNTs tested can cause botulism when administered intravenously or by inhalation and they differ markedly in potency by these routes (LeClaire and Pitt, 2005; Pitt and LeClaire, 2005). The different serotypes also vary in clinical properties including time to onset of symptoms, autonomic effects, severity and duration of the disease and time to recovery (Koenig, 1971; Woodruff et al., 1992; Hatheway and Dang, 1994; CDC, 1998). In general, BoNT/A causes the most severe and long-lasting and BoNT/E the shorter-lasting human botulism.

When BoNT/A-complex is injected in striated muscle, paresis commences within 2–8 days and lasts for 2–3 months when its effects begin to diminish (Dressler and Saberi, 2005; Johnson et al., 2006). The onset and duration of paralysis not only varies with toxin serotype and animal model, but also among patients. The extent of paresis is correlated with the dose of BoNT injected and these parameters can be optimized for the pathological condition to be treated

(Dressler and Rothwell, 2000). The duration of action shows a correlation primarily at low doses injected (Dressler and Saberi, 2005). When high doses are used, the duration appears to maximize at ~3 months for BoNT/A. Different preparations of BoNT/A and BoNT/B may have different potencies and therapeutic effects. Certain BoNT/A-complexes prepared and formulated by different methods are approved for clinical use and these appear to differ in their therapeutic efficacy (e.g., see recent review by Wenzel et al., 2007).

Some autonomic symptoms appear to be more prominent with BoNT/B than with BoNT/A (Dressler and Benecke, 2003). Autonomic effects between BoNT/A-complex and BTX/B-complex were compared in a double-blind, randomized trial in patients being treated for cervical dystonia (Tintner et al., 2005). BoNT/B-complex exhibited greater autonomic effects than BoNT/A-complex as indicated by decreased saliva production and increased severity of constipation, but not other autonomic functions such as orthostatic hypotension, heart rate or heart rate variation with respiration. Factors contributing to the difference in autonomic symptoms between the two serotypes may include the different quantities of BoNT/B to BoNT/A (50:1) used, which in turn determines toxin spreading from the site of injection, increased susceptibility of the cholinergic autonomic terminals to BoNT/B than BoNT/A and a greater systemic response to BoNT/B. One study in dogs indicated that BoNT/A-complex inhibited cholinergic ganglionic neurotransmission in dog heart (Tsuboi et al., 2002), but other studies showed no effects on heart rate (Claus et al., 1995; Meichsner and Reichel, 2005).

11.4. Toxicity and antitoxins

BoNTs are the most potent protein toxins known and their toxicity depends on the route of entry into the human body. They can enter the blood through the intestine, wounds and mucosal membranes. The estimated intravenous and intramuscular human lethal doses of BoNTs are 0.1–1 ng per kg body weight (Gill, 1982; Schantz and Johnson, 1992; Hatheway and Dang, 1994), whilst more than a one thousand times lower toxicity is detected using the oral route (Morton, 1961; Hatheway and Dang, 1994; Larson and Johnson, unpublished review 2005); for aerosol exposure, the lethal dose has been estimated to be 1–75 ng per kg depending on the serotype and efficiency of exposure (Pitt and LeClaire, 2005). These estimates are based mainly on primate exposure studies and investigations of quantities of toxin in foods implicated in foodborne botulism (Lamanna, 1959; Morton, 1961; Schantz and Johnson, 1992; Hatheway and Dang, 1994; Larson and Johnson, unpublished review 2005). It is expected that infants and children have substantially increased sensitivity to intoxication due to the reduced size of certain NMJs, including those innervating the respiratory diaphragm, paralysis of which can be linked to death.

The number of BoNT molecules required to cause intoxication, morbidity and death of animals and humans is an intriguing subject. For example, when considered in terms of total body cells, it has been estimated that 20–70 ng or $\sim 8 \times 10^{10}$ to 3×10^{11} molecules of botulinum neurotoxin (molecular mass = 150 kDa) is sufficient to produce lethality in a ca. 70 kg adult human (Lamanna, 1959). It has been estimated that ca. 10 molecules of BoNT/A are sufficient to cause blockade of neurotransmitter releases at a NMJ (Boroff et al., 1974). It is well known that stimulation of nerve activity enhances the toxicity of BoNT in tissue preparations which can influence toxicity (Hughes and Whaler, 1962). It should be noted that much lower amounts of BoNTs are sufficient to lead to death of animals in the wilderness as even minor impairment of functions can be lethal, as in the case of the diplopia of birds with respect to flight and landing abilities. With the recent elucidation of receptor molecules for certain serotypes, it has become evident that presynaptic activity is necessary for uptake and poisoning of cells and nerves in vivo (reviewed in Verderio et al., 2006). Once synaptic activity subsides, additional BoNT cannot enter the poisoned neuron and will be directed to non-poisoned nerve cells. Thus, *C. botulinum* has evolved a cunning means of pathogenesis through synthesis of BoNT, since when synaptic activity is shut down by intracellular toxin activity, uptake by poisoned nerves is also prevented and this in turn promotes spread of BoNT to active neurons.

Antitoxins to botulinum neurotoxins have been used extensively for characterization of BoNTs and for treatment of individuals with botulism (Tacket et al., 1984; Arnon et al., 2006). In 1897, Kempner first demonstrated that antitoxins against BoNT/A could be raised in goats by injection of inactivated crude botulinum toxin. His findings that BoNTs are antigenic proteins and that antitoxins against the toxins could reduce the severity of botulism has been confirmed and forms the mainstay of prophylaxis on intoxication (Kempner, 1897). Antitoxin antisera prepared against different BoNTs neutralize the homologous but not the heterologous serotype and a polyvalent antitoxin antiserum appears necessary for therapy. Antitoxins are presently used to confirm the identity of BoNTs and intravenous administration provides a first-line defense that is protective against botulism, lessens the symptoms and shortens the hospital stay (Tacket et al., 1984; Mayers et al., 2001; Chang and Ganguly, 2003; Arnon et al., 2006).

Recently, BabyBig® has been approved in the USA for administration to infants with botulism (Arnon et al., 2006).

Since botulism occurs rarely in humans, it is not practical to routinely immunize human populations. Natural immunity has not been observed in humans, even in individuals who have experienced repeated botulism. However, antibody formation is an important consideration in the treatment of humans with BoNT for therapeutic uses (Borodic, 2007). The possibility of developing an immune response is related to the quantity of antigen presented and the frequency of exposure (Hatheway and Dang, 1994; Borodic et al., 1996; Borodic, 2007). The dose of toxin required to elicit an antibody response is not known, yet clinical experience indicates that it is higher than the lethal dose. Humans who routinely handle BoNTs can be effectively immunized by pentavalent toxoid available from the CDC and other international governmental agencies (CDC, 1998).

11.5. General properties of botulinum neurotoxin

Biochemical and structural investigations of BoNT and TeNT and their domains have provided considerable insight into their evolution and mode of action (DasGupta, 1989; Schiavo et al., 2000; Hanson and Stevens, 2002; Swaminathan and Eswaramoorthy, 2002). BoNTs are synthesized as inactive single chain molecules of 150 kDa, that assume their characteristic high toxicity by proteolytic activation into a ca. 100 kDa heavy chain (HC) and a ca. 50 kDa light chain (LC) that remain linked by a single disulfide bond (DasGupta, 1989; Schiavo et al., 2000). Reduction of the disulfide bond is necessary for maximal catalytic activity in vitro and in vivo and may be a rate-limiting step in catalytic action (Schiavo et al., 1990; de Paiva et al., 1993; Antharavally et al., 1998). The carboxy-terminus HC comprises the structural domain for receptor binding to gangliosides and to proteins (Schiavo et al., 2000; Nishikawa et al., 2004; Chai et al., 2006; Jin et al., 2006; Uotso et al., 2006; Baldwin and Barbieri, 2007) and the N-terminus of the HC contains the function for channel formation and translocation of the LC from endosomes to the neuronal cytosol (Hoch et al., 1985; Schiavo et al., 2000; Koriazova and Montal, 2003). The LC is a Zn^{2+}-dependent endopeptidase that selectively cleaves neuronal substrates involved in synaptic vesicle trafficking and membrane fusion (Montecucco and Schiavo, 1995; Schiavo et al., 2000).

BoNTs occur naturally in toxin complexes, commonly referred to as progenitor toxins (Sakaguchi, 1983; Schantz and Johnson, 1992; Johnson and Bradshaw, 2001). In these complexes, BoNT is associated with non-toxic proteins, primarily non-toxic non-hemagglutinin (NTNH), hemagglutinin in certain serotypes, uncharacterized proteins and RNA (Sakaguchi, 1983; Schantz and Johnson, 1992; Inoue et al., 1996; Johnson and Bradshaw, 2001; Dineen et al., 2003, 2004). The structure of the complexes seems dependent upon the genetic composition and expression of the complex components (Sakaguchi, 1983; Johnson and Bradshaw, 2001; Dineen et al., 2003, 2004) as well as purification methods (Sakaguchi, 1983; Schantz and Johnson, 1992). The biochemical, biophysical and structural aspects of the toxin complexes are only beginning to become known (Hanson and Stevens, 2002). The non-toxic proteins in the complexes provide protection during experimental manipulations and from acid and proteases during passage through the gastrointestinal tract (Sugii et al., 1977; Sakaguchi, 1983; Schantz and Johnson, 1992). The different complexes also differ in their safety margins and therapeutic indexes from the perspective as a protein drug for human treatment (Aoki, 2002; Yoneda et al., 2005).

Purified BoNTs can be purified from the toxin complexes by established techniques (DasGupta and Rasmussen, 1983; DasGupta and Sathyamoorthy, 1984; Malizio et al., 2000). An important factor contributing to the structure and function of BoNTs has been the availability of highly purified neurotoxins free of accessory proteins, contaminating proteases or other enzymes. The isolated neurotoxin can be unstable, prone to aggregation, zinc removal, protease modification and auto-fragmentation under certain conditions (DasGupta and Dekleva, 1990; DasGupta et al., 2005; DasGupta, 2006; Keller, 2006; Paik et al., 2006). The loss of activity of BoNTs has negatively affected in vitro and animal studies as well as use of BoNTs for therapy (Schantz and Johnson, 1992; Gartlan and Hoffman, 1993; McLellan et al., 1996).

Three-dimensional structures have been obtained by crystallography for the holotoxins of types BoNT/A and BoNT/B in solution (Lacy and Stevens, 1999; Swaminathan and Eswaramoorthy, 2000, 2002; Hanson and Stevens, 2002) and for all seven serotypes of L chains (not including subtypes) (e.g., Arndt et al., 2006a). The structure of BoNT/A was initially achieved by Stevens and colleagues in 1998 at 3.3-Å resolution (Lacy et al., 1998) (Fig. 11.1) and BoNT/B by Swaminathan and colleagues in 2000 at 1.8-Å (Swaminathan and Eswaramoorthy, 2000). BoNT/A and BoNT/B have similar structures consisting of three distinct domains (Lacy and Stevens, 1999; Swaminathan and Eswaramoorthy, 2000, 2002; Hanson and Stevens, 2002). These domains represent the carboxy terminus of the heavy chain (HCC) (binding domain),

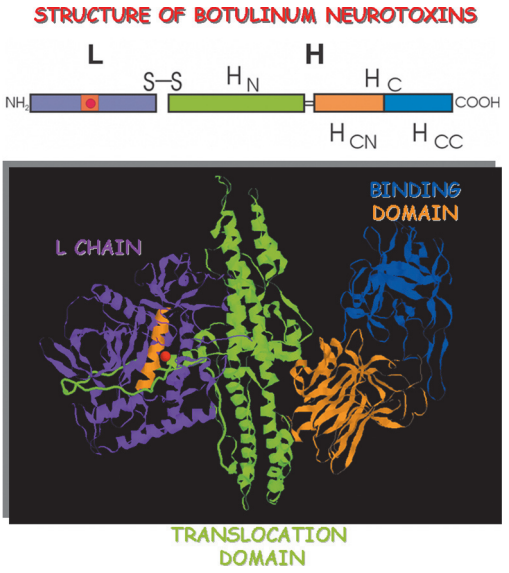

Fig. 11.1. Linear depiction (top) and three-dimensional structure (bottom) of the 150 kDa botulinum neurotoxin type A. The toxin is produced as a single chain protein, which is activated to the di-chain form by proteolysis. Three primary domains, receptor binding domain, translocation domain and the catalytic domain, are present in the neurotoxin.

amino terminus of the heavy chain (HCN) (translocation or channel domain) and the light chain (LC) (catalytic domain). The structural depiction of functional domains is consistent with the toxic activity of the BoNTs determined by various biochemical and physiological approaches and with its mechanism of cell intoxication. The major differences in 3-D structure between the two serotypes are mainly in the receptor binding and catalytic domains, which are anticipated by the differences in the amino acid sequences in these regions and their known binding to different protein receptors and catalytic activity on different SNARE proteins. Subtle differences in the translocation domain and the belt surrounding the catalytic site have also been recognized. The structure complexity has increased with the findings of subtypes of BoNTs (Smith et al., 2005; Arndt et al., 2006b) and it will be interesting to determine whether these newly found subtypes have biological properties that are distinct from the prototype BoNTs, thus increasing the pathogenic spectrum of BoNTs.

11.6. Clinical aspects

11.6.1. Types of botulism

The primary target of BoNTs is the NMJ of the peripheral nervous system and BoNT also binds to preganglionic sympathetic and parasympathetic nerve endings, postganglionic parasympathetic nerve endings and efferent motor nerve endings (Simpson, 2000). Botulism is a blood-borne toxicosis and the susceptibility of animals to different serotypes varies considerably across species. In humans, the primary serotypes of BoNT responsible for botulism are A, B, E and, rarely, F. The different forms of botulism have been categorized mainly by the route by which BoNT enters the bloodstream and the age of the subject. The times of onset, duration of action, symptoms and clinical course varies depending on the serotype of BoNT and the type of botulism, with serotype A causing a more dangerous and long-lasting disease.

11.6.2. Foodborne botulism

Foodborne botulism is caused by the consumption of pre-formed BoNT-complexes in foods. In some foodborne outbreaks, several persons are intoxicated by sharing contaminated food which can be of value in recognizing an outbreak, while the majority of outbreaks affect a single person (CDC, 1998; Shapiro et al., 1998; Sobel et al., 2004). In the United States, botulism is one of the most precisely recognized foodborne illnesses, since all hospitalized cases must be reported to the Centers for Disease Control and Prevention in Atlanta (CDC, 1998). Fortunately, foodborne botulism is quite rare worldwide, but similar to other acute diseases it is undoubtedly under-reported in that mild cases probably are not diagnosed (Woodruff et al., 1992). Certain countries currently do not have adequate public health facilities for botulism diagnosis and treatment, but efforts are being made to develop surveillance and treatment programs in these areas (Shapiro et al., 1998; Villar et al., 1999). The establishment of networks to provide access to botulinum antitoxins could also reduce morbidity and mortality (Abgueguen et al., 2003).

The incidence of foodborne botulism varies markedly by geographic region worldwide (Hauschild and Dodds, 1993; Hatheway, 1995; Johnson and Goodnough, 1998; Shapiro et al., 1998; Sobel, 2005). This regional incidence is related to the serotype and prevalence of spores in the soil and associated foods, as well as food preservation and consumption practices within a region. The principal regions of the world with reported foodborne botulism have been reviewed (Hauschild and Dodds, 1993; Johnson and Goodnough, 1998). Worldwide, case fatality rates from foodborne botulism have been estimated to range from 5–15%, with a mean of 10% (Varma et al., 2004). In the United States, type A has caused the most severe botulism and incidence of fatalities (5–7% deaths), compared to types B and E (1–3% deaths). The incidence and characteristics of foodborne botulism in the United Kingdom (McLauchlin et al., 2006), Thailand (Ungchusak et al.,

2007), South Africa (Arntzen et al., 2004), Republic of Georgia (Varma et al., 2004) and Argentina (Villar et al., 1999) have recently been described. With an increasingly globalized food supply and changes in food consumption, countries are observing types of botulism previously uncommon for that region (e.g., Boyer et al., 2001).

11.6.3. Wound botulism

Wound botulism results from the infection of a wound with *C. botulinum* and subsequent localized production of BoNT in the wound and its entry into the bloodstream (Dezfulian, 1989; Maselli et al., 1997; Werner et al., 2000; Gordon and Lowy, 2005). Wound botulism is suspected following trauma or a superficial wound without obvious infection and no history of consumption of botulinogenic food. Although adult tetanus and wound botulism occur under similar predisposing conditions, wound botulism is much more rare than tetanus, even though the general population is not immunized against BoNT. *C. botulinum* strains appear to have a weak ability to grow in wounds compared to C. tetani. Like other forms of botulism, wound botulism is quite rare and only about 1000 cases have been reported since its recognition in 1943 (Davis, 1951; Werner et al., 2000). Wound botulism has recently increased in incidence and geographical distribution due to its association with intranasal use of cocaine and subcutaneous injection of heroin (MacDonald et al., 1986; Elston et al., 1991; Werner et al., 2000). An unfortunate case of wound botulism was reported in a 5-year-old boy who died from toxin produced in a tooth abscess (Weber et al., 1993). Most wound botulism cases are caused by *C. botulinum* type A producing BoNT/A in the wound, which is absorbed into the bloodstream. A distinguishing feature of wound botulism compared to foodborne botulism is the longer incubation time of 4–14 days (Merson and Dowell, 1973), presumably reflecting the need for *C. botulinum* to colonize the wound and grow to sufficient levels to produce quantities of toxin required for illness.

Rare cases of wound botulism cases have occurred following intestinal surgery and disruption of the integrity of the intestine and composition of intestinal microflora (Chia et al., 1986; McCroskey and Hatheway, 1991). This form of botulism was initially suspected in a patient who had undergone ileojejunal bypass for obesity (English et al., 1981). Botulinum toxin is produced in these intestinal lesions and absorbed into the blood. Since wound botulism is rare, misdiagnosis and delay or failure in treatment have been encountered (Werner et al., 2000; Reller et al., 2006). Like other forms of botulism, predictors of survival in wound botulism are rapid diagnosis, prompt administration of antitoxin and mechanical ventilation for persons with respiratory failure (Werner et al., 2000; Sandrock and Murin, 2001; Reller et al., 2006).

11.6.4. Infant botulism

In 1976, infant botulism was suspected in two babies in California that had clinical signs of weakness characteristic of floppy babies (Pickett et al., 1976). The clinical diagnosis of botulism was confirmed by the detection of *C. botulinum* and BoNT in the feces of the suspected infants (Midura and Arnon, 1976). Initially, it was considered that the babies had consumed food containing preformed BoNT, but such a food source could not be identified (Midura and Arnon, 1976; Marx, 1978). In vivo formation of BoNT in the intestine and absorption of BoNT into the blood was hypothesized (Midura and Arnon, 1976; Pickett et al., 1976). Subsequently it was recognized that infant botulism occurs from the ingestion of spores of neurotoxigenic clostridia that germinate, multiply and produce BoNT in the intestinal tract of the baby. BoNT is then absorbed through the intestine and enters the general circulation and is carried to peripheral cholinergic synapses. Thus, infant botulism is a type of infection rather than a true intoxication as in foodborne botulism. Infant botulism is not a new disease and retrospective analyses of idiopathic floppy baby syndromes indicated that it has occurred for many years (Arnon et al., 1979).

Infant botulism is now recognized as the most common cause of botulism in the USA, with about 70 hospitalized cases annually (Arnon, 2004; Sobel, 2005). Nearly half of the cases have been reported from California, probably due to the relatively high incidence of type A *C. botulinum* spores and the vigorous surveillance program for infant botulism in this state (Arnon, 2004). Infant botulism has been documented worldwide in all populated continents except Africa (Arnon, 2004). Certain risk factors have been identified including feeding of honey, which can be contaminated with spores (Tanzi and Gabay, 2002). Dust and other environmental sources are also known to harbor *C. botulinum* spores (Nevas et al., 2005). Infant botulism has occurred in distinct geographic regions, probably due to the incidence of spores and other factors such as genetic disposition (Long et al., 1985; Arnon, 2004). Nearly all cases of infant botulism are caused by *C. botulinum* strains producing BoNT/A and BoNT/B. In the United States, infant botulism caused by type A is most common in the Western United States, whilst

type B has been prevalent in regions of the Eastern United States (Long et al., 1985; Arnon, 2004). The severity of infant botulism varies according to the serotype of BoNT, with BoNT/A generally causing a more severe and long-lasting disease-syndrome than BoNT/B (Arnon, 2004).

Neurotoxigenic strains of *Clostridium butyricum* and *Clostridium baratii* producing variants of BoNT/E and BoNT/F, respectively, have also been implicated in infant botulism in the United States, Italy and other regions of the world (Hatheway, 1993, 1995; Franciosa et al., 2003). Neurotoxigenic *C. butyricum* and *C. baratii* have recently been detected in expanding geographical regions and may be associated with foodborne as well as infant botulism. Atypical neurotoxigenic variant strains have mostly been isolated from intestinal botulism (Tabita et al., 1991; Hatheway, 1995). These findings suggest that the intestinal tract under certain conditions may be a permissive environment for gene exchange and for evolution of new variants of neurotoxigenic clostridia and it is likely that more neurotoxigenic species may be discovered from this source in the future.

The ability of *C. botulinum* to colonize infants is probably related to perturbations of intestinal flora during infancy and during weaning, as well as the presence of inhibitory factors such as bile acids and innate immunity (Arnon, 2004). The study of *C. botulinum* colonization in animal models has supported the notion that the ability to colonize is dependent on the number and species of competitor microbes present in the intestinal tract, particularly the large bowel (Sugiyama and Mills, 1978; Sugiyama, 1979; Arnon, 2004). Infant botulism usually affects babies 1–6 months of age, but can also manifest only a few hours after birth and (rarely) as long as 10–12 months of age (Arnon, 2004; Fox et al., 2005; Keet et al., 2005). The median age at onset is 10–12 weeks and 95% of the cases have occurred prior to 6 months of age, although cases have been observed up to 10 months (Arnon, 2004; Fox et al., 2005). The illness affects both sexes and all major ethnic and racial groups (Arnon, 2004).

