12 Botulism and Tetanus

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Abstract
Botulism and tetanus are two severe neurological diseases in man and animals. While botulism is characterized by a descendant flaccid paralysis, tetanus consists in spastic paralysis. In the severe forms of both diseases, death occurs by respiratory distress. Botulism and tetanus are caused by neurotoxins, botulinum neurotoxin (BoNT), and tetanus toxin (TeNT), respectively, which are produced by anaerobic sporulating bacteria, *Clostridium botulinum* and *Clostridium tetani*, respectively. In contrast to *C. tetani*, which forms a homogeneous bacterial species, BoNT-producing Clostridia are divided into several bacterial species and groups. These Clostridia are widely distributed in the environment, including food notably for *C. botulinum*, where they can survive during long periods in the sporulating forms. BoNTs and TeNT share a common structural organization consisting in a light (L) chain (about 50 kDa) linked by a disulfide bridge to the heavy (H) chain (about 100 kDa). Only a unique TeNT is known, while BoNTs encompass seven toxinotypes (A to G, BoNT/A, B, and E mainly involved in human botulism, and BoNT/C and D mainly responsible for animal botulism), which are subdivided into several subtypes according to amino acid sequence variations. H chain, which contains a C-terminal receptor-binding domain and an N-terminal translocation domain, delivers the L chain into target neurons. BoNTs target the motoneuron endings or neuromuscular junctions, and TeNT is transported to central inhibitory interneurons through a retrograde axonal pathway along motoneurons. Both BoNT and TeNT block the release of neurotransmitter by an L chain-mediated proteolytic cleavage of SNARE proteins (synaptobrevin, SNAP25, or syntaxin) which are involved in the neuroexocytosis process. Blockage of acetylcholine release at the neuromuscular junctions by BoNTs induces a flaccid paralysis, whereas TeNT-dependent inhibition of glycine or

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GABA exocytosis in inhibitory interneurons results in spastic paralysis. Botulism is mainly acquired by ingestion of preformed BoNT in food, but it may also occur subsequently to intestinal or wound colonization by *C. botulinum*. Tetanus essentially results from a wound contamination by *C. tetani*. BoNT/A is the most potent toxin with a long activity duration in neurons, and it is also a therapeutic agent widely used to treat hypercholinergic diseases including localized muscle spasticity, dystonia, autonomic dysfunctions (hyperhidrosis, hypersalivation), and also pain such as migraine headaches.

Introduction

Among the large number of *Clostridium* species, which are Gram-positive, spore-forming bacteria from the environment, some of them produce potent neurotoxins causing neurological disorders in human and animals. *Clostridium botulinum* is defined as a *Clostridium* which produces one or several neurotoxins inducing flaccid paralysis, termed botulinum neurotoxins (BoNT), which are responsible for a rare but often severe disease with a high lethality rate, the botulism. In contrast, *Clostridium tetani* synthesizes a related neurotoxin called tetanus toxin (TeNT), which causes a dramatic spastic paralysis. Both, BoNT and TeNT exert the neurotoxicity by blocking the release of neurotransmitters. While BoNTs inhibit the evoked release of acetylcholine at the neuromuscular junctions causing a flaccid paralysis, TeNT impairs the neuroexocytosis at the central inhibitory interneurons resulting in the prevention of the inhibitory pathways and thus in a spastic paralysis. Albeit BoNT is the most potent toxic compound, it is widely used as therapeutic tool in the treatment of various cholinergic disorders such as dystonia.

Clostridia Producing Botulinum Neurotoxins

Clostridia capable of producing BoNT display heterogeneous bacteriological characters and are divided into several species and groups. The taxonomic position of the *C. botulinum* species was originally based on only one phenotype, the production of a BoNT, and nontoxic variant strains, although genetically related to *C. botulinum*, were assigned to different species such as *Clostridium sporogenes* and *Clostridium subterminale*. It appeared soon that BoNTs are seven different protein neurotoxins which are immunologically distinct and are termed with letters from A to G. More recently, sequence analysis permits to distinguish subtypes within BoNT types (see below and ❯ *Table 12.1*). However, all of them cause the typical flaccid paralysis of botulism in experimental animals, similar to that observed in human patients suffering from botulism. Later on, physiological differences between *C. botulinum* strains were identified, but the production of the different BoNT types does not necessarily correlate with the *C. botulinum* strain phenotypes. The species was divided into four physiological groups (❯ *Table 12.1*) (Smith 1975).