The severity of the clinical syndrome may vary from relatively mild observed in outpatients who show minor weakness and failure to thrive to a rapid and fatal illness resembling sudden infant death syndrome (SIDS) (Arnon et al., 1981; Byard et al., 1992; Böhnel et al., 2001; Bartram and Singer, 2004; Böhnel and Gessler, 2005; Fox et al., 2005; Mitchell and Tseng-Ong, 2005; Nevas et al., 2005). It has been postulated that fulminant cases of botulism may be responsible for a small percentage (5%) of sudden infant death syndrome cases (Marx, 1978; Arnon et al., 1981; Bartram and Singer, 2004; Fischer et al., 2004; Böhnel and Gessler, 2005). The involvement of infant botulism in SIDS has been a matter of considerable debate. Recent clinical data indicate that rapid onset infant botulism can be a catastrophic presentation of the disease, but with rapid recovery (Keet et al., 2005; Nevas et al., 2005; Tseng-Ong and Mitchell, 2005). Rapid onset of infant botulism has also occurred by BoNT/F produced by *C. baratii* in extremely young patients (9 and 14 days old) (Hoffman et al., 1982; Paisley et al., 1995). The typical presentation of infant botulism is familiar to most pediatric neurologists, but atypical symptoms and sudden and severe features may be obscure or delay the diagnosis (Keet et al., 2005; Mitchell and Tseng-Ong, 2005). Complications of infant botulism during hospitalization and treatment have included sepsis, otitis media, aspiration pneumonia, inappropriate secretion of antidiuretic hormone and adult respiratory distress syndrome (Long et al., 1985). Muscle and nerve biopsies were performed on an infant with botulism, which helped to exclude myopathy or metabolic causes of paralysis (Keet et al., 2005). Studies in animals have demonstrated atrophy of muscle fibers in severe botulism cases, which regenerated during the recovery phase. It has been suggested that chronic neuropathic changes may persist following botulism (Keet et al., 2005), but further investigation is needed in this area to confirm this observation.

11.6.5. Intestinal botulism in adolescents and adults

Intestinal botulism has also occurred in susceptible adolescents and adults, especially following intestinal surgery and antibiotic administration, which can alter the gut's microbial ecology and predispose the patient to colonization and possibly provide wounds for colonization. Infections by *C. botulinum* in adults has been supported by the finding of botulinum toxin and organisms in the feces over a several month period (Hatheway, 1995). Intense immunosuppression and bowel sterilizing regimens in a 3-year-old girl with neuroblastoma were postulated to enable colonization by *C. botulinum* types A and B with ensuing infant botulism (Shen et al., 1994). An intestinal botulism case in a 12-year-old girl associated with an unusual strain of *C. botulinum* type Ab was reported in Japan (Kobayashi et al., 2003).

In foodborne cases, a slow onset of botulism symptoms and a long delay of toxin detection in serum and stool may predispose adults to colonization of the large bowel with *C. botulinum* spores. Thus, there may be a transition between different forms of botulism. In a foodborne case involving chopped garlic in oil

that contained type BoNT/B and *C. botulinum* type B, relatively mild and progressive development of botulism occurred over several weeks in patients containing type B toxin and organisms (St. Louis et al., 1988). In a foodborne botulism case occurring in France, type B toxin was found in serum from individuals for up to 122 days after ingestion of toxic food (Sebald and Saimot, 1973). These observations suggest that *C. botulinum* type B could colonize the intestine of adults and continue to produce toxin over several weeks to months. *C. botulinum* type B may have a greater ability than type A to colonize the intestinal tract, which likely explains why more than 50% of the infant botulism cases in the USA involve type B, while type A foodborne botulism is three times more prevalent than type B. The ecology of intestinal botulism is complex and the disease may involve more than one species of toxigenic clostridia including C. difficile, C. perfringens, *C. botulinum*, *C. baratii* and *C. butyricum* that produce various toxins and change the gut flora. C. difficile colonization was found in cases of infant botulism (Schechter et al., 1999), suggesting that certain permissive conditions may promote intestinal colonization by pathogenic clostridia.

11.6.6. Botulism of undetermined etiology

Botulism of undetermined etiology refers to diagnosed cases in patients 1 year of age or older in whom no plausible food vehicle or intestinal or wound colonization can be demonstrated (Bartlett, 1986; Hatheway, 1993; Sobel et al., 2004). *C. botulinum* and its toxin have been observed in the stools of these patients for long time periods (3 weeks and in some cases up to 6 months) (Hatheway, 1993). This form of the disease has occurred in adults following colonization of the digestive tract by *C. botulinum* with production of BoNT and these infections may follow surgery or other procedures that disrupt the GI microflora or inflict intestinal wounds (Chia et al., 1986; Hatheway, 1993). Intestinal botulism and endogenous antibody production occurred in an adult with underlying Crohn's disease (Griffin et al., 1997). Intestinal botulism was also observed in a 3-year-old female with neuroblastoma following autologous bone marrow transplantation, possibly indicating immune suppression leading to susceptibility to botulism (Shen et al., 1994). Following an incident of foodborne botulism in a 12-year-old girl, obstinate constipation was endured for more than 6 months and *C. botulinum* and BoNT/A was detected in stool specimens for several months (Kobayashi et al., 2003). These cases indicate that intestinal colonization by *C. botulinum* can occur following foodborne botulism.

11.6.7. Inhalational botulism

Owing to their high toxicity, BoNTs have been considered as biological weapons including as aerosol preparations (Franz et al., 1993; Bigalke and Rummel, 2005; LeClaire and Pitt, 2005; Pitt and LeClaire, 2005). Since respiratory exposure is not a known natural route of intoxication, virtually no human data on susceptibility are available (Pitt and LeClaire, 2005). Exposure of non-human primates has demonstrated that intoxication can occur by the respiratory route through mucosal membranes (Pitt and LeClaire, 2005). As with foodborne botulism, crude toxin complexes are more potent than purified BoNTs. The toxicity varies with serotype and BoNT/F and BoNT/C appear to be slightly more toxic than BoNT/A (LeClaire and Pitt, 2005; Pitt and LeClaire, 2005). Extrapolations from animal data suggest that BoNT is exceedingly potent by the aerosol route with an estimate LD50 of 1–75 ng per kg with the potency decreasing in the following order: BoNT/F > BoNT/C = BoNT/A > BoNT/D >> BoNT/B in rhesus macaques; no data are available for BoNT/E and BoNT/G (LeClaire and Pitt, 2005; Pitt and LeClaire, 2005). Respiratory failure occurred in 5 hours to 2 days with 90% lethality. A case of human botulism was attributed to inhalation of BoNT in a laboratory accident (Holzer, 1962).

11.6.8. Iatrogenic botulism

Iatrogenic botulism, sometimes referred to as inadvertent botulism, refers to botulism resulting from therapeutic use of botulinum toxin (Chertow et al., 2006). Rare cases of systemic weakness and botulism have been observed in patients treated with BoNT for medicinal purposes (Bakheit et al., 1997; Bhatia et al., 1999; Duffey and Brown, 2006). Quantitation of systemic weakness was assessed in a 34-year-old amateur weight-lifter treated for a gait disorder (Duffey and Brown, 2006). Although the gait disorder was successfully treated with 1000 units (11 U/kg) of BoNT/A-complex, the individual experienced 35–50% reduction in upper body strength, which resolved over a period of ~12 weeks. This case illustrates the possibility that locally injected BoNT may spread systemically and cause generalized weakness. Recently, four cases of iatrogenic botulism were acquired in patients treated for cosmetic purposes with BoNT that had not been approved by the FDA (Chertow et al., 2006). The patients may have been injected with as much as 8 million U of

BoNT/A-complex, which is 2857 times the estimated lethal dose for humans. Available evidence indicates that the toxin was obtained from a wholesale source and was repackaged and marketed at a bargain price. This occurrence emphasizes the potency of toxin and extreme care and scrutiny must be taken in the use of BoNT in medical practice.

This recent incident of iatrogenic botulism provided vivid evidence of the course of severe botulism in humans (Chertow et al., 2006). Initially, four suspected cases of botulism were reported to the Centers for Disease Control and Prevention on November 4, 2004. The patients had been injected with a highly concentrated, unlicensed preparation of botulinum neurotoxin type A for treatment of wrinkles. The vial used to treat the patients contained ~100 μg of BoNT/A, which is an amount estimated to be sufficient to kill more than 1000 adult humans if disseminated evenly. A fatal case of botulism was suspected due to a lidocaine-BoNT-complex mixture (Li et al., 1999), although the presence of human serum albumin could have also caused an immunologic reaction.

11.6.9. Animal botulism

Botulism commonly strikes domestic and wild animal populations, particularly birds and fish, where it can cause devastating epidemics with deaths of thousands to millions of animals (Smith and Sugiyama, 1988; Eklund, 1995; Lindström et al., 2004; Yule et al., 2006), and is the most prevalent form of botulism worldwide. BoNTs types C, D and F are involved as well as A, B and E, as seen in human botulism. Botulism of domestic animals not only causes economic losses but it is also a risk factor for BoNT transmission to humans. Food contaminated with BoNT has also been shown to cause botulism in various domestic or captive animals such as cattle, horses, lions and monkeys (Smith and Sugiyama, 1988). Depending on the animal species, botulism can be caused by contaminated food or water, ingestion of forage poisoned with BoNT from dead animals or by colonization of the intestinal tract by *C. botulinum*. Among fish and birds, botulism outbreaks can involve millions of cases and the diseases may take the appearance of an epidemic because insect larvae grow in the decomposing cadavers and accumulate BoNT, which is non-toxic to the invertebrates. Birds and fish are eager to eat the larvae and consequently become intoxicated and die, providing a rich anaerobic medium for the growth of *C. botulinum* and with consequent toxin accumulation in insect eggs and larvae. A self-perpetuating and escalating cycle is thus established and many animals can die rapidly, particularly among dense populations. In regions where animal botulism is common, animals often carry *C. botulinum* in their digestive tract which rapidly grows in the cadavers and becomes highly toxic and rich in spores (Smith and Sugiyama, 1988).

11.7. Clinical presentation

Irrespective of the type of botulism, the primary clinical signs are similar:

- Symmetrical cranial neuropathies;
- Difficulty swallowing, dry mouth, difficulty speaking, facial ptosis;
- Blurred near vision, blurred distant vision, dilated or non-reactive pupils, diplopia, drooping eyelids;
- Descending bilateral flaccid paralysis, generalized muscle weakness progressing to neck, limbs and torso.

The characteristic symptoms of botulism can principally be ascribed to the blockade of neurotransmission at neuromuscular junctions of skeletal muscle, with ensuing flaccid paralysis (Dickson, 1918; Koenig, 1971; Tacket and Rogawski, 1989; Cherington, 1998, 2004). The incubation period from exposure to BoNTs to onset of symptoms ranges from a few hours to as long as 10 days, but typically present within 12–72 hours in foodborne botulism (Hatheway and Dang, 1994; Cherington, 1998, 2004). The incubation period in foodborne botulism is inversely correlated with the quantity of toxin ingested (Gangarosa et al., 1971). The incubation time for wound and infant botulism is usually several days due to the time needed for *C. botulinum* to colonize the wound or intestine and produce toxin that is then absorbed into the circulation. There have been notable exceptions of rapid onset and rapid recovery (Fox et al., 2005; Keet et al., 2005). The time to onset for inhalational botulism has been reported to be 12–80 hours based on limited non-human primate experiments (LeClaire and Pitt, 2005).

After absorption into the blood, the neurotoxin binds to motor nerve endings as well as to parasympathetic nerve endings that release ACh and cause flaccid paralysis and preganglionic and parasympathetic postganglionic autonomic blockade. The first indications are usually bulbar and ocular weakness and generalized fatigue. The ocular symptoms include diplopia, mydriasis and loss of the light reflex and of accommodation. Occasionally there is also anisocoria. As the intoxication proceeds, weakness of the levator palpebrae and facial muscles becomes noticeable; speech becomes slurred and eventually anarthria ensues. Paralysis of pharyngeal, laryngeal and masticatory muscles results in dysphagia,

dysphonia, stridor, nasal regurgitation of liquids and a sensation of suffocation or actual suffocation. Paralysis of the facial muscles give the patient a dull appearance and they become unable to respond to stimuli. Weakness of the respiratory muscles and particularly of the diaphragm can result in respiratory failure and death. Descending weakness progresses for 4–8 days and then plateaus. Vomiting and abdominal pain may precede or follow the paralysis. The non-ocular autonomic symptoms include decreased salivation and a dry mouth, anhydrosis, particularly of the palms and the soles, obstipation, urinary retention and cardiovascular instability. The body temperature is generally normal or slightly subnormal. Representative portrayals of mild and severe botulism are shown in Fig. 11.2.

Despite the involvement of cranial nerves, most cases do not show cognitive or sensory abnormalities. The face often has an expression-less or sagging appearance because of the relaxed tone of the facial muscles. In severe cases the patient lies helpless, resembling a generalized paralysis or coma. The tendon reflexes are intact or slightly decreased. The patient may be able to initiate effective muscular contraction such as opening the eyes or raising the head or an extremity once or twice but cannot repeat the act. The inability of the patient to express their discomfort can cause hysterical attacks and mental depression. Occasionally patients experience a headache, which can persist. It is unusual for the patient to suffer any pain. Dryness of the mouth and impaired salivary and lacrimal secretions can persist for several months (Jenzer et al., 1975; Goode and Shearn, 1982; Dressler and Benecke, 2003; Chertow et al., 2006). The severe muscular weakness, the anxiety and helplessness, the difficulty in swallowing, the attacks of strangling, the struggle for breath and the unsuccessful attempts to articulate constitute an unforgettable clinical picture.

The progressive muscle weakening is a striking hallmark of botulism. Humans with mild cases of botulism also may exhibit poor coordination of the arms and legs. The patient becomes gradually weaker, fatigued and finally death occurs, generally by respiratory failure (Gangarosa et al., 1971; Koenig, 1971; Tacket and Ragawski, 1989; Shapiro et al., 1998). The requirement for endotracheal intubation and ventilatory support is an indicator of the severity of the disease in patients (Woodruff et al., 1992; Hatheway and Dang, 1994; Sandrock and Murin, 2001; Anderson et al., 2002). Woodruff et al. (1992) found that intubation was necessary for 67% of patients with type A botulism, 52% with type B and 39% with type E. Of patients that were intubated, 72% represented isolated cases, while only 54% were intubated from outbreak-associated illness. This suggests that certain patients with mild cases of botulism are often not hospitalized and may remain undiagnosed.

The severity of symptoms and duration of botulism in cell and animal models depends on the serotype of BoNT. In cultured primary neuronal cells

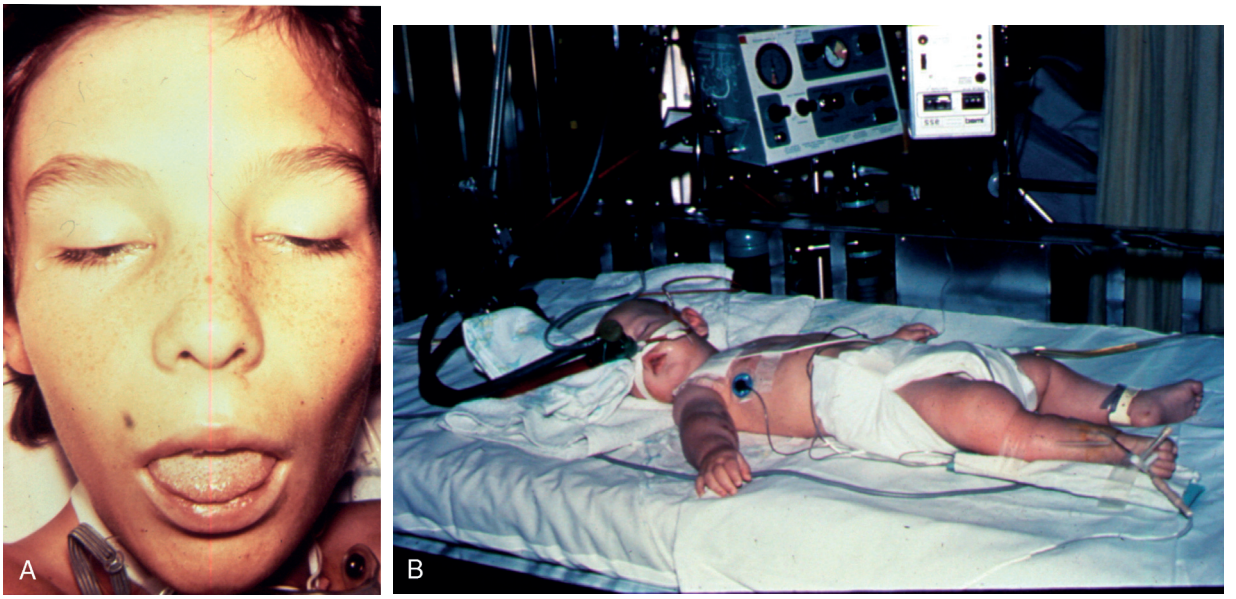

Fig. 11.2. Portrayal of typical symptoms of mild and severe botulism. (A) Photograph of boy is from the CDC collection and provided by Charles Hatheway (deceased). (B) Photograph of infant is courtesy of Stephen Arnon.

(Keller et al., 1999), animal models (Adler et al., 2001; Billante et al., 2002; Foran et al., 2003; Keller, 2006) and in humans with natural botulism or treated for medicinal purposes (Sellin et al., 1983a, b; Sloop et al., 1997; Brin et al., 2002; Eleopra et al., 2004; Gupta et al., 2005) the severity and duration of symptoms relates to the type of toxin in the following order: BoNT/A = BoNT/C1 > BoNT/B > BoNT/F > BoNT/E in cultured primary neuronal cells (Keller et al., 1999), in animal models (Adler et al., 2001; Billante et al., 2002; Foran et al., 2003; Keller, 2006) (Table 11.1) and in humans with natural botulism or after being treated for medicinal purposes (Sellin et al., 1983a, b; Sloop et al., 1997; Brin et al., 2002; Eleopra et al., 2004; Gupta et al., 2005).

Although the different forms of botulism show similar clinical signs and symptoms, there are certain distinctions. Early in foodborne botulism, patients may have gastrointestinal symptoms such as nausea, vomiting, abdominal cramps and diarrhea (Hughes et al., 1981). These symptoms are probably not due to BoNT but from food contaminants. Infant botulism differs from other forms of botulism in the ages of the affected individuals and the infection process by neurotoxigenic clostridia within the large bowel. The incubation period is often several days to weeks, reflecting the ability of *C. botulinum* to colonize, grow and produce toxin within the large intestine. In some infants an acute onset over several hours is followed by rapid recovery (Keet et al., 2005). The disease usually begins with severe constipation lasting 3 days or longer that precedes the appearance of other neurologic signs by one or more days (Arnon, 2004). Following constipation, the infant develops cranial nerve palsies resulting in oculomotor weakness, eyelid ptosis, a weak suck and hypotonia. In the more severe cases, the infant becomes lethargic and loses head control. The disease progresses to a generalized flaccid paralysis which may involve the respiratory muscles and culminate in apnea. Type A toxin is associated with greater morbidity and a slower recovery than type B or E toxin (Arnon, 2004).

Table 11.1

Potencies and durations of different serotypes of botulinum neurotoxins (BoNTs) acting on cerebellar granule neurons from rats

BoNT[a] blockade of transmitter release	BoNT concentration causing 50% of inhibition [pico Molar (pM)]	Duration (t1/2) (days ± SD)
BoNT/A	10	>>31
BoNT/B	100	9.84 ± 2.12
BoNT/C1	13	>>25
BoNT/E	43	0.73 ± 0.11
BoNT/F	1350	1.76 ± 0.28
TeNT	6.5	Not studied

[a]BoNTs were purified as 150 kDa BoNTs and were completely activated.
After Foran et al. (2003).

11.8. Diagnosis of botulism

The diagnosis of botulism relies on the clinical findings which include prominent oculobulbar signs and laboratory detection of BoNT from appropriate specimens (Table 11.2) (Hughes et al., 1981; Cherington, 1998, 2004; Shapiro et al., 1998; Arnon, 2004). The initial diagnosis of botulism is based on the characteristic clinical presentation as described in the previous section (CDC, 1998; Cherington, 1998, 2004).

The definitive laboratory diagnosis of botulism requires detection of BoNT in plasma, serum and gastrointestinal contents (stool and vomitus) and food or other sources by the mouse bioassay as described above. Isolation of *C. botulinum* from foods and clinical specimens including vomitus and feces is also supportive in the diagnosis of botulism (Hatheway, 1988; CDC, 1998). Since botulism is a rare disease, toxin and organism diagnostic tests should be performed in a suitable reference laboratory that has the necessary experience and reference toxins, antitoxins and standard cultures for the procedures, such as the Communicable Disease Center National Botulism Laboratory (404-639-2206; 404-639-2888) or qualified local and state public health laboratories (http://www.cdc.gov/other.htm#states or http://www.astho.org/state.html).

Regrettably, for many botulism cases, laboratory studies are of no aid in establishing a diagnosis since the sensitivity of detection of BoNT decreases rapidly

Table 11.2

Diagnosis of botulism

Clinical diagnosis	Recognition of clinical symptoms and signs
	Neurological examination, including electromyography
Laboratory confirmation	Demonstration of botulinal neurotoxin in serum, stool, gastric aspirate, or food.
	Culturing *C. botulinum* from stool, wound, or food

with time (Koenig, 1971; Woodruff et al., 1992). Due to delays in specimen collection and analysis or to the presence of mouse lethal substances in samples other than BoNT, laboratory detection of BoNT is often not conclusive (a positive test is present in ~30% of samples obtained more than 2 days after toxin exposure) (Cherington, 1998). A detailed survey of 494 cases of foodborne botulism in the United States from 1975–1988 illustrates the complexities of a definitive diagnosis of botulism (Woodruff et al., 1992). In this study, BoNT/A caused botulism illness in 148 patients (48%), type B in 89 (29%) and type E in 72 (23%). Among the type B outbreaks, 55 cases occurred from a single restaurant outbreak and affected patients mostly under 40 years of age (Terranova et al., 1978), whereas the largest outbreaks for type A and E affected 27 and seven persons, respectively. The median onset period for all patients was 1 day (ranges: type A, 0–7 days; type B, 0–5 days; type E, 0–2 days). Overall, type E patients had the shortest incubation period and type B patients the longest. Type A patients were more likely to require intubation. Of the 105 patients with an onset time of less than 1 day, 59% required intubation and artificial respiration compared to 44% of the 54 patients with longer incubaton periods. Overall, the greater severity of botulism of the reported sporadic cases suggests that sporadic cases are frequently misdiagnosed or not reported to health authorities (Woodruff et al., 1992).

Of patients for which adequate clinical specimens were submitted to the CDC, 67% were confirmed as having botulism by toxin assay or culture. Specimens from patients with type A botulism were more frequently positive for toxin in serum and stool than were those patients with types B and E botulism (Woodruff et al., 1992). BoNT testing results were positive in 126 (37%) of 349 sera and 65 (23%) of 288 stool samples; 27 stool samples gave non-specific deaths in the mouse bioassay (these samples were not neutralized by antitoxin). Serum toxin assays were more likely to be positive if sera were collected soon after toxin ingestion; BoNT was detected in >60% of serum samples obtained during the first 2 days but in only 13–28% of specimens obtained thereafter. Similarly, BoNT was detected in 50% of stool samples obtained within 1 day, but in <20% after 5 days. These results support the notion that different types of botulinal toxins produce distinct clinical manifestations and are diagnosed in the laboratory with different degrees of success. Type A botulism is more severe, as indicated by the increased need for intubation. However, type E botulism patients had the shortest incubation period. These observations are consistent with other studies using indicators of severity other than respiratory support (USPHS, 1979; Hughes et al., 1981; CDC, 1998).

The clinical and pharmacokinetic mechanisms governing these differences are not clear. It is not known if there are race differences in toxin absorption, activation and stability or action. However, short incubation times are common in type E cases and were mostly observed in Alaskan Natives. An investigation of a type B outbreak recorded more severe illness among patients of Chinese descent (St. Louis et al., 1988). After intraperitoneal injection of mice, the onset of symptoms was most rapid with type E toxin and slowest with type B toxin (Sugiyama, 1980; Woodruff et al., 1992). In rats, larger doses of types B and E than type A toxin are required to induce paralysis (Sellin et al., 1983a, b). The administration of antitoxin as well as the pharmacokinetics of absorption, distribution through the lymphatic system and rate and affinity of binding to and internalization within target nerves (Habermann and Dreyer, 1986) likely affect the chance of detecting BoNT in serum samples. The toxin titers in clinical specimens are often too little to definitively confirm the presence of BoNT with serotype specific antisera. Detection of neurotoxigenic organisms also supports the diagnosis, but a positive culture alone does not confirm it since spores are ubiquitously distributed and can be occasionally isolated from the stools of healthy humans and from foods and other environmental sources (Hatheway, 1988; CDC, 1998; Shapiro et al., 1998).

Electrodiagnostic testing can provide presumptive evidence of botulism and is particularly useful in those patients with clinical signs of botulism but with negative mouse bioassay results (Hatheway and Dang, 1994; Cherington, 1998, 2004; Dumitru et al., 2002; Crawford, 2003). EMG abnormalities are observed as the disease progresses but may not be present at onset of symptoms. The amplitude of the compound muscle action potential (CMAP) gradually decreases in the clinically affected muscles, but motor and sensory nerve conduction are usually normal. The most common electrophysiological abnormality in an affected muscle is a low amplitude CMAP in response to a single supra-maximal nerve stimulus (Maselli et al., 1997; Dumitru et al., 2002; Crawford, 2003; Cherington, 2004). Electrodiagnostic findings typical of botulism include normal motor nerve velocity, normal sensory nerve amplitudes, velocities and latencies and a pattern of brief, small amplitude and polyphasic motor unit potentials, and generally an incremental response (facilitation) to repetitive stimulation at high frequencies, with a decremental response at lower frequencies. Single-fiber EMG studies often show

increased jitter in posterior cervical and extremity muscles, which diminish on blocking (Cherington, 1988, 2004; Dumitru et al., 2002). Guidelines for electrodiagnostic testing for botulism have been outlined (Cherington, 1990, 1998, 2004; Maselli et al., 1997; Crawford, 2003). Acute care pediatric electromyography can be useful in diagnosing infant botulism among floppy baby syndromes (Gutierrez et al., 1994; Jones and Darras, 2000; Crawford, 2003; Cherington, 2004; Swoboda and Jones, 2006), although pitfalls may occur, particularly in early evaluation (Sheth et al., 1999). Unlike certain other NMJ diseases such as myasthenia gravis and Lambert–Eaton syndrome, botulism is an acute intoxication caused solely by exposure to BoNT; therefore genetic tests or a search for anti-acetylcholine receptor or anti-MuSK antibodies are futile.

11.8.1. Differential diagnosis

The definitive diagnosis of botulism requires differentiation from other neuromuscular disorders including sepsis, meningitis, myasthenia gravis, Guillain–Barré syndrome, Miller–Fisher syndrome, Lambert-Eaton syndrome, stroke, tick paralysis, snake venom poisoning, diphtheritic neuropathy, nerve agent poisoning, Streptococcus pharyngitis, diabetic complications, inflammatory myopathy and CNS infections or tumors, particularly of the brainstem (Table 11.3) (Cherington, 1998, 2004; Dimitru et al., 2002; Arnon, 2004; Caya et al., 2004). Acute flaccid paralysis from polioviruses and other enteroviruses have been misdiagnosed as infant botulism (Kelly et al., 2006). A summary of illnesses requiring differential diagnosis from botulism is presented in Table 11.3. In a recent study, clinical mimics of infant botulism were classified into five broad categories: muscular atrophy, metabolic disorders, other infectious diseases, miscellaneous and probable infant botulism lacking laboratory confirmation (Francisco and Arnon, 2007).

Table 11.3

Differential diagnosis of botulism from other disorders (Caya et al., 2004; Meriggioli et al., 2004; CDC, 2006)

Adults and children	Infants
Meningitis	Sepsis, meningitis
Guillain–Barré syndrome (GB)	Guillain–Barré syndrome (GB)
Myasthenia gravis	Myasthenia gravis
Lambert–Eaton syndrome	Acute infantile neuropathy
Cerebrovascular accidents	Meningitis/encephalitis
Acute intermediate porphyria	Metabolic disorders, e.g., electrolyte imbalance
Carcinomatosis of cranial nerves	Reye's syndrome
Neoplasm of CNS	Neoplasm
Tick paralysis	Congenital myopathy
Diphtheritic neuropathy	Enteric virus
Polymyelitis	Poliomyelitis
Miller–Fisher variant of GB	Werdnig–Hoffman disease
Food poisoning (e.g., saxitoxin)	Leigh disease
Chemical neurotoxin exposure	Chemical neurotoxin exposure
Mushroom poisoning	Food poisoning
Neuronal viral infection	Neuronal viral infection

11.9. Treatment of botulism

The mainstay of treatment is intensive nursing care, with careful attention to respiratory failure, need for enteric feeding and cardiac arrest (Woodruff et al., 1992; Arnon, 2004; Cherington, 2004; CDC, 2006). Passive immunization has long been known to lessen the symptoms of botulism and length of clinical course and reduce the incidence of fatalities (Dack and Wood, 1928; Hatheway et al., 1984; Tacket et al., 1984; Mayers et al., 2001; Chang et al., 2003; Arnon et al., 2006). When administered within hours following intoxication, antitoxin can markedly reduce morbidity and mortality. Until recently antitoxin treatment has relied on equine-derived products with associated side-effects in about 9% of patients (Black and Gunn, 1980). Equine antitoxin has a half-life in serum of about 5–7 days (Hatheway et al., 1984), whilst human-derived antitoxins may have a longer half-life (Arnon et al., 2006). Recently, human-derived botulism immune globulin (BabyBig®) was shown to reduce the severity and duration of the symptoms, length of hospital stay and associated hospital costs (Arnon et al., 2006). BabyBig® was derived from human donors who had been immunized with botulinum toxoid and the antibody fraction was isolated and formulated for parenteral administration (Arnon et al., 2006). Its supply is currently limited and use is restricted to infants, but efforts are under way to produce additional quantities of antibodies from immunized human donors or to develop antibody producing cell lines with human-compatible antibodies to avoid side-effects caused by the equine antitoxin. New technologies are in progress to develop humanized antibodies of low immunogenicity and side-effects compared to rodent-derived antibodies (Dessain et al., 2004; Lonberg, 2005).

The severity of botulism has been increased with concomitant treatment with certain antimicrobial agents, particularly aminoglycosides and macrolides. These should be avoided in myasthenic illnesses including botulism since they can potentiate muscle paralysis (Pittinger and Adamson, 1972; L'Hommedieu et al., 1979; Santos et al., 1981; Wang et al., 1984; Howard, 1990; Barclay and Begg, 1994). Consequently, botulinum toxin treatment for pharmaceutical purposes is contraindicated in patients with myasthenia gravis, Lambert–Eaton syndrome and possibly other myasthenic disorders. Injection of BoNT-complex in patients has unmasked underlying myasthenic and other neurological disorders (Erbguth et al., 1993; Mezaki et al., 1996; Tuite and Lang, 1996; Tarsy et al., 2000; Thobois et al., 2001; Gioltzoglou et al., 2005; Iwase and Iwase, 2006). Adverse heart rate variability was also detected in a set of patients treated with BoNT/A-complex or BoNT/B-complex (Meichsner and Reichel, 2005). Other cardiovascular and rheumatologic drugs known to adversely affect patients with MG and LES should also be avoided in patients with botulism or in healthy individuals treated with BoNT for cosmetic or therapeutic purposes (Howard, 1990).

11.10. Recovery from botulism and clinical predictors of mortality

Recovery from botulism is slow and tedious. A retrospective review of cases in the USA found a mean of 58 days of mechanical ventilation for type A and 26 days for type B (Colice, 1987). Recovery of speech and the ability to swallow recurs relatively early. Muscular weakness, vertigo and constipation diminish more slowly and may persist for several months. The oculobulbar disturbances are usually the last symptoms to clear. Some patients continue to experience weakness, fatigue and symptoms of impaired autonomic nervous system dysfunction such as dry mouth, constipation and impotence even after 1–2 years following onset of botulism (Goode and Shearn, 1982; Colebatch et al., 1989; Cherington, 1990; Dressler and Benecke, 2003). Communication during recovery is impaired, including inability to speak or write due to ventilatory support and extreme weakness. Agitation, depression and anxiety are common among patients with severe botulism (Cohen and Anderson, 1986).

Clinical predictors of morbidity and mortality from botulism have been evaluated (Mann et al., 1981; Woodruff et al., 1992; Hatheway and Dang, 1994; Nishiura, 2007). The incubation period is related to case fatality and index and early onset cases show the greatest incidence of death (Nishiura, 2007). Early administration of antitoxin reduces morbidity and deaths and is a predictor of the need for mechanical respiration (Tacket et al., 1984; Sandrock and Murin, 2001). Patients with shortness of breath and impaired gag reflex were 23 times more likely to succumb than patients without these signs (Varma et al., 2004). Death from botulism has generally been attributed to ventilatory weakness and respiratory arrest (Hughes et al., 1981; Wilcox et al., 1990; Woodruff et al., 1992). In severe cases of botulism, ventilator muscles may require up to a year to regain full strength (Wilcox et al., 1990). However, deaths also occur by causes not directly involved in airway sufficiency. In a study of causes of death among 19 patients with type A botulism, seven were attributed to respiratory arrest or associated ventilator malfunction and aspiration pneumonia, while surprisingly six were attributed to cardiac arrest (Tacket et al., 1984). It appears that reduction of fatalities related to botulism would benefit from more detailed scrutiny of the causes involved. In common with foodborne botulism, infant botulism caused by BoNT/A is often the most severe in symptoms and time required for patient recovery. In California, a study showed that the mean hospital stay for patients with type A infant botulism was 5.6 weeks, whereas the mean hospital stay for babies with type B infant botulism was 3.7 weeks (Arnon, 2004). Fortunately death in hospitalized cases has been rare and babies recover completely with no permanent weakness or neurologic abnormalities. When death or chronic morbidity has occurred, it has usually resulted from infections or other secondary complications.

Cases of severe botulism with neuropathic features have been reported (Chang and Robinson, 2000; Mackle et al., 2001). Disease sequelae including infectious diseases such as pneumonia have been reported. Stimulation of murine B cells to secrete immunoglobulins by "lipoteichoic acid-like" molecules from *C. botulinum* has been suspected (Campos-Neto et al., 1995), but this needs confirmation. Muscle and nerve biopsies have rarely been performed on patients with botulism, though such analyses can help to exclude myopathy or metabolic causes of paralysis (Keet et al., 2005). Studies in animals have demonstrated atrophy of muscle fibers in severe botulism cases, which regenerated during the recovery phase. It has been suggested that chronic neuropathic changes may persist following botulism (Keet et al., 2005).

11.11. Pathophysiology of botulism and cellular mechanisms of botulinum neurotoxins

Animal models and tissue preparations have traditionally been employed to study the pathophysiology of botulism (Drachman, 1971; Habermann and Dreyer, 1986; Simpson, 2000). The specificity and action of

BoNTs for different tissues and cell types depends on the receptor systems, the trafficking mechanisms and the isoforms of SNARE proteins present in the cells (Schiavo et al., 2000). In this section of the chapter, molecular and tissue effects are described with an emphasis on new developments. It follows the series of actions in the sequence that is believed to occur in botulism poisoning.

11.11.1. Adsorption of botulinum toxin into the lymphatic system

Poisoning caused by botulism involves entry of BoNT into the general circulation either through absorption from the gastrointestinal system, in wound infections or by inhalation. An intriguing aspect of BoNTs is their high oral toxicity as occurs in traditional food-borne botulism (Bonventre, 1979; Sakaguchi, 1983). The estimated lethal dose for humans of BoNT/A by oral ingestion is 0.1–1 μg per kg body weight (Schantz and Johnson, 1992; Hatheway and Dang, 1994), while the systemic toxicity by intramuscular injections in monkeys has been estimated at 39 U/kg (~400 pg) (Scott and Suzuki, 1988). These data suggest about one-thousandth of the toxin ingested is effectively absorbed into systemic circulation, while other estimates have suggested that the ratio of oral to parenteral toxicity in various species of animals is 10^4:10^6 (Smith, 1977). Oral toxicity varies considerably depending on the serotype and the animal species. It has been estimated that ~10^{11}–10^{12} (~1–2 μg) molecules of BoNT/A that enter the systemic circulation is sufficient to produce neurologic symptoms and death (Lamanna, 1959; Bonventre, 1974). Boroff et al. (1984) estimated that ~10 molecules of BoNT are necessary to cause intoxication of a NMJ in the frog, but this is certainly an estimate as the quantitative dose would depend on the size and safety margin of the particular NMJs and motor endplate.

Although oral poisoning is the major cause of human botulism (Shapiro et al., 1998), surprisingly little is known regarding the mechanisms of BoNT passage through and absorption from the GI tract and its entry into the lymph and blood of general circulation (Bonventre, 1979; Simpson, 2000). It has been demonstrated that the toxin complexes (progenitor toxins) are more stable during oral passage than is the purified neurotoxin (Ohishi et al., 1977; Sugiyama, 1980; Sakaguchi, 1983; Fujinaga, 2006). The non-toxic proteins of the toxin complexes including hemagglutinins (HA) and non-toxic, non-hemagglutinin protect the toxin from digestive enzymes and low pH. During passage, activation of single chain BoNT precursor proteins from non-proteolytic types such as BoNT/E probably occurs by limited proteolysis in the stomach or in the brush border of the enterocytes (Bonventre, 1979). Such activation of types B, D, E, F and G increases toxicity by 10–100-fold (Sugiyama, 1980; DasGupta, 1989). BoNTs are also inactivated with loss of toxicity by exposure to high concentrations and/or extended times to trypsin and certain other proteases and thus controlled conditions and the use of soybean trypsin inhibitor are needed for careful cleavage to the dichain form in vitro (Sugiyama, 1980; DasGupta, 1989).

How such a large protein (150 kDa) penetrates the intestinal barrier is an interesting question that has not been resolved (Simpson, 2000; Fujinaga, 2006). The quantity of BoNT needed to induce neurologic symptoms is extremely low (fM levels) and it is possible that a sufficient quantity crosses the polarized epithelial monolayer from the apical side to the basolateral side (transcytosis) via non-specific mechanisms similar to absorption of certain other proteins. BoNTs are mainly absorbed in the upper small intestine and lesser amounts from the ileum and stomach in various animal models (Dack, 1926; Heckly et al., 1960; Maksymowych et al., 1999; Simpson, 2000). It has been shown that intestinal crypt cells may be a preferential site of transcytosis (Coueson et al., 2008). Small quantities of BoNT may also enter the circulation from other locations within the gastrointestinal tract such as mucosal surfaces of the mouth, esophagus and through intestinal lesions (Lamanna et al., 1967). In inhalational botulism, BoNT is absorbed from the upper respiratory tract by poorly understood mechanisms. *C. botulinum* colonizes the large bowel in infant and adult intestinal botulism, but it is unclear if the ileum is the major site of absorption. In a mouse model, as few as 10 botulinum spores were able to colonize the ileum and toxin was detected in the feces, but the mice remained asymptomatic, suggesting that BoNT is poorly absorbed from the ileum (Sugiyama, 1979).

Bonventre (1979) presented evidence that BoNT is absorbed by an endocytic process in which the toxin reaches the microvillus membrane and is engulfed in pinocytic vesicles. Some vesicles fuse with lysosomes and the protein is subsequently degraded, while other vesicles will reach the surface of lateral cells and reach the interstitial regions. This pinocytic process is analogous to the mechanism by which neonates absorb immunoglobulins from colostrum and is less prevalent in children and adults than in infants. Bonventre (1979) further proposed that there may be specific receptors for BoNTs in the lumen of the gut and this hypothesis has been supported in more recent studies from various investigators (Maksymowych and Simpson, 2004; Ahsan et al., 2005). Indeed, it has been documented that polysialogangliosides and the protein SV2 are involved

in the apical uptake of BoNT/A (Coueson et al., 2008) and that the transcytosis of BoNTs via specific receptors is energy-dependent (Ahsan et al., 2005). The traversal process was visualized in the human gut epithelial cell line (T-84) by labeling BoNT/A with Alexa dye 488 (Ahsan et al., 2005).

Very little is known of the mechanisms involved in the absorption of BoNT into the vasculature from mucosal membranes, from wounds and following intramuscular injection. BoNT likely enters directly into the bloodstream by diffusion from wounds and on intramuscular injection, but the actual mechanism is unclear. Circulating BoNT has been detected in less than 50% of human wound botulism cases (Merson and Dowell, 1973; Hikes and Manoli, 1981), suggesting that the infection is relatively short or that sufficient quantities of BoNT to elicit an immune response are not produced.

11.11.2. Systemic distribution of BoNT

BoNT enters the lymphatic system from the intestine, wounds, mucous membranes or from intramuscular locations and travels via blood to neuronal targets. The different specificity of the serotypes for different cell types mainly depends on the receptor systems (Schiavo et al., 2000; Simpson, 2000). Since BoNT dissociates from the complex proteins at pH $\geq$7.2 (Sakaguchi et al., 1984), it would occur free in the bloodstream following absorption. The pharmacokinetics of BoNTs in the bloodstream have been poorly studied, particularly in humans. Information is lacking on the mechanisms for transport, metabolism and elimination. In our laboratory, on injections of high concentrations of BoNT 10^6 and 10^5 LD50s into the tail vein of mice, symptoms of botulism are noticeable in ~30 and ~60 minutes, respectively (Malizio et al., 2000). Thus, compared to certain other toxins, especially small molecular weight organic chemical toxins such as saxitoxin and tetrodotoxin (where mice die in minutes following injection) (Schantz and Johnson, 1992), the onset requires several hours, probably due to the large size of BoNT and the multiple steps involved in intoxication.

Few studies have addressed pharmacokinetic properties of BoNTs including mechanisms of metabolism, elimination and detoxification. Such studies have been hindered by the extraordinary potency of BoNTs, which are physiologically active and potentially lethal at femtomolar concentrations, which imposes limitations in detection in in vivo and PK evaluations. Borodic et al. (1994) investigated regional toxin spread in a rabbit-back model showing that diffusion gradients are formed at 5–10 U (1 U = 1 mouse LD50), but the gradient collapsed at 1 U (Borodic et al., 1994). The diffusion and tissue distribution of BoNT/A and the 900 kDa BoNT/A-complex labeled with ^{125}I were investigated following injection into the gastrocnemius muscle of rats and the eyelids of rabbits (Tang-Liu et al., 2003). No generalized symptoms or systemic effects were observed. In rats, most of the toxin, whether complex or neurotoxin, remained in the regions of the injection site. Radioactivity detected in distant tissues including thyroid, skin and contralateral muscle appeared to be attributed to low molecular weight peptides or free ^{125}I-iodide, suggesting that breakdown of the toxin occurred. Very low concentrations of ^{125}I-iodide were detected even in the brain. Following injection into rabbit eyelids, very low levels of ^{125}I-BoNT/A-complex or free ^{125}I-BoNT/A were detected in distant tissues, including the eye (Tang-Liu et al., 2003). These results suggested that BoNT/A and BoNT/A-complex, when administered at physiological levels, do not diffuse significantly from the injection site, reducing the potential for systemic effects. Illicit injection of high amounts of BoNT/A-complex into the forehead in cosmetic procedures led to systemic botulism in humans, but these levels are much higher than occurs in natural botulism or in proper medical treatments (Chertow et al., 2006).