- Group I: *C. botulinum* A and proteolytic strains of *C. botulinum* B and F
- Group II: *C. botulinum* E and glucidolytic strains of *C. botulinum* B and F
- Group III: *C. botulinum* C and D
- Group IV: *C. botulinum* G (or *C. argentinense*).

The latter group which also includes nontoxic strains previously identified as *C. subterminale* and *C. hastiforme* is metabolically distinct from the other groups and has been assigned to a different species called *C. argentinense* (Suen et al. 1988).

The taxonomic position of *C. botulinum* became more ambiguous since it has been found that BoNTs can be produced by *Clostridium* strains clearly distinct from already defined *C. botulinum*, and biochemically and genetically related to different species such as *C. butyricum* and *C. baratii* (Hall et al. 1985; McCroskey et al. 1986, 1991). In each group, all the strains regardless of toxin types are closely related according to their phenotypic properties, DNA/DNA homology and 16s rRNA analysis. All the BoNT-producing *Clostridia* have a G+C content between 26% and 28% (Cato et al. 1986) and belong to the group I of 23S rRNA homology (Johnson and Francis 1975), which contains the type species *C. butyricum*. 16S rRNA comparison corroborated that the group I strains are homogeneous and form a distinct phylogenetic branch. It has been proposed that the genus *Clostridium* should be retained only for this taxon (Hippe et al. 1992; Lawson et al. 1993).

Morphological Aspects

BoNT-producing *Clostridium* is usually straight to slightly curved rods, 0.6–2 wide and 2–22 µm long. They are usually motile and peritrichous, except *C. baratii* which is nonmotile. Spores are oval and subterminal and swell the cell. Type G strains sporulate poorly, and *C.butyricum* spores are central to subterminal and usually do not swell the cell (Cato et al. 1986).

These bacteria grow well in usual anaerobic liquid media with production of gas. Surface colonies can be grown on blood agar plates, incubated under anaerobic conditions; they can be circular (1–6 mm in diameter) or irregular, with a scalloped or lobate margin, translucent to semiopaque, gray-white. They are surrounded by a narrow zone of partial hemolysis, while those of *C. butyricum* are nonhemolytic.

Physiological Properties

Group I (*C. botulinum* A, and Proteolytic Stains of Types B and F)

The strains of this group are characterized by a proteolytic and lipase activity and by the non or weak acidification of carbohydrates (❯ *Table 12.1*). Glucose is acidified weakly. The cell wall contains glucose as sugar. The optimal temperature of growth is 37 °C. The strains of group I usually do not grow and do not form toxin at 10 °C or below. But large inoculum and long incubation period might result in sufficient growth and toxin

Table 12.1

Groups of botulinum neurotoxin-producing Clostridia, produced toxins, and main properties.[a]According to (Peck 2006, 2009; Peck et al. 2011)