An assessment of the systemic pharmacokinetics of BoNT was recently studied in mice, rats and rabbits (Ravichandran et al., 2006). Native BoNT and toxin modified by diethyl pyrocarbonate to inactivate histidine residues in the active site (Rossetto et al., 1992) and by iodination were used. Ex vivo experiments involving incubation of BoNT in blood indicated that the toxin was not proteolytically modified or did not undergo major structural changes. BoNT recovered from rat blood was not diminished in its metalloprotease activity and retained its ability to block exocytosis in the phrenic nerve-hemidiaphragm preparations (Ravichandran et al., 2006). These experiments supported the conclusion that BoNT retained full functional activity and biologic structure during incubation in rat blood. In order to reach nerve terminals, BoNT must exit from the bloodstream, which probably depends on the toxin being free and not bound to albumin or other blood proteins. It was estimated that ~70% was free and available for traversal from the blood. The half-life of ^{125}I-BoNT administered intravenously in the tail vein of mice was extrapolated to be ~239 minutes in blood and 231 minutes in serum, and that elimination half-life was ca. 4 hours. In rats, the half-lives in blood and serum of rats was 260 and 255 minutes, respectively. As expected, incubation of BoNT with neutralizing antibodies prior to injection

prevented botulinum intoxication for more than 4 days. In antibody chase experiments of 10 or 20 minutes post-challenge decreased the survival time to ca. 22 and 4 hours, respectively, compared to the time to death of 100 minutes in mice challenged without an antibody chase. The tissue disposition of the antiserum-toxin accumulated in the liver was 30–40% and 5–7% in the spleen. These results supported the notion that toxin-specific antibodies strongly promote clearance of toxin from the circulation. This ability would be expected to depend on the affinity and number of antibodies and may be slower with monoclonals against BoNT. BoNT alone was also found in the liver (ca. 7%), spleen (ca. 1%) and kidney (ca. 2%), whereas negligible toxin was detected in the heart or brain. Blood is an important conduit for delivery of the toxin to its sites of action including cholinergic nerve endings. This study also confirmed that the time window of neutralization by antisera administration is small.

11.12. Cellular mechanisms of action of BoNTs at nerve terminals

11.12.1. Background

The cellular mechanisms of BoNTs of activity remained enigmatic for several decades, began to be revealed in the 1950s and 1960s and were gradually elucidated by new techniques and concepts, particularly advanced techniques for imaging tissue, electrophysiological methods, the theory of quantal release of acetylcholine at the synaptic membrane and eventually genetic analyses and structural analyses of the BoNTs (Heuser, 1976; Katz, 1966; Thesleff, 1976; Niemann, 1991; Schiavo et al., 2000). The morphology and structural basis of synaptic vesicle discharge and reformation was illuminated by electron microscopy and freeze fracture, particularly at the frog neuromuscular junction (Heuser, 1976). These studies provided a "panoramic view" of the morphology of the nerve terminal and were important in defining the active zone. The classic study of Burgen et al. (1949) demonstrated that partially purified BoNT/A-complex produced an irreversible paralysis of the isolated rat phrenic-nerve diaphragm after a latent period. They also showed that much higher concentrations (500 times) of BoNT/B-complex were necessary compared to BoNT/A-complex to paralyze the phrenic nerve-diaphragm of the rat, but that guinea pigs and isolated guinea pig phrenic nerve preparations were both highly sensitive to both type A and B toxins. Importantly, they showed that conduction in the nerve was unaffected by BoNT, that the motor endplates remained sensitive to applied acetylcholine, that the output of acetylcholine on motor nerve stimulation was greatly reduced and that the toxin does not affect the enzymes acetylating choline or cholinesterase. This seminal study showed that BoNT bound irreversibly to nerve fibers and prevented release of acetylcholine, resulting in the neuromuscular block (Burgen et al., 1949). Brooks (1953, 1954) subsequently confirmed that conduction in nerve trunks or in muscle fibers was not affected by the toxin even on direct stimulation and that the toxin produced neuromuscular paralysis by interfering with "conduction" proximal to the site of ACh release. The results of these studies implied that botulinum toxin interferes directly with the release of ACh from nerve endings.

The cellular mechanisms of BoNT toxicity and blockade of neurotransmitter release follows a complex multistep process (Montecucco et al., 1994): (a) receptor binding; (b) receptor mediated endocytosis inside synaptic vesicles; (c) translocation of the L chain from the acidic vesicle lumen into the cytoplasm; and (d) catalytic activity of the BoNT LCs on SNARE substrates (Fig. 11.3). Several excellent reviews have described these steps, particularly the intracellular activity of the toxins on SNARE substrates (Schiavo et al., 2000; Jahn and Scheller, 2006); therefore the sections described below will focus mainly on new developments.

11.12.2. Receptors for BoNTs in neuronal cells

Several protein toxins are known to have specific protein cellular receptors, with cholera and diphtheria being classic examples (van Heyningen, 1974; Chang et al., 1975). In addition, polysialogangliosides often participate as membrane receptors for toxins (van Heyningen, 1974). The mode of BoNT binding to nerve terminals is still poorly understood (Montecucco et al., 2004), particularly in comparison to other steps such as intracellular cleavage of SNARE substrates (Jahn and Scheller, 2006). The binding of BoNTs A and B to murine motoneurons was initially studied quantitatively by Black and Dolly (1986). Using iodinated BoNTs, they demonstrated that the toxins bound specifically to unmyelinated regions of the nerve terminals of mouse hemidiaphragms. Binding only occurred at the presynaptic membrane and was not detected on other cell types including muscle, blood vessels, connective tissue, Schwann cells or noradrenergic terminals. Binding was found to be temperature-dependent and was considerably reduced at 4°C compared to 22°C. Transfer of radioactivity across the nerve plasma membrane was detected within 20 minutes and the extent of internalization reached a maximum after

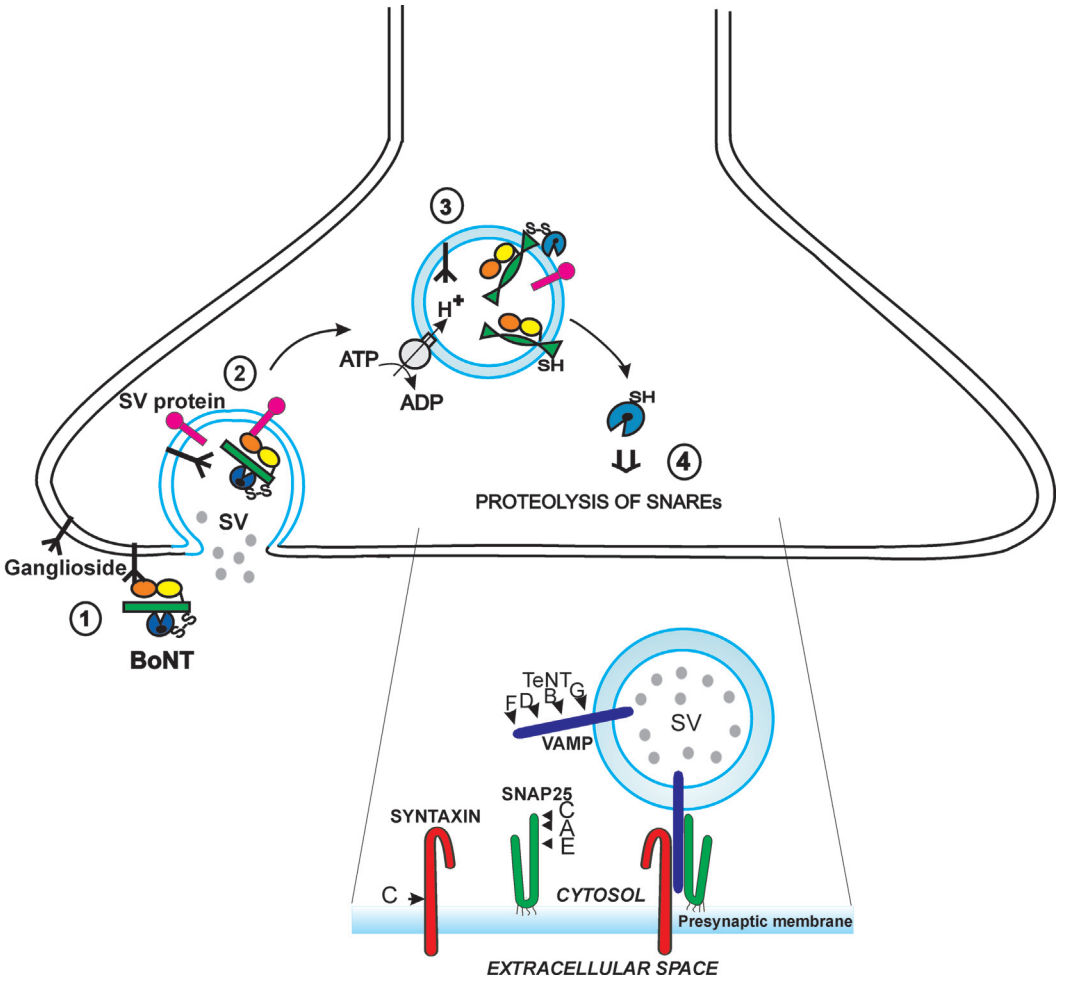

Fig. 11.3. Portrayal of the molecular mechanism of BoNTs at nerve terminals acting on the SNARE apparatus. See text for details.

90 minutes. Lowering the temperature and inhibitors of ATP production such as DNP abolished transfer. It was shown that the larger 97 kDa subunit inhibited binding, indicating that the receptor region was located on the HC. TeNT competed for binding but internalization was not affected in the presence of TeNT; 150–500 binding sites per μm^2 of membrane were estimated. These seminal findings showed that BoNT bound specifically in a saturable manner to presynaptic membranes at specialized target sites and that at least two distinguishable and sequential steps of binding and internalization are involved in BoNT intoxication.

A double receptor concept for BoNTs has been presented to explain specificity and avidity to the nerve terminals (Montecucco, 1986). Primary receptors are polysialogangliosides, generally of the GD1b and GT1b series, and certain proteins at the nerve terminus (Dong et al., 2003, 2006; Montecucco et al., 2004; Rummel et al., 2004a, b). Phospholipids, and possibly lipid rafts, have been considered as primary molecules involved in binding for certain serotypes, particularly for BoNT/D (Tsukamoto et al., 2005; Geny and Popoff, 2006). Much progress has been made in recent years in identifying protein receptors for BoNTs. Recently, using gain-of-function and loss-of-function as well as other genetic, molecular and morphological approaches has led to strong evidence implicating protein receptors for BoNT/A (Rummel et al., 2004a, 2007; Dong et al., 2006; Mahrhold et al., 2006), BoNT/B (Nishiki et al., 1994, 1996; Dong et al., 2003; Jin et al., 2006; Baldwin and Barbieri, 2007; Rummel et al., 2007), /C, /D and /G (Rummel et al., 2004a, 2007; Dong et al., 2007) entry into neuronal cell lines. Molecular structures have recently been achieved showing the interaction of BoNT/B with polysialogangliosides and synaptotagmin II (Chai et al., 2006; Jin et al., 2006).

Synaptic vesicle (SV) proteins participate with their luminal domains to the neurospecific binding of BoNT/A, /B and /G and this is probably true for the other BoNTs (Verderio et al., 2006). BoNT/B and BoNT/G interact with the luminal domain of the SV proteins synaptotagmin I (Syt-I) and Syt-II. BoNT/B has a higher affinity for Syt-II (Nishiki et al., 1996; Dong et al., 2003), whereas BoNT/G interacts preferentially with Syt-I (Rummel et al., 2004a; Dong et al., 2007). The crystal structures of BoNT/B in complex with the luminal domain of Syt-II (Chai et al., 2006; Jin et al., 2006) shows that HCC accommodates the α-helical segment 45–59 of Syt-II in a cleft adjacent to the polysialoganglioside-binding site of the toxin. Mutations in the synaptotagmin-binding cleft and in the polysialoganglioside-binding pocket greatly reduce the toxicity of BoNT/B and /G and double mutants at the two binding sites abolish neurotoxicity (Rummel et al., 2007; Dong et al., 2007).

SV2 is a membrane protein found associated with secretory vesicles of neural and endocrine cells of vertebrates (Scranton et al., 1993; Custer et al., 2006). It exists in three isoforms, SV2A, SV2B and SV2C, which differ in distribution in neuronal and endocrine tissues (Bajjalieh et al., 1992, 1993, 1994). Recently, the three isoforms of SV2 have been shown to serve as specific protein receptors for BoNT/A. The primary receptor for BoNT/A is SV2C, although SV2A and SV2B can also act as receptors (Dong et al., 2006; Mahrhold et al., 2006). The distribution of SV2 in neuronal and endocrine tissue has not been clearly delineated, but its location as well as that of other protein receptors will dictate the distribution of the toxins. The differential distribution of synaptic vesicle proteins in various neuronal tissues leads to the potential of using different serotypes of BoNTs for the treatment of peripheral and central nervous system diseases by targeting neuronal cell populations (Verderio et al., 2006). The NMJ also contains several proteins modified by glycosylation such as agrin, neural cell adhesion molecule, dystroglycan and SV2 (Kröger and Schröder, 2002; Martin, 2003) and the overall structure and organization of the NMJ contributes to binding of BoNTs by mechanisms that have not yet been elucidated.

An intriguing aspect of receptor binding for BoNT/A, /B and /G is that it depends on vesicle recycling and that their internalization within the nerve terminals is mediated by the synaptic vesicles themselves acting as sort of "Trojan horses" for the neurotoxins (Dong et al., 2006; Verderio et al., 2006). This mechanism favors toxin attack on the most active terminals. Theoretically a toxin-penetrated terminal will shut down exocytosis, allowing toxin molecules to enter actively exocytosing terminals yet to be poisoned. It is presently thought that synaptic vesicle recycling is involved in the uptake of most, if not all, serotypes.

11.12.3. Trafficking and internalization of BoNT-LCs into the neuronal cytosol

During or following internalization, both the H and L chains of BoNT/A, /B, /E and TeNT are phosphorylated by the Src family of tyrosine kinases (Ferrier-Montiel et al., 1996). Phosphorylation enhances thermal stability and increases catalytic activity of BoNTs, possibly by transitioning the structure to a more helical and compact form (Encinar et al., 1998). The primary stabilizing and thermal effects appear to be on the L chains (Blanes-Mira et al., 2001; Ibañez et al., 2004), suggesting that the phosphorylated L chains may be the dominant physiological form within the nerve terminals. Since phosphorylation and dephosphorylation have been implicated in regulation of synaptic transmission (Lee, 2006) it also seems possible that BoNT may act as an integral or surrogate component in the regulation of synaptogenesis.

An inherent property of bacterial protein toxins with intracellular targets is their ability to insert into and translocate across membranes (Montecucco et al., 1988; London, 1992; Parker and Pattus, 1993; Montecucco and Schiavo, 1995; Lesieur et al., 1997; Neale, 2003; Ménétrey et al., 2005). Once in the intracellular acidic compartment, the H chain is believed to form a channel in lipid bilayers through which the L chain enters into the cytosol following acidification of the vesicle lumen (Schiavo et al., 2000; Koriazova and Montal, 2003; Fischer and Montal, 2007b). Membrane translocation is the least understood step of the cellular mechanism of action of BoNTs. It has been proposed that the H chain acts as a transmembrane chaperone for the light chain to ensure a translocation competent conformation during its transit from the endosome (Koriazova and Montal, 2003; Fischer and Montal, 2007b). The mechanisms of trafficking and localization of LCs in the cytosol appear to differ according to the serotype and structure (Aikawa et al., 2006; Lawrence et al., 2007). Critical to entry of the catalytically active L chain of BoNTs into the neuronal cytosol is acidification of the lumen, which is thought to trigger a structural change of the toxin and initiation of membrane insertion (Montecucco et al., 1988; Koriazova and Montal, 2003; Puhar et al., 2004). To reach the cytosol the L chain must cross the hydrophobic barrier of the vesicle membrane. The pH gradient across the membrane is instrumental in such a movement (Simpson et al., 1994). In fact,

the internal acidity causes a conformational change from a water-soluble "neutral" structure to an "acid" structure, with the surface exposure of hydrophobic patches which mediate the interaction of the H and L chains with the hydrocarbon core of the lipid bilayer (Montecucco et al., 1988, 1989; Puhar et al., 2004). The release of the L chain requires reduction of the interchain disulfide bond and an intact S-S bond is an absolute requirement for toxicity and membrane translocation (Schiavo et al., 1990; Fischer and Montal, 2007a).

Once internalized the LCs may locate to different compartments in neuronal cells. LC/A locates to the plasma membrane and LC/E to the cytoplasm within PC12 cells (Fernández-Salas et al., 2004). Mutations in the LC revealed amino acid sequences at the N terminus as well as a dileucine domain in the C terminus that affected localization. The location of the LCs was postulated as one factor affecting the duration of action of the toxins, which can be several months to a year for LC/A, while generally only a few weeks for LC/E. Certain other factors likely contribute to the duration of action and recovery from cellular intoxication including processing and degradation (Keller et al., 1999; Adler et al., 2001; Keller, 2006), as well as regeneration of a productive SNARE apparatus (Raciborska and Charlton, 1999; Meunier et al., 2003; Bajohrs et al., 2004). There is evidence that the long duration of action of BoNT/A and BoNT/C (Eleopra et al., 1997, 2004) is significantly contributed by the presence of a SNAP-25 molecule deprived of only a few C-terminal residues which act as a strong dominant negative in the assembly of the SNARE supercomplex which mediates the binding of the synaptic vesicle to the presynaptic membrane (Montecucco et al., 2005). Further elucidation of the mechanism of translocation and intracellular activities could lead to novel treatments for botulism and targeted drug delivery to neuronal cells.

11.12.4. Intracellular mechanisms of BoNT-LCs

The intracellular activities of BoNT-LCs in nerve terminals remained enigmatic until the 1990s. The determination of the nucleotide and amino acid sequences of BoNTs and TeNT (Fairweather and Lyness, 1986; Binz et al., 1990; Niemann, 1991) revealed that the light chain contains a zinc-binding motif and this led to the demonstration that BoNTs and TeNTs have zinc metalloprotease activity (Schiavo et al., 1992a, b, 1993a, b; Montecucco et al., 1993). They were demonstrated to act on specific isoforms of the SNARE proteins SNAP-25 synaptobrevin and syntaxin (Schiavo et al., 1992b, 1993a, b, 1995, 2000; Blasi et al., 1993a, b; Montecucco and Schiavo, 1995), which are integral for trafficking of synaptic vesicles and their fusion with the plasma membrane.

The BoNT-LCs are a unique group of proteases that recognize 9-residue SNARE motifs within the natural substrates (Montecucco and Schiavo, 1993, 1995; Rossetto et al., 1994; Schiavo et al., 2000); the recognition is structurally based and contributes to the high specificity of the toxins for recognizing and cleaving their substrates. The interactions of the catalytic domain of BoNT with the neuronal substrates have been studied for all seven serotypes. TeNT, BoNT/B, /D, /F and /G cleave the vesicle-associated membrane protein VAMP, at different single peptide bonds; BoNT/C cleaves both syntaxin and SNAP-25, two proteins of the presynaptic membrane; whilst BoNT/A and /E cleave SNAP-25 at different sites within the COOH-terminus (Montecucco and Schiavo, 1995; Schiavo et al., 2000). Proteolysis of SNARE proteins prevents the formation of an active membrane fusion complex (Schiavo et al., 2000).

The findings that BoNT-LCs enzymatically cleaved SNARE proteins through recognition of the SNARE motif provided an explanation to morphological studies which showed, in some cases, accumulation of synaptic vesicles at the plasma membrane in BoNT poisoned nerves (Fig. 11.3). This clearly demonstrated that a primary mechanism of BoNTs in blocking exocytosis is to block exocytosis by disruption of SNARE trafficking and neurotransmitter release. However, other functions likely exist for these intriguing proteins. A summary of the current understanding of the cellular mechanism of action of the BoNTs at nerve terminals is illustrated in Fig. 11.4.

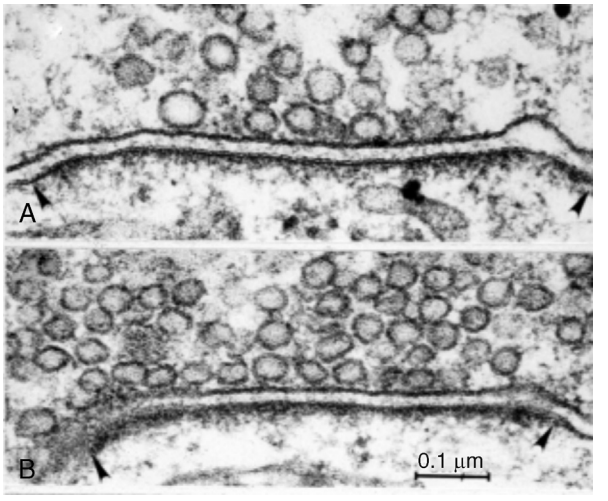

Fig. 11.4. Representation of blockade of vesicle fusion with the presynaptic membrane by BoNT action. (A) Control; (B) BoNT-treated.

11.13. Synaptic and postsynaptic effects

The synaptic and postsynaptic pathophysiology of BoNTs and secondary actions on muscle have been much less studied than presynaptic activities at nerve terminals. The onset, duration of paralysis, time for recovery and effects in distal neuromuscular regions is highly dependent on the serotype of BoNT, muscle activity, muscle stimulation and other factors (Hughes and Whaler, 1962; Eleopra et al., 1997, 2004, 2006; Sloop et al., 1997; Hesse et al., 1998; Davletov et al., 2005). The toxin's efficacy also depends strongly on the serotype and its method of preparation and composition (Schantz and Johnson, 1992; Mclellan et al., 1996; Eleopra et al., 1997; Sampaio et al., 2004; Rosales et al., 2006). In humans, muscle paralysis generally reaches its highest severity in 4–8 days depending on the serotype and dose of toxin, plateaus for several weeks to months and then muscle function gradually recovers (Sloop et al., 1997; Eleopra et al., 1998, 2006; Billante et al., 2002; Foran et al., 2003; Rosales et al., 2006). These studies demonstrated that local therapeutic injection into humans of BoNT/A, /B, /C and /E cause paralysis for durations of ~3–6, 3, 2–4 and 1–2 months, respectively.

The pathophysiology of synaptic and postsynaptic effects from BoNT has been most thoroughly studied in animal models. Local injections or application of the toxin to tissue preparations are well known to cause denervation and associated events including motor paralysis, spread of AChR-sensitive areas, neuronal sprouting, muscle fiber atrophy and some other changes in muscle morphology and composition (Thesleff, 1960, 1976, 1991; Drachman and Johnston, 1975; Pestronk et al., 1976; Alderson, 1993; Hassan et al., 1995; Dodd et al., 2005). In animal models, two primary morphological changes are observed at the NMJ following BoNT treatment. In proximal muscles, motor axon sprouting is prominent, which probably results from a signal related to denervation (Duchen, 1972; Pestronk and Drachman, 1978; Brown et al., 1981; Alderson, 1993; English, 2003). In more distal muscles, expansion of the endplate region is more evident, although both changes may occur in each muscle as observed in humans (Alderson, 1993). In animal models, particularly rat hemidiaphragm preparations, muscle activity increases uptake of BoNTs into the terminal nerve endings (Hughes and Whaler, 1962). Periodic electrical stimulation in patients being treated with BoNT for blepharospasm and hemifacial spasm increased the neuromuscular blockade (Eleopra et al., 1997). Reduction of the CMAP was also greater for the stimulated muscles. These results indicate that muscle activity can enhance the clinical effects of the toxin. Although quantal and spontaneous release of neurotransmitter is blocked nearly completely by BoNT, the nerve terminals and neuromuscular junctions do not degenerate (Duchen and Strich, 1968; Duchen, 1971, 1972; Holland and Brown, 1981; Gomez and Queiroz, 1982; Yee and Pestronk, 1987). Paralysis by BoNT has also revealed trophic secretions at the NMJ (Thesleff, 1993), supporting exchange of pre- and postsynaptic information following the functional denervation.

Botulinum-treated muscles are supersensitive to ACh activation (Thesleff, 1960). Other than sprouting, the ultrastructure of nerve terminals in botulinum-intoxicated frog and cat muscles is not altered even after periods of 3–4 weeks' treatment (Thesleff, 1960). The muscle preparations did not show any miniature endplate potentials (mepps) or responses to nerve stimulation. In cat tenuissimus muscle neuromuscular blockade by a single application of botulinum toxin reached its maximum after 5 days and then remained at a constant level for several months (Thesleff, 1976). At high concentrations of toxin, spontaneous mepps were usually absent and nerve stimulation evoked no endplate potentials (epps). With lower quantities of toxin, the mepp frequency was about 100 times less than in normal muscle. Nerve stimulation was either ineffective or evoked tiny epps (Thesleff, 1960). The rate of recovery from the blocked state of the neuromuscular junction increased with age (Gutmann and Habnzlíková, 1972).

Elegant studies by Drachman (1964, 1971) demonstrated the utility of botulinum toxin for studying the nervous system. Injection of large intravenous doses of BoNT/A-complex to 7- and 12-day chick embryos resulted in skeletal muscle atrophy and only slight structural defects in heart and liver (Drachman, 1964, 1971). The significance and utility of the chick model has been reviewed (Drachman and Coulombre, 1962; Drachman, 1971; Johnson, 1999). The administration of enormous BoNT doses is possible in chick embryos because their respiratory gas exchange is by passive diffusion across the chorioalloantoic membrane (Drachman, 1964). Drachman administered doses which would kill 20 000 or more hatched chickens at 7 and 12 days. The embryos injected with BoNT/A-complex showed multiple joint contractures on hatching (arthrogryposis congenita multiplex) due to the immobility during embryonic development (Drachman, 1964) and had a slightly shortened upper beak. All skeletal muscles were markedly shrunken and fatty, an appearance most evident in the chick embryos injected on prenatal day 7. Heart and liver weights were reduced this was about 27% and 17%, respectively, whereas in body (and limb) muscle this was about 45% and 80%, respectively. Degenerative

and fatty changes were more prominent in muscles of embryo injected on day 7, consistent with age-dependent susceptibility of the neuromuscular system to BoNT. The observed histologic changes are like those of physical denervation and atrophy, but on a greater scale in the less mature animals (Adams et al., 1960; MacIntosh et al., 2006). The results were interpreted to show a "trophic" influence of neurally released acetylcholine on skeletal muscle form and function (Drachman, 1964).

Physical denervation produces a physical separation of nerve and muscle membranes at the synapse within 4–5 days (Thesleff, 1976). Chemical denervation by BoNT is rapidly followed by copious sprouting of the motor nerve terminals as well as by some nodal sprouting (Duchen, 1971, 1972; Duchen and Strich, 1971; Brown et al., 1981; Alderson, 1993; English, 2003). In the experimental studies, terminal nerve sprouts appeared erratically on the surface of the muscle fibers and it is not clear whether these sprouts are attracted to the functionally denervated endplates; perhaps more likely, are seeking muscle sites for establishing new synaptic contacts. It is clear that nerve terminal sprouting is governed by the Schwann cells activated by the lack of supply of acetylcholine (Son and Thompson, 1995). In BoNT/A denervated muscle, uncharacterized biochemical changes were also detected (Gutmann, 1962; Watson, 2006). Determinants of sprout growth possibly include electrical stimulation, when applied, and pre- and postsynaptic secretion of neurotrophic growth factors, cytokines and proteases, including the ciliary neurotrophic factor and insulin-like growth factors I and II (Brown et al., 1981; Thoenen, 1995; English, 2003). The age of the animal also affects the propensity of sprout formation (Gutmann and Habnzlíková, 1972). In BoNT-treated muscle, the immature sprouts temporarily establish neuromuscular transmission (de Paiva et al., 1999; English, 2003). However, these immature connections tend not to reach full maturity nor establish permanent motor endplates; instead, in a second phase of reinnervation the original motor endplate becomes reactivated and the superfluous sprouts are eliminated (de Paiva et al., 1999).

The remodeling process occurs with alterations in neural plasticity (de Paiva et al., 1999; Sanes and Lichtman, 2001; Kummer et al., 2006). Sprouting and repair is also associated with intriguing physiological activities (Brown et al., 1981; de Paiva et al., 1999; English, 2003). Sprouts induced by BoNT denervation exhibit enhanced uptake and retrograde transport of adenoviral particles and increased uptake of exogenously introduced genes (Millecamps et al., 2001, 2002; Federici and Boulis, 2006), pointing to possible novel approaches for gene therapy of neuronal diseases, such as amyotrophic lateral sclerosis (ALS). Drachman and colleagues found that botulinum toxin and denervation increased sensitivity of skeletal muscle to Coxsackie A2 virus infection (Andrew et al., 1984).

The stability and regeneration of functional NMJ and myogenesis also depends on the half-life of acetylcholine receptors. In normally innervated muscle, a relatively long half-life of ~12 days is observed (Avila et al., 1989). Denervation leads to a much more rapid turnover of AChRs after a lag period. Avila et al. (1989) examined the effects of presynaptic and postsynaptic denervation on the stability on AChRs in soleus and flexor digitorum brevis muscles by repeated injection of BoNT/A complex and by continuous application of α-bungarotoxin in the soleus. Treatment with BoNT caused accelerated turnover of AChRs, like that after surgical denervation, but with a lag phase, while exposure to α-bungarotoxin led to AChR loss with the same time course as that observed after surgical denervation. These experiments indicated that neurotransmission regulates the stability of AChRs at the NMJ (Avila et al., 1989).

Muscle and nerve biopsies have been performed to assess the pathology of BoNT-induced functional denervation (Maselli et al., 1997; Keet et al., 2005). Animals exposed to BoNT display varying degrees of muscle atrophy depending on the dose applied (Drachman, 1971; Duchen, 1971; Capra et al., 1991; Hassan et al., 1995). Use of acetylcholinesterase staining and muscle fiber alterations have been employed to evaluate the potency of BoNT preparations and to study diffusion characteristics of the toxin (Borodic et al., 1994). The affected muscle fibers are abnormal in size, with most fibers atrophic and shrunken, and may also have a basophilic sarcoplasm, altered nuclei and nucleoli and shifts in myosin composition (Rosales et al., 1996; Dodd et al., 2005; Keet et al., 2005).

The molecular pathways of myogenesis, myofiber remodeling and muscle activity restoration following BoNT exposure are in the early stages of investigation. Extraocular muscles are highly sensitive to BoNT, probably due to their specialized innervation and muscle composition and topography, as well as supersensitivity to certain myasthenic diseases (Porter and Baker, 1996; Ruff, 2002; Buttner-Ennever et al., 2003; Conti-Fine et al., 2006). BoNT/A injection in the superior rectus muscles of rabbits resulted in a significant increase in the number of bromodeoxyuridine-positive nuclei and satellite cells. MyoD expression in satellite cells and myonuclei was also significantly increased following injection (Ugalde et al., 2005). Several genes were upregulated in skeletal muscle tissue in response to BoNT treatment. Transcription of

the AChR α-subunit is enhanced, though not as rapidly as in surgically denervated muscles (Lipsky et al., 1989). In juvenile (1-month-old) rats, the expression of mRNA was determined following injection of BoNT/A-complex into the gastrocnemius muscle (Ma et al., 2005; Shen et al., 2005, 2006). mRNA was extracted from muscle extracts and real-time PCR and gene microarrays were used to identify genes involved in remodeling and stabilization of the NMJ and muscle functional recovery. Key genes that were upregulated included those encoding nAChR subunits, SNAP-25, GAP-43, plasminogen activator and members of the MyoD family (Tian et al., 1995; Ma et al., 2005; Shen et al., 2005). In an extensive study, Shen et al. (2006) examined more than 28 000 rat genes and observed that ~9000 genes were expressed in gastrocnemius muscle. Among these, 105 genes were up-regulated and 59 genes were down-regulated significantly in gastrocnemius muscle extracts in BoNT-treated muscle. As expected, the genes were expressed in a temporal order, suggesting that a sequence of cellular events is involved in the recovery process. Shen et al. (2006) suggested that recovery occurred in two major stages, which they designated as aneural and neural, and that IFG-1 was integral in the signaling process. Extensive proteomic studies are being performed on the systematic analysis of genes required for synapse structure and function (Sieburth et al., 2005) and this approach should yield valuable insights into the genetic regulation of neuromuscular function in response to denervation or other perturbations.

In therapeutic applications, BoNT affects classes of muscle tissue differently. When BoNT/A is injected into smooth detrusor muscle of the bladder and certain other smooth muscle systems, such as the esophagus, its effects last 6–9 months (occasionally up to 12 months) (Grosse et al., 2005; Schurch, 2006), compared to 3–6 months observed in striated muscles. The longer duration suggests that BoNT may act by different mechanisms in the lower urinary tract including the bladder, proximal urethra and external urethral sphincter (Smith et al., 2003). The reason for this is unclear, but lack of axonal sprouting and delayed innervation could be important factors (Schurch, 2006); a different rate of replacing the BoNT/A-cleaved SNAP-25 in different types of neurons may also play an important role in determining the duration of the effects. Additional mechanisms could include long-lasting atrophy or inactivation of smooth muscle, while in striated muscle repeated and long courses of injection do not appear to cause permanent muscle atrophy or weakening (Borodic et al., 1994).

Smith et al. (2003) suggested that autonomic nerves and parasympathetic and sympathetic nerves in the urethra may have different sensitivity to BoNT/A. A rat model was used to investigate the release of ^{14}C-choline and ^{3}H-norepinephrine at various frequencies of nerve stimulation (2, 4 and 20 Hz) in the urinary tract system. The fractional release of ACh in BoNT/A-treated animals was significantly inhibited at a higher frequency of stimulation (20 Hz), but not at lower frequencies (2 Hz) 5 days after injection. However, ACh release increased to SHAM-injected values 30 days after toxin injection. Although no significant differences were observed in the fractional release of norepinephrine from injected bladders, norepinephrine release was inhibited in the urethra for at least 30 days (Smith et al., 2003). These results indicated that BoNT/A injected into the bladder could depress the release of neurotransmitters in a frequency and time-dependent manner. No significant changes were observed in nerve density. Due to the heightened effect at high frequencies of stimulation, Smith et al. (2003) suggested that BoNT may affect intracellular signaling mechanisms involving protein kinase C. Furthermore, protein kinase C phosphorylation of SNAP-25 affects phorbol-12-myristate-13-acetate stimulation of norepinephrine release from PC12 cells. It has also been shown that BoNT/A depressed the facilitation of evoked release of norepinephrine and serotonin release by the protein kinase C activator 4-β-phorbol-12,13-dibutyrate in rabbit hippocampal slices (Nakov et al., 1989). These results indicate that BoNT may participate in signaling pathways, but further studies are needed to evaluate the toxin's involvement.

Distal temporal neuromuscular effects have been observed following injection of BoNT. Increased jitter was initially reported following periorbital treatment for blepharospasm (Sanders et al., 1986). Most other studies have evaluated distal effects on treatment of cervical dystonia, in which relatively high levels of BoNT/A are generally injected (e.g., 100–500 U) (Olsney et al., 1988). Modest increases in the magnitude of jitter and mean fiber density were observed (Lange et al., 1987; Olsney et al., 1988). Single-fiber EMG studies on distal muscles including biceps brachii to measure jitter and fiber density changes have been investigated. In most studies, patients did not develop weakness or decrement of muscle response to nerve stimulation in muscles distant from the injection site. Significant presynaptic blockage measured electrophysiogically were not observed. These data support the notion that relatively high doses can be well tolerated in clinical practice.

11.14. Central effects of botulinum neurotoxins

For many years there has been considerable debate about whether physiological concentrations of BoNT can enter the CNS (e.g., Koenig, 1971; Boroff and Chen, 1975; Habermann and Dreyer, 1986; de Groot

et al., 2002; Abbruzzese and Berardelli, 2006). This is an intriguing area of study since BoNT injections have been tried to alleviate CNS-related syndromes including pain, epilepsy, migraine, visual function and psychological disorders such as depression (Aoki, 2003; Benecke et al., 2003; Lang, 2003; Luvisetto et al., 2003, 2004, 2006; Ashkenazi and Silberstein, 2004; Costantin et al., 2005; Johnson et al., 2006; Caleo et al., 2007). BoNTs have been shown to affect synaptic activity of central neurons in tissue preparations at high doses or after direct intracranial injection (Boroff and Chen, 1975; Habermann and Dreyer, 1986; Ashton and Dolly, 1988; Habermann, 1989). Presumably because of its large size of 150 kDa, BoNT is not able to permeate the blood–brain barrier or to be retrogradely transmitted to the CNS at physiological concentrations (Simpson, 2000). However, limited clinical observations in humans and animals suggest that BoNT affects the central nervous systems at physiologically relevant doses (Koenig, 1971; Habermann and Dreyer, 1986; Luvisetto et al., 2003, 2004; Abbruzzese and Berardelli, 2006; Bozzi et al., 2006). It is currently unclear if BoNT affects cortical excitability and plasticity in humans (Abbruzzese and Berardelli, 2006). The ability of BoNT to affect brain function remains controversial and needs further study. If BoNT entered the CNS and brain at physiological levels or elicited indirect effects from the periphery, this might open important therapeutic opportunities.

11.15. Possible role of BoNT in neuronal plasticity and learning

Motor systems throughout life within an organism have a dynamic capacity for adaptive remodeling and plasticity changes (Sanes and Donoghue, 2000; Franchi, 2002). For several years, certain physicians have reported that treatment of children for cerebral palsy with BoNT sometimes leads to positive adaptation of muscle function over time. Motor cortex reorganization has been proposed to occur following injection of BoNT/A into various muscles (Franchi, 2002). Adaptive changes in motor control have been observed after localized injections of BTX, suggesting remodeling of cortical activity. This has been postulated to occur in musicians who have developed and maintained high levels of skills in muscular activity and motor control (Peschel and Altenmuller, 2004; Watson, 2006). Musicians are also prone to suffer from focal dystonia. In patients with upper limb dystonia, patients treated with BoNT/A were found to transiently alter the excitability of the inhibitory and excitatory intracortical circuit activity, which was postulated to originate through peripheral mechanisms (Gilio et al., 2000). Besides relieving spasticity, functional improvement by BoNT could involve synaptic plasticity of the muscle afferents (Krishnan, 2005). It was reported that BoNT generates synaptic plasticity in spinal α-motoneurons and facilitates relearning by Hebbian and Contrastive Hebbian modes. It is postulated that BoNT can be used as a tool for relearning of motor activity in various neural disorders (Krishnan, 2005).

11.16. Emergency information

The seriousness of botulism led to the establishment of a National Botulism Laboratory at the Centers for Disease Control and Prevention in the United States and similar laboratories in certain other countries. In suspected cases of botulism, the CDC can be contacted at www.cdc.gov and the emergency 24-hour phone number for state health departments is 770-4888-7100. Medical care providers who suspect botulism in patients should immediately call their state health department's emergency 24-hour telephone number. The state health department can arrange for clinical consultation and, if indicated, release of botulinum neurotoxin. State health departments can be located by consulting the local telephone operator or law enforcement agency, the telephone directory under "government listings" or the Internet at http://www.cdc.gov/other.htm#states or http://www.astho.org/state.html (Hatheway and Dang, 1994).

Acknowledgments

EAJ acknowledges support from the Pacific Southwest Regional Center of Excellence (grant U54 AI065359) and the Great Lakes Regional Center of Excellence (U54 AI57153), the University of Wisconsin – Madison, and sponsors of the Food Research Institute; and that in CM's laboratory by a Telethon grant and the Armenise-HMS Foundation. The authors are grateful to members of their laboratories over the years and to collaborators and mentors on various projects involving neurotoxigenic clostridia and botulinum neurotoxin.

References

Abbruzzese G, Berardelli A (2006). Neurophysiological effects of botulinum toxin type A. Neurotox Res 9: 109–114.

Abgueguen P, Delbos V, Chennebault JM, et al. (2003). Nine cases of foodborne botulism type B in France and literature review. Eur J Clin Microbiol Infect Dis 22: 749–752.

Adams CE (1960). Embryonic mortality induced experimentally in the rabbit. Nature 188: 332–333.

Adler M, Keller JE, Sheridan RE, et al. (2001). Persistence of botulinum neurotoxin A demonstrated by sequential administration of serotypes A and E in rat EDL muscle. Toxicon 39: 233–243.

Ahsan CR, Hajnóczky, Maksymowych, et al. (2005). Visualization of binding and transcytosis of botulinum toxin by human intestinal cells. J Pharmacol Exp Therapeut 315: 1028–1035.

Aikawa Y, Lynch KL, Boswell KL, et al. (2006). A second SNARE role for exocytic SNAP25 in endosome fusion. Molec Biol Cell 17: 2113–2124.

Alderson K (1993). Motor nerve terminal morphology following botulinum A toxin injection in humans. In: BR DasGupta (Ed.), Botulinum and Tetanus Neurotoxins. Plenum Press, New York, pp. 53–62.

Anderson TD, Shah UK, Schreiner MS, et al. (2002). Airway complications of infant botulism: ten year experience with 60 cases. Otolaryngol Head Neck Surg 126: 234–239.

Andrew CG, Drachman DB, Pestronk A, et al. (1984). Susceptibility of skeletal muscle to cosackie A2 virus infection. Effects of botulinum toxin and denervation. Science 223: 714–716.

Antharavally B, Tepp W, DasGupta BR (1998). Status of cys residues in the covalent structure of botulinum neurotoxins A, B, and E. J Prot Chem 17: 187–196.

Aoki KR (2002). Botulinum neurotoxin serotypes A and B preparations have different safety margins in preclinical models of muscle weakening efficacy and systemic safety. Toxicon 40: 923–928.

Aoki KR (2003). Evidence for antinociceptive activity of botulinum toxin type A in pain management. Headache 43(Suppl. 1): S9–S15.

Arndt JW, Chai Q, Christian T, et al. (2006a). Structure of botulinum neurotoxin type D light chain at 1.65 ansgstrom resolution: repercussions for VAMP-2 substrate specificity. Biochemistry 45: 3255–3262.

Arndt JW, Jacobson MJ, Abole EE, et al. (2006b). A structural perspective of the sequence variability within botulinum neurotoxin subtypes A1–A4. J Molec Biol 362: 733–742.

Arnon SS (2004). Infant botulism. In: RD Feigen, JD Cherry (Eds.), Textbook of Pediatric Infectious Diseases. 5th ed. WB Saunders, Philadelphia, pp. 1758–1766.

Arnon SS, Werner SB, Faber HK, et al. (1979). Infant botulism in 1931—discovery of a misclassified case. Am J Dis Child 133: 580–582.

Arnon SS, Damus K, Chin J (1981). Infant botulism: epidemiology and relation to sudden infant death syndrome. Epidemiol Rev 3: 45–66.

Arnon SS, Schechter R, Inglesby TW, et al. (2005). Botulinum toxin as a biological weapon. Medical and public health management. JAMA 285: 1059–1070.

Arnon SS, Schechter R, Maslanka SE, et al. (2006). Human immune globulin in for the treatment of infant botulism. N Engl J Med 354: 462–471.

Arntzen FJ, van den Heever J, Perovic O (2004). Fatal type A botulism in South Africa, 2002. Trans R Soc Trop Med Hyg 98: 290–295.

Ashkenazi A, Silberstein SD (2004). Botulinum toxin and other approaches to migraine therapy. Annu Rev Med 55: 505–518.

Ashton AC, Dolly JO (1988). Characterization of the inhibitory activity of botulinum neurotoxin type A on the release of several transmitters from rat cerebrocortical synaptosomes. J Neurochem 50: 1808–1816.

Avila OL, Drachman DB, Pestronk A (1989). Neurotransmission regulates stability of acetylcholine receptors at the neuromuscular junction. J Neurosci 9: 2902–2906.

Bajjalieh SM, Peterson K, Shinghal R, et al. (1992). SV2, a brain synaptic vesicle protein homologous to bacterial transporters. Science 257: 1271–1273.

Bajjalieh SM, Peterson K, Linial M, et al. (1993). Brain contains 2 forms of synaptic vesicle protein-2. Proc Natl Acad Sci U S A 90: 2150–2154.

Bajjalieh SM, Frantz GD, Weimann JM, et al. (1994). Differential expression of synaptic vesicle protein-2 (SV2) isoforms. J Neurosci 14: 5223–5235.

Bajohrs M, Rickman C, Binz T, et al. (2004). A molecular basis underlying differences in the toxicity of botulinum serotypes A and E. EMBO Rep 5: 1090–1095.

Bakheit AM, Ward CD, McLellan DL (1997). Generalised botulism-like syndrome after intramuscular injections of botulinum toxin type A: a report of two cases. J Neurol Neurosurg Psychiatry 62: 198.

Baldwin MR, Barbieri JT (2007). Association of botulinum neurotoxin serotypes A and B with synaptic vesicle protein complexes. Biochemistry 46: 3200–3210.

Barclay ML, Begg EJ (1994). Aminoglycoside toxicity and relation to dose regimen. Adverse Drug React Toxicol Rev 13: 207–234.

Bartlett JC (1986). Infant botulism in adults. N Engl J Med 315: 254–255.

Bartram U, Singer D (2004). Infant botulism and sudden infant death syndrome. Klin Padiatr 216: 26–30.

Benecke R, Dressler D, Kunesch E, et al. (2003). Botulinum toxin in the treatment of muscle pain. Schmerz 17: 450–458.

Bhatia KP, Munchau A, Thompson PD, et al. (1999). Generalized muscular weakness after botulinum toxin injections for dystonia: a report of three cases. J Neurol Neurosurg Psychiatry 67: 90–93.

Bigalke H, Rummel A (2005). Medical aspects of toxic weapons. Toxicol 214: 210–220.

Billante CR, Zealear DL, Billante M, et al. (2002). Comparison of neuromusculur blockade and recovery with botulinum neurotoxins A and F. Muscle Nerve 26: 395–403.

Binz T, Kurazano H, Wille M, et al. (1990). The complete sequence of botulinum neurotoxin type-A and comparison with other clostridial neurotoxins. J Biol Chem 265: 9153–9158.

Black RE, Gunn RA (1980). Hypersensitivity reactions associated with botulinal antitoxin. Am J Med 69: 567–570.

Black JD, Dolly JO (1986). Interaction of 125I-labeled botulinum neurotoxins with nerve terminals. Autoradiographic evidence for its uptake into motor nerves by acceptor-mediated endocytosis. J Cell Biol 103: 535–544.

Blanes-Mira C, Ibañez Fernández-Ballester G, et al. (2001). Thermal stabilization of the catalytic domain of botulinum neurotoxin E by phosphorylation of a single tyrosine residue. Biochemistry 40: 2234–2242.

Blasi J, Chapman ER, Link E, et al. (1993a). Botulinum neurotoxin-A selectively cleaves the synaptic protein SNAP-25. Nature 365: 160–163.

Blasi J, Chapman ER, Yamasaki S, et al. (1993b). Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. EMBO J 12: 4821–4828.

Blasi J, Binz T, Yamasaki S, et al. (1994). Inhibition of neurotransmitter release by clostridial neurotoxins correlates with specific proteolysis of synaptosomal proteins. J Physiol (Paris) 88: 235–241.

Böhnel H, Gessler F (2005). Botulinum toxins—cause of botulism and systemic diseases? Veter Res Commun 29: 313–345.

Böhnel H, Behrens S, Loch P, et al. (2001). Is there a link between infant botulism and sudden infant death? Bacteriological results obtained in Central Germany. Eur J Pediatr 160: 623–628.

Bonventre PF (1979). Absorption of botulinal toxin from gastrointestinal tract. Rev Infect Dis 1: 663–667.

Borodic G (2007). Botulinum toxin: immunologic considerations with long-term repeated use, with emphasis on cosmetic applications. Facial Plastic Surg Clin North Amer 15: 11–16.

Borodic GE, Ferrante RJ, Pearce LB, et al. (1994). Pharmacology and histology of the therapeutic application of botulinum neurotoxin. In: J Jankovic, M Hallett (Eds.), Therapy with Botulinum Toxin. Marcel Dekker, Inc., New York, pp. 119–157.

Borodic G, Johnson E, Goodnough M, et al. (1996). Botulinum toxin therapy, immunologic resistance, and problems with available materials. Neurology 46: 26–29.

Boroff DA, Chen GS (1975). On the question of permeability of the blood brain barrier to botulinum toxin. Int Arch Allergy Appl Immunol 48: 495–504.

Boroff DA, del Castillo J, Evory WH, et al. (1974). Observations on the action of type A botulinum toxin on the frog neuromuscular junctions. J Physiol 240: 227–253.

Boyer A, Girault C, Bauer F, et al. (2001). Two cases of foodborne botulism type E and review of epidemiology in France. Eur J Clin Microbiol Infect Dis 20: 192–195.

Bozzi Y, Costantin L, Antonucci F, et al. (2006). Action of botulinum neurotoxins in the central nervous system: antiepileptic effects. Neurotox Res 9: 197–203.

Brin MF, Hallett M, Jankovic J (Eds.) (2002). Scientific and therapeutic aspects of botulinum toxin. Lippincott Williams and Wilkins, New York.

Brooks VB (1953). Motor nerve filament block by botulinum toxin. Science 117: 334–335.

Brooks VB (1954). The action of botulinum toxin on motor-nerve filaments. J Physiol (London) 123: 501–515.

Brown MC, Holland RL, Hopkins WG (1981). Motor nerve sprouting. Annu Rev Neurosci 4: 17–42.

Burgen ASV, Dickens F, Zatman LJ (1949). The action of botulinum toxin on the neuromuscular junction. J Physiol (Lon) 109: 10–24.

Buttner-Enneyer JA, Eberhorn A, Horn ADE (2003). Motor and sensory innervation of extraocular eye muscles. Ann N Y Acad Sci 1004: 40–49.

Byard RW, Moore L, Bourne AJ, et al. (1992). *Clostridium botulinum* and sudden infant death syndrome—a 10 year prospective study. J Paediatr Child Health 28: 156–157.

Caleo M, Restani L, Gianfranceschi L, et al. (2007). Transient synaptic silencing of developing striate cortex has persistent effects on visual function and plasticity. J Neurosci 27: 4530–4540.

Campos-Neto A, Mengel JO, Oliveira-Silva DA, et al. (1995). Potent stimulation of murine B cells to proliferate and to secrete immunoglobulins by a lipoteichoic acid-like molecule produced by *Clostridium botulinum* C and D. Braz J Med Biol Res 28: 575–584.

Capra NF, Bernanke JM, Porter JD (1991). Ultrastructural changes in the masseter muscle of *Macaca fasciluaris* resulting from intramuscular injections of botulinum toxin type A. Arch Oral Biol 36: 827–836.

Caya JG, Rashmi A, Miller JE (2004). *Clostridium botulinum* and the clinical laboratorian. A detailed review of botulism, including biological warfare ramifications of botulinum toxin. Arch Pathol Lab Med 128: 653–662.

Centers for Disease Control and Prevention (CDC) (1998). Botulism in the United States 1899–1996: Handbook for Epidemiologists, Clinicians, and Laboratory Workers. Centers for Disease Control and Prevention, Atlanta, GA. www.cdc.gov/ncidod/dbmd/diseaseinfo/files/botulinism.pdf (accessed March 2008).

Centers for Disease Control and Prevention (CDC) (2006). CDC Botulism. Available online at: http://www.bt.cdc.gov/agent/botulism, accessed March 2008.

Chai Q, Arndt JW, Dong M, et al. (2006). Structural basis of cell surface receptor recognition by botulinum neurotoxin B. Nature 444: 1096–1100.

Chang GY, Ganguly G (2003). Early antitoxin treatment in wound botulism results in better outcome. Eur Neurol 49: 151–153.

Chang KJ, Bennett V, Cuatrecasas P (1975). Membrane receptors as general markers for plasma membrane isolation procedures. The use of ^{125}I-labeled wheat germ agglutinin, insulin, and cholera toxin. J Biol Chem 250: 488–500.

Chang VH, Robinson LR (2000). Serum positive botulism with neuropathic features. Arch Phys Med Rehabil 81: 122–126.

Cherington M (1990). Botulism. Semin Neurol 10: 27–31.

Cherington M (1998). Clinical spectrum of botulism. Muscle Nerve 21: 701–710.

Cherington M (2004). Botulism: update and review. Seminar Neurol 24: 155–163.

Chertow DS, Tan ET, Maslanka SE, et al. (2006). Botulism in 4 adults following injections with an unlicensed, highly concentrated botulinum preparation. J Am Med Assoc 296: 2476–2479.

Chia JK, Clark JK, Ryan CA, et al. (1986). Botulism in an adult associated with foodborne intestinal infection with *Clostridium botulinum*. N Engl J Med 315: 239–241.

Claus D, Druschky A, Ergbuth F (1995). Botulinum toxin—influence on respiratory heart-rate variation. Mov Disord 10: 574–579.

Cohen RE, Anderson DL (1986). Botulism: emotional impact on patient and family. J Psychom Res 30: 321–326.

Colebatch JG, Wolff AH, Gilbert RJ, et al. (1989). Slow recovery from severe foodborne botulism. Lancet 2: 1216–1217.

Colice GL (1987). Prolonged intubation versus tracheostomy in the adult. J Intens Care Med 2: 85–102.

Conti-Fine BM, Milani M, Kaminski HJ (2006). Myasthenia gravis: past, present, future. J Clin Invest 116: 2843–2854.

Costantin L, Bozzi Y, Richichi C, et al. (2005). Antiepileptic effects of botulinum neurotoxin E. J Neurosci 25: 1943–1951.

Couesnon A, Pereira Y, Popoff MR (2008). Receptor-mediated transcytosis of botulinum neurotoxin A through intestinal cell monolayers. Cell Microbiol 10: 375–387.

Crawford TO (2003). Infantile botulism. In: HR Jones, Jr, DC DeVito, BT Barnes (Eds.), A Clinician's Approach Neuromuscular Diorders of Infancy, Childhood, and Adolescence. Butterworth Heinemann, Elsevier Science, Philadelphia, PA, pp. 547–554.

Custer KL, Austin NS, Sullivan JM, et al. (2006). Synaptic vesicle protein 2 enhances release probability at quiescent synapses. J Neurosci 26: 1303–1313.

Dack GM (1926). Behavior of botulinum toxin in alimentary tract of rats and guinea pigs. J Infect Dis 38: 174–181.

Dack GM, Wood WL (1928). Serum therapy for botulism in monkeys. J Infect Dis 42: 209–212.

DasGupta BR (1989). The structure of botulinum neurotoxin. In: LL Simpson (Ed.), Botulinum Toxin and Tetanus Toxin. Academic Press, San Diego, pp. 53–67.

DasGupta BR (2006). Botulinum neurotoxins: perspective on their existence and as polyproteins harboring viral proteases. J Gen Appl Microbiol 52: 1–8.

DasGupta BR, Dekleva ML (1990). Botulinum neurotoxin type A—sequence of amino acids at the N-terminus and around the nicking site. Biochimie 72: 661–664.

DasGupta BR, Rasmussen S (1983). Purification and amino acid composition of type E botulinum neurotoxin. Toxicon 21: 535.

DasGupta BR, Sathyamoorthy V (1984). Purification and amino acid composition of type A botulinum neurotoxin. Toxicon 22: 415–424.

DasGupta BR, Antharavally BS, Tepp W, et al. (2005). Botulinum neurotoxin types A, B, and E: fragmentations by autoproteolysis and other mechanisms including O-phenantrholine—dithiothreitol, and association of the dinucleotides NAD(+)/NADH with the heavy chain of the three neurotoxins. Protein J 24: 337–368.

Davis JB (1951). *Clostridium botulinum* in a fatal wound infection. JAMA 146: 646.

Davletov B, Bajohrs M, Binz T (2005). Beyond BOTOX: advantages and limitations of individual botulinum neurotoxins. Trends Neurosci 28: 446–452.

de Groot M, Schubert M, Rothe B, et al. (2002). Central effects of botulinum toxin type A. Klin Neurophysiol 33: 207–212.

de Paiva A, Poulain B, Lawrence GW, et al. (1993). A role for interchain disulfide or its participating thiols in the internalization of botulinum neurotoxin A revealed by a toxin derivative that binds to ecto-acceptors and inhibits transmitter release intracellularly. J Biol Chem 28: 20838–20844.

de Paiva A, Meunier FA, Molgó J, et al. (1999). Functional repair of motor endplates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci U S A 96: 3200–3205.

Dessain SK, Adekar SP, Carpenter KA, et al. (2004). High efficiency creation of human monoclonal antibody-producing hybridomas. J Immunol Meth 291: 109–122.

Devriese PP, Devriese LA (2001). Historical aspects of botulinum toxin. Neurology 57: 1144.

Dezfulian M (1989). Animal models of botulism. In: LL Simpson (Ed.), Botulinum Neurotoxin and Tetanus Toxin. Academic Press, Inc., San Diego, CA, pp. 335–350.

Dickson EC (1918). Botulism. A clinical and experimental study. Monograph of the Rockefeller Institute, No. 8, New York, pp. 1–117.

Dineen SS, Bradshaw M, Johnson EA (2003). Neurotoxin gene clusters in *Clostridium botulinum* type A strains: sequence comparison and evolutionary implications. Curr Microbiol 46: 345–352.

Dineen SS, Bradshaw M, Karasek C, et al. (2004). Nucleotide sequence and transcriptional analysis of the type A2 neurotoxin gene cluster in *Clostridium botulinum*. FEMS Microbiol Lett 235: 9–16.

Dodd SL, Selsby J, Payne A, et al. (2005). Botulinum neurotoxin type A causes shift in myosin heavy chain composition in muscle. Toxicon 46: 196–203.

Dolman EC (1964). Botulism as a world health problem. In: KH Lewis, K Cassel (Eds.), Botulism. US Pub Health Serv, Washington, DC, pp. 5–32.

Dong M, Richards DA, Goodnough MC, et al. (2003). Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. J Cell Biol 162: 1293–1303.

Dong M, Yeh F, Tepp WH, et al. (2006). SV2 is the protein receptor for botulinum neurotoxin A. Science 312: 592–596.

Dong M, Tepp WH, Liu H, et al. (2007). Mechanism of botulinism neurotoxin B and G entry into hippocampal neurons. J Cell Biol 31: 1511–1522.

Drachman DB (1964). Atrophy of skeletal muscle in chick embryos treated with botulinum toxin. Science 145: 719–721.

Drachman DB (1971). Botulinum toxin as a tool for research on the nervous system. In: LL Simpson (Ed.), Neuropoisons. Their Pathophysiological Actions, Vol. 1. Poisons of Animal Origin. Plenum, New York, pp. 325–347.

Drachman DB, Coulombre AJ (1962). Method for continuous infusion of fluids into the chorioallantoic circulation of the chick embryo. Science. 138: 144–145.

Drachman DB, Johnston DM (1983). Neurotrophic regulation of dynamic properties of skeletal muscle—effects of botulinum toxin and denervation. J Physiol (Lon) 252: 657–667.

Dressler D, Rothwell JC (2000). Electromyographic quantification of the paralysing effect of botulinum toxin in the sternocleidomastoid muscle. Eur Neurol 43: 13–16.

Dressler D, Benecke R (2003). Autonomic side effects of botulinum toxin type B treatment of cervical dystonia and hyperhydrosis. Eur Neurol 49: 34–38.

Dressler D, Saberi FA (2005). Botulinum toxin: mechanisms of action. Eur Neurol 53: 3–9.

Duchen LW (1971). An electron microscopic study of the changes induced by botulinum toxin in the motor end-plates of slow and fast skeletal muscle fibres of the mouse. J Neurol Sci 14: 47–60.

Duchen LW (1972). Motor nerve growth induced by botulinum toxin as a regenerative phenomenon. Proc R Soc Med 65: 10–11.

Duchen LW, Strich SJ (1968). The effects of botulinum toxin on the pattern of innervation of skeletal muscle in the mouse. Q J Exp Physiol Cogn Med Sci 53: 84–89.

Duchen LW, Strich SJ (1971). An electron microscopy study of the changes induced by botulinum toxin in the motor end-plates of skeletal muscle in the mouse. Q J Exp Physiol 53: 84–89.

Duffey P, Brown C (2006). Iatrogenic botulism in an amateur weight-lifter. Movement Disord 21: 1056.

Dumitru D, Amato AA, Zwarts M (Eds.) (2002). Electrodiagnostic Medicine, 2nd edn. Hanley and Belfus, Philadelphia.

Eklund MW (Ed.) (1995). Avian Botulism. Charles C. Thomas, Springfield, Illinois.

Eleopra R, Tugnoli V, de Grandis D (1997). The variability in the clinical effect induced by botulinum toxin type A: the role of muscle activity in humans. Movement Disord 12: 89–94.

Eleopra R, Tugnoli V, Rossetto O, et al. (1998). Different time courses in recovery after poisoning with botulinum neurotoxin serotypes A and E in humans. Neurosci Lett 256: 135–138.

Eleopra R, Tugnoli V, Quatrale R, et al. (2004). Different types of botulinum toxin in humans. Movement Disord 19(Suppl. 8): S53–S59.

Eleopra R, Tugnoli V, Quatrale R, et al. (2006). Clinical use of non-A botulinum toxins: botulinum toxin type C and botulinum type F. Neurotox Res 9: 127–131.

Elston HR, Wang M, Loo LK (1991). Arm abscesses caused by *Clostridium botulinum*. J Clin Microbiol 29: 2678–2679.

Encinar JA, Fernández A, Ferragut JA, et al. (1998). Structural stabilization of botulinum neurotoxins by tyrosine phosphorylation. FEBS Lett 429: 78–82.

English AW (2003). Cytokines, growth factors and sprouting at the neuromuscular junction. J Neurocytol 32: 943–960.

Erbguth FJ (2004). Historical notes on botulism, *Clostridium botulinum,* botulinum toxin, and the idea of the therapeutic use of the toxin. Movement Disord 19(Suppl. 8): S2–S6.

Erbguth F, Claus D, Engelhardt A, et al. (1993). Systemic effect of local botulinum toxin injections unmasks subclinical Lambert–Eaton syndrome. J Neurol Neurosurg Psychiatry 62: 198.

Fairweather NF, Lyness VA (1986). The complete nucleotide sequence of tetanus toxin. Nucl Acids Res 14: 7809–7812.

Federici T, Boulis NM (2006). Gene-based treatment of motor neuron diseases. Muscle Nerve 33: 302–323.

Fernández-Salas E, Steward LE, Ho H, et al. (2004). Plasma membrane localization signals in the light chain of botulinum neurotoxin. Proc Natl Acad Sci U S A 101: 3208–3213.

Ferrier-Montiel AV, Canaves JM, DasGupta BR, et al. (1996). Tyrosine phosphorylation modulates the activity of clostridial neurotoxins. J Biol Chem 271: 18322–18325.

Fischer D, Freislederer A, Jorch G (2004). Sudden death of twins: botulism because of contamination by pap vegetables. Klin Pediatr 216: 31–35.

Fischer A, Montal M (2007a). Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. J Biol Chem 282: 29604–29611.

Fischer A, Montal M (2007b). Single molecule detection of intermediates during botulinum neurotoxin translocation across membranes. Proc Natl Acad Sci U S A 104: 10447–10452.

Foran PG, Mohammed N, Lisk GO, et al. (2003). Evaluation of the therapeutic usefulness of botulinum neurotoxins B, C1, E and F compared with the long lasting type A. Basis for distinct durations of inhibition of exocytosis in central neurons. J Biol Chem 278: 1363–1371.

Fox CK, Keet CA, Strober JB (2005). Recent advances in infant botulism. Pediatr Neurol 32: 149–154.

Franchi G (2002). Time course of motor cortex reorganization following botulinum toxin injection into the vibrissal pad of the adult rat. Eur J Neurosci 16: 1333–1348.

Franciosa G, Aureli P, Schecter R (2003). In: MD Milliotis, JW Bier (Eds.), International Handbook of Foodborne Pathogens. Marcel Dekker, Inc., New York and Basel.

Francisco AMO, Arnon SS (2007). Clinical mimics of infant botulism. Pediatrics 119: 826–828.

Franz DR, Pitt LM, Clayton MA, et al. (1993). Efficacy of prophylactic and therapeutic administration of antitoxin for inhalational botulism. In: BR DasGupta (Ed.), Botulinum and Tetanus Neurotoxins. Neurotransmission and Biomedical Aspects. Plenum Press, New York, pp. 473–476.

Fujinaga Y (2006). Transport of bacterial toxins into target cells: pathways followed by cholera toxin and botulinum progenitor toxin. J Biochem (Tokyo) 140: 155–160.

Gangarosa EJ, Donadio JA, Armstrong RW, et al. (1971). Botulism in the United States, 1899–1969. Am J Epidemiol 93: 93–101.

Gartlan MG, Hofman HT (1993). Crystalline preparation of botulinum toxin type A (Botox): degradation in potency with storage. Otolaryngol Head Neck Surg 108: 135–140.

Geny B, Popoff MR (2006). Bacterial protein toxins and lipids: role in toxin targeting and activity. Biol Cell 98: 633–651.

Gilio F, Curra A, Lorenzano C, et al. (2000). Effects of botulinum toxin type A on intracortical inhibition in patients with dystonia. Ann Neurol 48: 20–26.

Gill DM (1982). Bacterial toxins: a table of lethal amounts. Microbiol Rev 46: 86–94.

Giménez DF, Giménez JA (1993). Serological subtypes of botulinal neurotoxins. In: BR DasGupta (Ed.), Botulism and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects. Plenum Press, New York, pp. 421–432.

Gioltzoglou T, Cordivari C, Lee PJ, et al. (2005). Problems with botulinum toxin treatment in mitochondrial cytopathy: case report and review of the literature. J Neurol Neurosurg Psychiatry 76: 1594–1596.

Gomez S, Queiroz LS (1982). The effects of black widow spider venom on the innervation of muscles paralyzed by botulinum toxin. Q J Exp Physiol Cog Med Sci 67: 495–506.

Goode GB, Shearn DL (1982). Botulism: a case associated with sensory abnormalities. Arch Neurol 39: 55.

Gordon RJ, Lowy FD (2005). Curent concepts—bacterial infections in drug users. N Engl J Med 353: 1945–1954.

Griffin PM, Hatheway CL, Rosenbaum R, et al. (1997). Endogenous antibody production to botulinum toxin in an adult with intestinal colonization botulism and underlying Crohn's disease. J Infect Dis 175: 633–637.

Grosse J, Kramer G, Stohrer M (2005). Success of repeated injections of botulinum A toxin in patients with severe neurogenic detrusor overactivity and incontinence. Eur Urol 47: 653–659.

Gupta A, Sumner CJ, Castor M, et al. (2005). Adult botulism type F in the United States, 1981–2002. Neurology 65: 1694–1700.

Gutierrez AR, Bonensteiner J, Gutmann L (1994). Electrodiagnosis of infantile botulism. J Child Neurol 9: 362–366.

Gutmann E (1962). Denervation and disuse atrophy in cross-striated muscle. Rev Can Biol 21: 353–365.

Gutmann E, Habnzlíková E (1972). Age Changes in the Neuromuscular Junction. Scientechnica Ltd., Bristol, UK.

Habermann E (1989). Clostridial neurotoxins and central nervous effects: functional studies on isolated preparations. In: LL Simpson (Ed.), Botulinum Neurotoxin and Tetanus Toxin. Academic Press, San Diego, CA, pp. 255–279.

Habermann E, Dreyer F (1986). Clostridial neurotoxins: handling and action at the cellular and molecular level. Curr Top Microbiol Immunol 129: 93–179.

Hanson MA, Stevens RC (2002). Structural views of botulinum neurotoxin in numerous functional states. In: MF Bain, J Jankovic, M Hallett (Eds.), Scientific and Therapeutic Aspects of Botulinum Toxin. Lippincott Williams & Wilkins, Philadelphia, pp. 9–27.

Hassan SM, Jennekens FGI, Veldman H (1995). Botulinum toxin-induced myopathy in the rat. Brain 118: 533–545.

Hatheway CL (1988). Botulism. In: WH Hausler, A Balows, M Ohashi, et al. (Eds.), Laboratory Diagnosis of Infectious Diseases. Vol. 1. Springer-Verlag, New York, pp. 111–133.

Hatheway CL (1993). *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin. In: AHS Hauschild, KL Dodds (Eds.), *Clostridium Botulinum*: Ecology and Control in Foods. Marcel Dekker, New York, pp. 3–20.

Hatheway CL (1995). Botulism: the present status of the disease. Curr Top Microbiol Immunol 195: 55–75.

Hatheway CL, Dang C (1994). Immunogenicity of the neurotoxins of *Clostridium botulinum*. In: J Jankovic, M Hallett (Eds.), Therapy with Botulinum Toxin. Marcel Dekker, Inc., New York, pp. 93–107.

Hatheway CL, Johnson EA (1998). Clostridium: the spore-bearing anaerobes. In: L Collier, A Balows, M Sussman (Eds.), Topley & Wilson's Microbiology and Infections. 9th ed. Vol. 2. Systematic Bacteriology. Arnold, London, pp. 731–782.

Hatheway CL, Snydjer JD, Seals JE, et al. (1984). Antitoxin levels in botulism patients treated with trivalent equine botulism antitoxin to type A, type B, and type E. J Infect Dis 150: 407–412.

Hauschild AHS, Dodds KL (Eds.) (1993). *Clostridium Botulinum*: Ecology and Control in Foods. Marcel Dekker, New York, pp. 3–20.

Heckly RJ, Hildebrand GJ, Lamanna C (1960). On the size of the toxic particle passing the intestinal barrier in botulism. J Exp Med 111: 745–759.

Hesse S, Reiter F, Konrad M, et al. (1998). Botulinum toxin type A and short term electrical stimulation in the treatment of upper limb flexor spasticity after stroke: a randomized, double-blind, placebo-controlled trial. Clin Rehabil 12: 381–388.

Heuser J (1976). Morphology of synaptic vesicle discharge and reformation at the frog neuromuscular junction. In: S Thesleff (Ed.), Motor Innervation of Muscle. Academic Press, London, pp. 51–115.

Hikes DC, Manoli A (1981). Wound botulism. J Trauma 21: 68–71.

Hoch DH, Romero-Mira M, Ehrlich BE, et al. (1985). Channels formed by botulinum, tetanus, and diphtheria toxins in planar lipid bilayers: relevance to translocation of proteins across membranes. Proc Natl Acad Sci U S A 82: 1692–1696.

Hoffman RE, Pincomb BJ, Skuls MR (1982). Type F infant botulism. Am J Dis Child 136: 270–271.

Holland RL, Brown MC (1981). Nerve growth in botulinum toxin poisoned muscles. Neuroscience 6: 1167–1179.

Holmes GL, Moshé SL, Royden Jones H, Jr. (Eds.) (2006). Clinical Neurophysiology of Infancy, Childhood, and Adolescence. Elsevier, Inc., Philadelphia, PA.

Holzer E (1962). Botulism dürch inhalation. Med Klin 41: 1735–1740.

Horowitz MA, Hatheway CL, Dowell VR, Jr. (1976). Laboratory diagnosis of botulism complicated by pyridostigmine treatment of the patient—a method for selectively removing interfering substances from clinical samples. Am J Clin Pathol 66: 97–107.

Howard JF (1990). Adverse effects on neuromuscular transmission. Semin Neurol 10: 89–102.

Hughes JM, Blumenthal JR, Merson MH, et al. (1981). Clinical features of types A and B food-borne botulism. Ann Intern Med 95: 442–445.

Hughes R, Whaler BC (1962). Influence of nerve-ending activity and of drugs on the rate of paralysis of rat diaphragm preparations of Cl. botulinum type A toxin. J Physiol 160: 221–233.

Ibañez C, Blanes-Mira C, Fernández-Ballester G, et al. (2004). Modulation of botulinum neurotoxin A catalytic domain stability by tyrosine phosphorylation. FEBS Lett 578: 121–127.

Inoue K, Fujinaga Y, Watanabe T, et al. (1996). Molecular composition of *Clostridium botulinum* type A progenitor toxins. Infect Immun 64: 1589–1594.

Iwase T, Iwase C (2006). Systemic effect of local and small-dose botulinum toxin injection to unmask subclinical myasthenia gravis. Graefe's Arch Clin Exp Ophthalmol 244: 415–416.

Jahn R, Scheller RH (2006). SNARESs—engines for membrane fusion. Nature Rev Molec Cell Biol 7: 631–643.

Jankovic J, Hallett M (Eds.) (1994). Therapy With Botulinum Toxin. Marcel Dekker, Inc., New York, pp. 93–107.

Jenzer G, Mumenthaler M, Ludin HP, et al. (1975). Autonomic dysfunction in botulism B: a clinical report. Neurology 25: 150–153.

Jin R, Rummel A, Binz T, et al. (2006). Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. Nature 444: 1092–1095.

Johnson EA (1999). Clostridial toxins as therapeutic agents: benefits of nature's most toxic proteins. Annu Rev Microbiol 53: 551–575.

Johnson EA (2005a). Clostridial neurotoxins. In: P Dürre (Ed.), Handbook on Clostridia. CRC Press, Boca Raton, FL, pp. 491–525.

Johnson EA (2005b). Bacteriophages encoding botulinum and diphtheria toxins. In: MK Waldor, DI Friedman, S Adhya (Eds.), Phages: Their Role in Bacterial Pathogenesis and Biotechnology. ASM Press, Washington, DC, pp. 280–296.

Johnson EA (2005c). *Clostridium botulinum* and Clostridium tetani. In: SP Borrellio, PR Murray, G Funke (Eds.), Topley & Wilson's Microbiology and Microbial Infections, 10th ed. Vol. 2. Bacteriology. Hodder Arnold, London, pp. 1035–1088.

Johnson EA (2006). Neurotoxigenic clostridia. In: VA Fischetti, RP Novick, JJ Feretti, et al. (Eds.), Gram-Positive Pathogens. 2nd ed. ASM Press, Washington, DC, pp. 688–702.

Johnson EA (2007). *Clostridium botulinum*. In: MP Doyle, LR Beuchat (Eds.), Food Microbiology. Fundamentals and Frontiers. 3rd ed. ASM Press, Washington, DC, pp. 401–421.

Johnson EA, Bradshaw M (2001). *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. Toxicon 39: 1703–1722.

Johnson EA, Goodnough MC (1998). Botulism. In: L Collier, A Balows, M Sussman (Eds.), Topley & Wilson's Microbiology and Microbial Infections. 98th ed. Vol. 2. Systematic Bacteriology. Arnold, London, pp. 723–741.

Johnson EA, Borodic GE, Acquadro MA (2006). Medical applications of botulinum neurotoxins. In: JE Alouf, MR Popoff (Eds.), Comprehensive Sourcebook of Bacterial Protein Toxins. 3rd ed. Elsevier, Inc., Philadelphia, PA, pp. 959–975.

Jones HR, Darras BT (2000). Acute care pediatric electromyography. Muscle Nerve Suppl 9: S53–S62.

Kaminski H (Ed.) (2003). Myasthenia Gravis and Related Disorders (Electronic Resource). Humana Press, Totowa, NJ.

Katz B (1966). Nerve Muscle, Synapse. McGraw Hill, New York.

Keet CA, Fox CK, Margeta M, et al. (2005). Infant botulism, type F, presenting at 54 hours of life. Pediatr Neurol 32: 193–196.

Keller JE (2006). Recovery from botulinum neurotoxin poisoning in vivo. Neuroscience 139: 629–637.

Keller JE, Neale EA, Oyler G, et al. (1999). Persistence of botulinum neurotoxin action in cultured spinal cord cells. FEBS Lett 456: 137–142.

Kelly H, Brussen KA, Lawrence A, et al. (2006). Polioviruses and other enteroviruses isolated from faecal samples of patients with acute flaccid paralysis in Australia, 1996–2004. J Paediatr Child Health 42: 370–376.

Kempner W (1897). Welterer Beitrag Zur Lehre von der Fleischvergiftung, Das Antitoxin des Botulismus. Z Hyg Infectionskr 26: 481–500.

Kerner C (1820). Neue Boebachtungen über die in Württenberg so häufig verfallenden tödtchen Vengiftungen durch den gennus geraucherter Würste, Tubingen, Osiander.

Kobayashi H, Fujisawa K, Saito Y, et al. (2003). A botulism case of a 12-year-old girl caused by intestinal colonization of *Clostridium botulinum* type Ab. Jpn J Infect Dis 56: 73–74.

Koenig MG (1971). The clinical aspects of botulism. In: LL Simpson (Ed.), Neuropoisons. Their Pathophysiological Actions, Vol. 1. Poisons of Animal Origin. Plenum Press, New York and London, pp. 283–301.

Koriazova LK, Montal M (2003). Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. Nat Struct Biol 10: 13–18.

Krishnan RV (2005). Botulinum toxin: from spasticity reliever to neuromotor re-learning tool. Int J Neurosci 115: 1451–1467.

Kröger S, Schröder JE (2002). Agrin in the developing CNS: new roles for a synapse organizer. News Physiol Sci 17: 207–212.

Kummer TT, Misgeld T, Sanes JR (2006). Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. Curr Opin Neurol 16: 74–82.

Lacy DB, Stevens RC (1999). Sequence homology and structural analysis of the clostridial neurotoxins. J Molec Biol 291: 1091–1104.

Lacy DB, Tepp W, Cohen AC, et al. (1998). Crystal structure of botulinum neurotoxin type A and implications for toxicity. Nature Struct Biol 5: 898–902.

Lamanna C (1959). The most poisonous poison. Science 130: 763–772.

Lamanna C, Hillowalla RA, Alling CC (1967). Buccal exposure to botulinal toxin. J Infect Dis 117: 327–331.

Lang AM (2003). Botulinum toxin type A therapy in chronic pain disorders. Arch Phys Med Rehabil 84(Suppl. 1): S69–S73.

Lange DJ, Brin MF, Warner CL, et al. (1987). Distance effect of local injection of botulinum toxin. Muscle Nerve 10: 552–555.

Lawrence G, Wang J, KwoChion CKN, et al. (2007). Two protein trafficking processes at motor nerve endings unveiled by botulinum neurotoxin E. J Pharmacol Exp Therap 320: 410–418.

LeClaire RD, Pitt MLM (2005). Biological weapons defense. Effect levels. In: LE Lindler, FJ Lebeda, GW Korch

(Eds.), Infectious Diseases: Biological Weapons Defense: Infectious Diseases and Counterterrorism. Humana Press, Inc., Totowa, NJ, pp. 41–61.

Lee HK (2006). Synaptic plasticity and phosphorylation. Pharmacol Therapeut 112: 810–832.

Lesieur C, Vécsey-Semjén C, Abrami L, et al. (1997). Membrane insertion: the strategies of toxins (Review). Mol Memb Biol 14: 45064.

L'Hommedieu C, Stough R, Brown L, et al. (1979). Potentiation of neuromuscular weakness in infant botulism by aminoglycosides. J Pediatr 95: 1065–1070.

Li LYJ, Kelkar P, Exconde RE, et al. (1999). Adult onset "infant" botulism: an unusual cause of weakness in the intensive care unit. Neurol 53: 891.

Lindström M, Nevas M, Kurki J, et al. (2004). Type C botulism due to toxic feed affecting 52,000 farmed foxes and minks in Finland. J Clin Microbiol 42: 4718–4725.

Lipsky NG, Drachman DB, Pestronk A, et al. (1989). Neural regulation of messenger RNA for the alpha subunit of acetylcholine receptors. Role of neuromuscular transmission. Exp Neurol 105: 171–176.

Lonberg N (2005). Human antibodies from transgenic animals. Nature Biotechnol 23: 1117–1125.

London E (1992). How bacterial toxins enter cells: the role of partial unfolding in membrane translocation. Molec Microbiol 6: 3277–3282.

Long SS, Gajewski JL, Brown LW, et al. (1985). Clinical, laboratory and environmental features of infant botulism in Southeastern Pennsylvania. Pediatrics 75: 935–941.

Luvisetto S, Rossetto O, Montecucco C, et al. (2003). Toxicity of botulinum neurotoxins in central nervous system of mice. Toxicon 41: 475–481.

Luvisetto S, Marinelli S, Rossetto O, et al. (2004). Central injection of botulinum neurotoxins: behavioural effects in mice. Behav Pharmacol 15: 233–240.

Luvisetto S, Marinelli S, Lucchetti F, et al. (2006). Botulinum neurotoxins and formalin-induced pain: central vs. peripheral effects in mice. Brain Res 1082: 124–131.

Ma J, Shen J, Lee CA, et al. (2005). Gene expression following botulinum toxin A injection: a study in rats. J Orthopaed Res 23: 302–309.

MacDonald KL, Cohen ML, Blake PA (1986). The changing epidemiology of adult botulism in the United States. Am J Epidemiol 124: 794–799.

MacIntosh BR, Gardiner PF, McComas AJ (Eds.) (2006). Skeletal Muscle. Form and Function. 2nd ed. Human Kinetics, Champaign, IL.

Mackle IJ, Halcomb E, Parr MJ (2001). Severe adult botulism. Anaesth Intensive Care 29: 297–300.

Mahrhold S, Rummel A, Bigalke H, et al. (2006). The synaptic vesicle protein 2C mediates the uptake of botulinum neurotoxin A into phrenic nerves. FEBS Lett 580: 2011–2014.

Maksymowych AB, Simpson LL (2004). Structural features of the botulinum neurotoxin molecule that govern binding and transcytosis across polarized human intestinal epithelial cells. J Pharmacol Exp Ther 310: 633–641.

Maksymowych AB, Reinhard M, Malizio CJ, et al. (1999). Pure botulinum neurotoxin is absorbed from the stomach and small intestine and produces peripheral neuromuscular blockade. Infect Immun 67: 4708–4712.

Malizio CJ, Goodnough MC, Johnson EA (2000). Purification of botulinum type A neurotoxin. Meth Molec Biol 145: 27–39.

Mann JM, Martin S, Hoffmann R, et al. (1981). Patient recovery from type A botulism: morbidity assessment following a large outbreak. Am J Public Health 71: 266–269.

Martin PT (2003). Glycobiology of the neuromuscular junction. J Neurocytol 32: 915–929.

Marx JL (1978). Botulism in infants: a cause of sudden death? Science 201: 799–801.

Maselli RA, Ellis W, Mandler RN, et al. (1997). Cluster of wound botulism in California: clinical, electrophysiologic, and pathologic study. Muscle Nerve 20: 1284–1295.

Mayers CN, Holley JL, Brooks T (2001). Antitoxin therapy for botulinum intoxication. Rev Med Microbiol 12: 29–37.

McCroskey LM, Hatheway CL (1988). Laboratory findings in four cases of adult botulism suggest colonization of the intestinal tract. J Clin Microbiol 26: 1052–1054.

McLauchlin J, Grant KA, Little CL (2006). Food-borne botulism in the United Kingdom. J Public Health 28: 337–342.

McLellan K, Das RE, Ekong TA, et al. (1996). Therapeutic botulinum type A toxin: factors affecting potency. Toxicon 34: 975–985.

Meichsner M, Reichel G (2005). Effect of botulinum toxin A and B on vegetative cardiac innervation. Fortschr Neurol Psych 73: 409–414.

Ménétrey J, Gillet D, Ménez A (2005). Structural features common to intracellularly acting toxins from bacteria. Toxicon 45: 129–137.

Merriggioli MN, Howard JE, Jr., Harper CM (2004). Neuromuscular Junction Disorders. Diagnosis and Treatment. Marcel Dekker, Inc., New York and Basel.

Merson MH, Dowell VR (1973). Epidemiologic, clinical and laboratory aspects of wound botulism. N Engl J Med 289: 1005–1010.

Meunier FA, Lisk G, Sesardic D, et al. (2003). Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation. Molec Cell Neurosci 22: 454–466.

Meyer KF (1956). The status of botulism as a world health problem. Bull World Health Org 15281–15298.

Mezaki T, Kaji R, Kohara N, et al. (1996). Development of generalized weakness in a patient with amyotrophic lateral sclerosis after focal botulinum toxin injection. Neurol 46: 845–846.

Midura TF, Arnon SS (1976). Infant botulism—identification of *Clostridium botulinum* and its toxin in feces. Lancet 2: 934–936.

Millecamps S, Nicolle D, Ceballos-Picot I, et al. (2001). Synaptic sprouting increases the uptake capacities of motoneurons in amyotrophic lateral mice. Proc Natl Acad Sci U S A 98: 7582–7587.

Millecamps S, Mallet J, Barkats M (2002). Adenoviral retrograde gene transfer in motoneurons is greatly enhanced

by prior intramuscular inoculation with botulinum toxin. Hum Gene Ther 13: 225–232.

Mitchell WG, Tseng-Ong L (2005). Catastrophic presentation of infant botulism may obscure or delay diagnosis. Pediatrics 116: E436–E438.

Montecucco C (1986). How do tetanus and botulinum neurotoxins bind to neuronal membranes? Trends Biochem Sci 11: 314–317.

Montecucco C, Schiavo G (1993). Tetanus and botulism neurotoxins: a new group of zinc proteases. Trends Biochem. Sci 18: 324–327.

Montecucco C, Schiavo G (1995). Structure and function of tetanus and botulinum neurotoxins. Q Rev Biophys 28: 423–472.

Montecucco C, Schiavo G, Gao Z, et al. (1988). Interaction of botulinum and tetanus toxins with the lipid bilayer surface. Biochem J 251: 379–383.

Montecucco C, Schiavo G, DasGupta BR (1989). Effect of pH on the interaction of botulinum neurotoxin A, neurotoxin B, and neurotoxin E with liposomes. Biochem J 259: 47–53.

Montecucco C, Papine E, Schiavo G (1994). Bacterial protein toxins penetrate cells via a 4-step mechanism. FEBS Lett 346: 92–98.

Montecucco C, Rosetto O, Schiavo G (2004). Presynaptic receptor arrays for clostridial neurotoxins. Trends Microbiol 12: 442–446.

Montecucco C, Schiavo G, Pantano S (2005). SNARE complexes and neuroexocytosis: how many, how close? Trends Biochem Sci 30: 367–372.

Moore P, Naumann M (Eds.) (2003). Handbook of Botulinum Toxin Treatment, 2nd ed. Blackwell Publishing, Malden, MA.

Morton HE (1961). The toxicity of *Clostridium botulinum* type A toxin for various species of animals, including man. The Institute for Cooperative Research, University of Pennsylvania, Philadelphia.

Nakov R, Habermann E, Hertting G, et al. (1989). Effects of botulinum toxin on presynaptic modulation of evoked transmitter release. Eur J Pharmacol 164: 45–53.

Neale EA (2003). Moving across membranes. Nat Struct Biol 10: 2–3.

Nevas M, Lindström M, Virtanen A, et al. (2005). Infant botulism acquired from household dust presenting as sudden infant death syndrome. J Clin Microbiol 43: 511–513.

Niemann H (1991). Molecular biology of clostridial neurotoxins. In: JE Alouf, JH Freer (Eds.), A Sourcebook of Bacterial Protein Toxins. Academic Press Ltd., London, pp. 303–348.

Nishikawa A, Uotsu N, Arimitsu H, et al. (2004). The receptor and transporter for internalization of *Clostridium botulinum* type C progenitor toxin into HT-29 cells. Biochem Biophys Res Commun 319: 327–333.

Nishiki T, Kamata Y, Nemoto Y, et al. (1994). Identification of protein receptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptosomes. J Biol Chem 269: 10498–10503.

Nishiki T, Tokuyama Y, Kamata Y, et al. (1996). The high-affinity binding of *Clostridium botulinum* type B neurotoxin to synaptotagmin II associated with gangliosides GT1b/GD1a. FEBS Lett 378: 253–257.

Nishiura H (2007). Incubation period as a clinical predictor of botulism: analysis of previous Izushi-borne outbreaks in Hokkaido, Japan from 1951 to 1965. Epidemiol Infect 135: 126–130.

Ohishi I, Sugii S, Sakaguchi G (1977). Oral toxicities of *Clostridium botulinum* toxins in response to molecular size. Infect Immun 16: 107–109.

Olsney RK, Aminoff MJ, Gelb DJ, et al. (1988). Neuromuscular effects distant from the site of botulinum toxin injection. Neurol 38: 1780–1783.

Paik NJ, Seo K, Eun HC (2006). Reduced potency after refrigerated storage of botulinum toxin A: human extensor digitorum brevis muscle study. Mov Disord 21: 1759–1763.

Paisley JW, Lauer BA, Arnon SS (1995). A second case of infant botulism caused by Clostridium baratii. Pediatr Infect Dis 14: 912–914.

Park J-P, Simpson LL (2003). Inhalational poisoning by botulinum toxin and inhalational vaccination with its heavy chain component. Infect Immun 71: 1147–1154.

Parker MW, Pattus F (1993). Rendering a membrane soluble in water: a common packing motif in bacterial protein toxins. TIBS 39: 1–395.

Pellett S, Tepp WH, Clancy CM, et al. (2007). A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection ofneutralizing serum antibodies. FEBS Lett 581: 4803–4808.

Peschel T, Altenmuller E (2004). Altered movement-related brain potentials in musicians suffering from action induced focal dystonia. Klin Neurophysiologie 35: 96–103.

Pestronk A, Drachman DB (1978). Motor nerve sprouting and acetylcholine receptors. Science 199: 1223–1225.

Pestronk A, Drachman DB, Griffin JW (1976). Effect of botulinum toxin on trophic regulation of acetylcholine receptors. Nature 264: 787–789.

Pickett J, Berg B, Chaplin E, et al. (1976). Syndrome of botulism in infancy—clinical and electrophysiological study. N Engl J Med 295: 770–772.

Pitt MLM, LeClaire RD (2005). Pathogenesis by aerosol. In: LE Lindler, FJ Lebeda, GW Korch (Eds.), Infectious Diseases: Biological Weapons Defense: Infectious Diseases and Counterterrorism. Humana Press, Inc., Totowa, NJ, pp. 65–78.

Pittinger C, Adamson R (1972). Antibiotic blockade of neuromuscular function. Ann Rev Pharmacol 12: 169.

Popoff MR (1995). Ecology of neurotoxigenic strains of clostridia. In: C Montecucco (Ed.), Clostridial Neurotoxins. 195. Curr Top Microbiol Immunol, Springer, Berlin, pp. 1–29.

Porter JD, Baker RS (1996). Muscles of a different "color": the unusual properties of the extraocular muscles may predispose them in neurogenic and myogenic disease. Neurol 46: 30–37.

Puhar A, Johnson EA, Rosetto O, et al. (2004). Comparison of pH-induced conformational change of different clostridial neurotoxins. Biochem Biophys Res Commun 319: 66–71.

Raciborska DA, Charlton MP (1999). Retention of cleaved synaptosome-associated protein of 25 kDa (SNAP-25) in neuromuscular junctions: a new hypothesis to explain persistence of botulinum A poisoning. Can J Physiol 77: 679–688.

Ravichandran E, Gong Y, Al Saleem FH, et al. (2006). An initial assessment of the systemic pharmacokinetics of botulinum toxin. J Pharmacol Exp Ther 318: 1343–1351.

Reller ME, Douce RW, Maslanka SE, et al. (2006). Wound botulism acquired in the Amazonian rain forest of Ecuador. Am J Trop Med Hyg 74: 628–631.

Rosales RL, Arimura K, Takenaga S, et al. (1996). Extrafusal and intrafusal muscle effects in experimental botulinum toxin A injection. Muscle Nerve 19: 488–496.

Rosales RL, Bigalke H, Dressler D (2006). Pharmacology of botulinum toxin: differences between type A preparations. Eur J Neurol 13(Suppl. 1): 2–10.

Rossetto O, Schiavo G, Polverino de Laureto P, et al. (1992). Surface topography of histidine residues of tetanus toxin probed by immobilized metal ion affinity chromatography. Biochem J 285: 9–12.

Rossetto O, Schiavo G, Montecucco C, et al. (1994). SNARE motif and neurotoxins. Nature 372: 415–416.

Ruff RL (2002). More than meets the eye: extraocular muscle is very distinct from extremity skeletal muscle. Muscle Nerve 25: 311–313.

Rummel A, Karnath T, Henke T, et al. (2004a). Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. J Biol Chem 279: 30865–30870.

Rummel A, Mahrhold S, Bigalke H, et al. (2004b). The Hcc-domain of botulinum neurotoxin A and B exhibit a singular ganglioside binding site displaying serotype specific carbohydrate interaction. Mol Microbiol 51: 631–643.

Rummel A, Eichner T, Weil T, et al. (2007). Identification of the protein receptor binding site of botulinum neurotoxins B and G proves the double-receptor concept. Proc Natl Acad Sci U S A 104: 359–364.

Sakaguchi G (1983). *Clostridium botulinum* toxins. Pharmacol Ther 19: 165–194.

Sakaguchi G, Kosaki S, Ohishi S (1984). Structure and function of botulinum toxins. In: JE Alouf (Ed.), Bacterial Protein Toxins. Academic Press, London, pp. 435–443.

Sampaio C, Cost J, Ferreira JJ (2004). Clinical comparison of marketed formulations of botulinum toxin. Movement Disord 19(Suppl. 8): S129–S136.

Sanders DB, Massey EW, Buckley EG (1986). Botulinum toxin for blepharospasm: single fiber EMG studies. Neurology 36: 545–547.

Sandrock CE, Murin S (2001). Clinical predictors of respiratory failure and long-term outcome in black tar heroin-associated wound botulism. Chest 120: 562–566.

Sanes JN, Donoghue JP (2000). Plasticity and primary motor cortex. Annu Rev Neurosci 23: 393–415.

Sanes JR, Lichtman JW (2001). Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2: 791–805.

Santos JI, Swensen P, Glasgow LA (1981). Potentiation of *Clostridium botulinum* toxin by aminoglycoside antibiotics—clinical and laboratory observations. Pediatrics 68: 50–54.

Schantz EJ, Johnson EA (1992). Properties and use of botulinum neurotoxin and other microbial neurotoxins in medicine. Microbiol Rev 56: 80–99.

Schechter R, Peterson B, McGee J, et al. (1999). Clostridium difficile colitis associated with infant botulism: near-fatal case analogous to Hirschsprung's enterocolitis. Clin Infect Dis 29: 367–374.

Schiavo G, Papini E, Genna G, et al. (1990). An intact interchain disulfide bond is required for the neurotoxicity of tetanus toxin. Infect Immun 58: 4136–4141.

Schiavo G, Benfenati F, Poulain B, et al. (1992a). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature 92: 832–835.

Schiavo G, Poulain B, Rosetto O, et al. (1992b). Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release depends on zinc. EMBO J 11: 3577–3583.

Schiavo G, Rosetto O, Catsicas S, et al. (1993a). Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E. J Biol Chem 268: 23784–23787.

Schiavo G, Santucci A, DasGupta BR, et al. (1993b). Botulinum neurotoxins serotype A and serotype E cleave SNAP-25 at distinct COOH terminal peptide bonds. FEBS Lett 335: 99–103.

Schiavo G, Shone CC, Bennett MK, et al. (1995). Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. J Biol Chem 270: 10566–10570.

Schiavo G, Matteoli M, Montecucco C (2000). Neurotoxins affecting neuroexocytosis. Physiol Rev 80: 717–766.

Schneider HJ, Fisk R (1939). Botulism: demonstration of toxin in blood and tissues. J Am Med Assoc 113: 2299–2300.

Schurch B (2006). Botulinum toxin for the management of bladder dysfunction. Drugs 66: 1301–1318.

Scott AB (1989). Clostridial toxins as therapeutic agents. In: LL Simpson (Ed.), Botulinum Toxin and Tetanus Neurotoxin. Academic Press, Inc., San Diego, pp. 399–412.

Scott AB, Suzuki D (1988). Systemic toxicity of botulinum toxin by intramuscular injection in the monkey. Movement Disord 3: 333–335.

Scranton TW, Wwata M, Carlson SS (1993). The SV2 protein of synaptic vesicles is a keratan sulfate proteoglycan. J Neurochem 61: 29–44.

Sebald M, Saimot G (1973). Circulating toxin, an aid in the diagnosis of type B botulism in man. Ann Microbiol (Paris) 124: 61–69.

Sellin LC, Kauffman JA, DasGupta BR (1983a). Comparison of the effects of botulinum neurotoxin types A and E at the rat neuromuscular junction. Med Biol 61: 120–125.

Sellin LC, Thesleff S, DasGupta BR (1983b). Different effects of types A and B botulinum toxin on neurotransmitter release at the rat neuromuscular junction. Acta Physiol Scand 119: 127–133.

Setlow P, Johnson EA (2007). Spores and their significance. In: MP Doyle, LR Beuchat (Eds.), Food Microbiology. Fundamentals and Frontiers. 3rd ed. ASM Press, Washington, DC, pp. 35–67.

Shapiro RL, Hatheway C, Swerdlow DL (1998). Botulism in the United States: a clinical and epidemiologic review. Ann Intern Med 129: 221–228.

Shen J, Ma J, Elsaida GA, et al. (2005). Gene expression of myogenic regulatory factors following intramuscular injection of botulinum A toxin in juvenile rats. Neurosci Lett 381: 207–210.

Shen J, Ma J, Lee C, et al. (2006). How muscles recover from paresis and atrophy after intramuscular injection of botulinum toxin A: study in juvenile rats. J Orthopaed Res 24: 1128–1135.

Shen WPV, Felsing N, Land D, et al. (1994). Development of infant botulism in a 3-year-old female with neuroblastoma following autologous bone-marrow transplantation. Potential use of human botulism immune globulin. Bone Marrow Transplant 13: 345–347.

Sheth RD, Lotz BP, Hecox KE, et al. (1999). Infantile botulism: pitfalls in electrodiagnosis. J Child Neurol 14: 156–158.

Sieburth D, Ch'ng Q, Dybbs M, et al. (2005). Systematic analysis of genes required for synapse structure and function. Nature 436: 510–517.

Simpson LL (2000). Identification of the characteristics that underlie botulinum toxin potency: implications for designing novel drugs. Biochimie 82: 943–953.

Simpson LL, Coffield JA, Bakry N (1994). Inhibition of vacuolar adenosine triphosphatase antagonizes the effects of clostridial neurotoxins but not phospholipase A2 neurotoxins. J Pharmacol Exp Ther 269: 256–262.

Sloop R, Cole A, Escutin E (1997). Human response to botulinum toxin injection: type B compared with type A. Neurology 49: 189–194.

Smith CP, Franks ME, NcNeil BK, et al. (2003). Effect of botulinum toxin A on the autonomic nervous system of the rat lower urinary tract. J Urol 169: 1896–1900.

Smith LDS (1977). Botulism. The Organism, Its Toxins, the Disease Charles C. Thomas, Springfield, Illinois.

Smith LDS, Sugiyama H (1988). Botulism. The Organism, Its Toxins, the Disease. Charles C Thomas, Springfield, Illinois.

Smith TJ, Lou J, Geren I, et al. (2005). Sequence variation with botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect Immun 73: 5450–5457.

Sobel J (2005). Botulism. Clin Infect Dis 41: 1167–1173.

Sobel J, Tucker T, Sulka A, et al. (2004). Foodborne botulism in the United States, 1990–2000. Emerg Infect Dis 10: 1606–1611.

Solomon HM, Johnson EA, Bernard DT, et al. (2001). *Clostridium botulinum* and its toxins. In: FP Downes, K Ito (Eds.), Compendium for the Microbiological Examination of Foods. 4th ed. American Public Health Association, Washington, DC, pp. 317–324.

Son YJ, Thompson WJ (1995). Schwann cell processes guide regeneration of peripheral axons. Neuron 14: 125–132.

St. Louis ME, Peck SH, Bowering D, et al. (1988). Botulism from chopped garlic: delayed recognition of a major outbreak. Ann Intern Med 108: 363–368.

Sugii S, Ohishi I, Sakaguchi G (1977). Correlation between oral toxicity and in vitro stability of *Clostridium botulinum* type-A and type-B toxins of different molecular sizes. Infect Immun 16: 910–914.

Sugiyama H (1979). Animal models for the study of infant botulism. Rev Infect Dis 1: 683–688.

Sugiyama H (1980). *Clostridium botulinum* neurotoxin. Microbiol Rev 44: 419–448.

Sugiyama H, Mills DC (1978). Intraintestinal toxin in infant mice challenged intragastrically with *Clostridium botulinum* spores. Infect Immun 21: 59–63.

Swaminathan S, Eswaramoorthy S (2000). Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. Nat Struct Biol 7: 1751–1759.

Swaminathan S, Eswaramoorthy S (2002). Crystal structure of *Clostridium botulinum* serotype B. In: MF Brin, J Jankovic, M Hallett (Eds.), Scientific and Therapeutic Aspects of Botulinum Toxin. Lippincott Williams & Wilkins, Philadelphia, pp. 29–40.

Swoboda KJ, Jones HR, Jr. (2006). The floppy infant. In: GL Holmes, SL Moshé, HR Jones, Jr. (Eds.), Clinical Neurophysiology of Infancy, Childhood, and Adolescence. Elsevier, Inc., Philadelphia, PA, pp. 505–518.

Tabita K, Sakaguchi S, Kozaki S, et al. (1991). Distinction between *Clostridium botulinum* type A strains associated with foodborne botulism and those with infant botulism in Japan. FEMS Microbiol Lett 79: 251–256.

Tacket CO, Rogawski CO (1989). Botulism. In: LL Simpson (Ed.), Botulinum Neurotoxin and Tetanus Toxin. Academic Press, Inc., San Diego, pp. 351–378.

Tacket CO, Shandera WX, Mann JM, et al. (1984). Equine antitoxin use and other factors that predict outcome in type-A foodborne botulism. Am J Med 76: 794–798.

Tang-Liu DDS, Aoki KR, Dolly JO, et al. (2003). Intramuscular injection of 125I-botulinum neurotoxin complex versus 125I-botulinum-free neurotoxin: time course of tissue distribution. Toxicon 42: 461–469.

Tanzi MG, Gabay MP (2002). Association between honey consumption and infant botulism. Pharmacotherapy 22: 1479–1483.

Tarsy D, Bhattacharya N, Borodic G (2000). Myasthenia gravis after botulinum toxin for Meige syndrome. Movement Disord 15: 736–738.

Terranova W, Breman JG, Locey RP, et al. (1978). Botulism type B: epidemiologic aspects of an extensive outbreak. Am J Epidemiol 108: 150–156.

Thesleff S (1960). Supersensitivity of skeletal muscle produced by botulinum toxin. J Physiol 151: 598–607.

Thesleff S (Ed.) (1976). Motor Innervation of Muscle. Academic Press, London.

Thesleff S (1991). Highlights from 40 years' research on the neuromuscular junction. Physiol Res 40: 381–394.

Thesleff S (1993). Paralysis by botulinum neurotoxins uncovers trophic secretions at the neuromuscular junction. In: BR DasGupta (Ed.), Botulinum and Tetanus Neurotoxins. Plenum Press, New York, pp. 45–52.

Thobois S, Broussolle Toureille L, et al. (2001). Severe dysphagia after botulinum toxin injection for cervical dystonia in multiple system atrophy. Movement Disord 16: 764–765.

Thoenen H (1995). Neurotrophins and neuronal plasticity. Science 270: 593–598.

Tian W-H, Festoff BW, Blot S, et al. (1995). Synaptic transmission blockade increases plasminogen activator activity in mouse skeletal muscle poisoned with botulinum toxin type A. Synapse 20: 20–32.

Tintner R, Gross R, Winzer UF, et al. (2005). Autonomic function after botulinum toxin type A or B: a double-blind, randomized trial. Neurology 65: 765–767.

Tseng-Ong L, Mitchell WG (2005). Catastrophic presentation of infant botulism may obscure or delay diagnosis. Pediatrics 116: 436–438.

Tsuboi M, Furukawa Y, Kuroguchi F, et al. (2002). Botulinum neurotoxin A blocks cholinergic ganglionic neurotransmission in the dog heart. Jpn J Pharmacol 89: 249–254.

Tsukamoto K, Kohda T, Mukamoto M, et al. (2005). Binding of *Clostridium botulinum* type C and D neurotoxins to ganglioside and phospholipid. J Biol Chem 280: 35164–35171.

Tuite PJ, Lang AE (1996). Severe and prolonged dysphagia complicating botulinum toxin A injections for dystonia in Machado-Joseph disease. Neurology 46: 846.

Ugalde I, Christiansen SP, McLoon LK (2005). Botulinum toxin treatment of extraocular muscles in rabbits results in increased myofiber remodeling. Invest Ophthamol Visual Sci 46: 4114–4120.

Ungchusak K, Chunsuttiwat S, Braden C, et al. (2007). The need for global planned mobilization of essential medicine: lessons from a massive Thai botulism outbreak. Bull World Health Org 85: 238–240.

United States Public Health Service (USPHS) (1979). Botulism in the United States, 1899–1977: Handbook for Epidemiologists, Clinicians and Laboratory Workers. Centers for Disease Control, Atlanta, GA.

Uotso N, Nishikawa A, Watanabe T, et al. (2006). Cell internalization and traffic pathway of *Clostridium botulinum* type C neurotoxin into HT-29 cells. Biochim Biophys Acta 1763: 120–128.

van Ermengem EP (1897a). Contribution à l'étude des Intoxications Alimentaires. Recherches sur des accidents à caractéres botuliniques par du jammbon. Travaux du Laboratorie d'Hygiène et de Bactériologie, Université de Gan, J. van In & Cie Lierre/H. Engelcke, Gand, pp. 5–269. Extrat des Archives de Pharmacodynamie, Vol III.

van Ermengem EP (1897b). Ueber einen neuen anaëroben Bacilus und seine Beziehungen zum Botulismus. Zeitschrift für Hygiene und Infektionskrankheitein (Leipzig) 26: 1056.

van Ermengem EP (1979). Classics in infectious disease. A new anaerobic bacillus and its relation to botulism. Rev Infect Dis 1: 701–719.

van Heyningen S (1974). Gangliosides as membrane receptors for tetanus toxin, cholera toxin and serotonin. Nature 249: 415–417.

Varma JK, Katsitadze G, Moiscrafishvili M, et al. (2004). Signs and symptoms predictive of death in patients with foodborne botulism—Republic of Georgia, 1980–2002. Clin Infect Dis 39: 357–362.

Verderio C, Rossetto O, Grumelli C, et al. (2006). Entering neurons: botulinum toxins and synaptic vesicle recycling. EMBP Rep 7: 995–999.

Villar RG, Shapiro RL, Busto S, et al. (1999). Outbreak of type A botulism and development of a botulism surveillance and antitoxin release program in Argentina. JAMA 281: 1334–1338.

Wang YC, Burr DH, Korthals GJ, et al. (1984). Acute toxicity of aminoglycoside antibiotics as an aid in detecting botulism. Appl Environ Microbiol 48: 951–955.

Watson AHD (2006). What can studying musicians tell us about motor control of the hand? J Anatomy 208: 527–542.

Weber JT, Goodpasture HC, Alexander H, et al. (1993). Wound botulism in a patient with a tooth abscess—case report and review. Clin Infect Dis 16: 635–639.

Wenzel R, Jones D, Borrego JA (2007). Comparing two botulinum toxin type A formulations using manufacturers' product summaries. J Clin Pharm Ther 32: 387–402.

Werner SB, Passarro D, McGee J, et al. (2000). Wound botulism in California, 1951–1998: recent epidemic in heroin injectors. Clin Infect Dis 31: 1018–1024.

Wilcox PG, Morrison NJ, Pardy RL (1990). Recovery of ventilatory and upper airway muscles and excercise performance after type A botulism. Chest 98: 620–626.

Woodruff BA, Griffin PM, McCroskey LM, et al. (1992). Clinical and laboratory comparison of botulism from types A, B, and E in the United States, 1975–1988. J Infect Dis 166: 1281–1286.

Wright GP (1955). The neurotoxins of *Clostridium botulinum* and Clostridium tetani. Pharmacol Rev 7: 413–465.

Yee WC, Pestronk A (1987). Mechanisms of postsynaptic plasticity: remodeling of the junctional acetylcholine receptor cluster induced by motor nerve terminal outgrowth. J Neurosci 7: 2019–2024.

Yoneda S, Shimazawa M, Kato M, et al. (2005). Comparison of the therapeutic indexes of different molecular forms of botulinum toxin type A. Eur J Pharmacol 508: 223–229.

Yule AM, Barker IK, Austin JW, et al. (2006). Toxicity of *Clostridium botulinum* type E neurotoxin to Great Lakes fish: implications for infant botulism. J Wildlife Dis 42: 479–493.