Neurotoxin-producing *Clostridium*	Group I	Group II	Group III	Group IV *C. argentinense*	C. butyricum	*C. Baratii*
Toxin type	A proteolytic B, F	E nonproteolytic B, F	C, D	G	E	F
Subtype	A1, A2, A3, A4, A5 B1, B2, B3 bivalent B (Ba, Bf, Ab) F1, F2, F3, F4, F5	E1, E2, E3, E6, E7, E8 nonproteolytic B F6	C, D, C/D, D/C	G	E4, E5	F7
Proteolysis	+	–		+	–	
Lipase production	+			–		
Growth temperature[a]						
Optimum	30–40 °C	25–37 °C	37–40 °C	30–37 °C	30–37 °C	30–45 °C
Minimum	10–12 °C	2.5–3 °C	15 °C		10 °C	
Minimum pH for growth[a]	4.6	5.0	5.1		4.8	
Minimum water activity for growth[a]	0.96	0.97	0.97	0.94		
NaCl concentration preventing growth[a]	10%	5%		6.5%		
Spore thermoresistance[a]	$D_{121°C}$=0.21 min	$D_{82.2°C}$=2.4–231 min	$D_{104°C}$=0.1–0.9 min	$D_{104°C}$=0.8–1.12 min	$D_{100°C}$<0.1 min	
Botulism	Human		Animal		Human, animal	
Related non-botulinum toxin-producing *Clostridium*	*C. sporogenes*		*C. novyi* *C. hemolyticum*	*C. subterminale* *C. proteolyticus* *C. schimacherense*	*C. butyricum*	*C. baratii*

formation at low temperature such as 12 °C within 3–4 weeks (Peck 2006). Growth of group I strains is inhibited by acidic pH (<4.6) and NaCl concentrations ≥10%. The thermoresistance of the spores is usually high (above 120 °C) but varies according to the strains and also of the heating buffer and recovery culture medium (Hinderink et al. 2009; Peck 2009; Smith 1975). Botulism was a major problem at the beginning of the canning industry, and the thermoresistance of *C. botulinum* strains has been extensively investigated in order to recommend safety rules of heat treatment. According to the most heat-resistant strains which require a heating at 121 °C for 0.21 min to reduce the number of viable spores by a factor of 10^{12} (12-D or *D* value), heating at 121 °C for 3 min has been recommended for commercial and low-acid canned foods (Peck 2009).

Group II (*C. botulinum* E and Glucidolytic Strains of Types B and F)

Group II strains acidify various carbohydrate substrates (amygdalin, dextrin, fructose, galactose, glucose, glycogen, maltose, ribose, sorbitol, sucrose, and trehalose but not lactose, mannitol, melibiose, and salicin) and produce mainly butyric and acetic acids from trypticase-yeast extract-glucose broth. They hydrolyze gelatin, but they are nonproteolytic (Cato et al. 1986). The strains of this group contain glucose and galactose as the cell wall sugars. They have a lower optimal temperature of growth (around 25–30 °C), and they can growth and produce toxins at very low temperature (*Table 12.1*). Growth and toxin production have been reported to occur at temperature as low as 2.5–3.0 °C in 5–7 weeks. However, most of the strains grow poorly below 5 °C (Peck 2006, 2009; Peck et al. 2011). Spores are only moderately resistant to heat, not withstanding 10 min at 90 °C (Smith 1992). The highest temperature resistance of spores in phosphate buffer was reported to be 82.2 °C. Spores of group II strains are lysozyme-dependent for their germination. Lysozyme diffuses in the spore coat and induces the peptidoglycan hydrolysis in the cortex permitting the first step of germination. Lysozyme increases the spore recovery after heat treatment and thus increases heat resistance. In the presence of lysozyme, the resistance time to heat treatment at 82.2 °C is 100-fold increased to 231 min instead of 2.31 min without lysozyme (Peck 2009). Moreover, the presence of high protein and fat content in raw material may also confer a higher heat resistance of spores (Lindstrom et al. 2006a). The BoNT produced by group II strains is mainly type E and is not fully activated by endogenous protease. Therefore, trypsinization enhances the toxicity of

these cultures. In addition, strains from group II are most sensitive than proteolytic *C. botulinum* strains to NaCl (inhibitory concentration 5% and above) and pH (no growth below pH 5).

Group III (*C. botulinum* C and D)

The organisms of this group are nonproteolytic or very slightly. They ferment glucose, glycerol, inositol, ribose, and xylose. Their cell wall do not contain sugar or only traces of glucose (Smith 1975). The main end products of metabolism are acetate and butyrate. They grow at higher temperature than the other *C. botulinum* strains, optimum temperature for growth being 37–40 °C, with most strains growing well at 45 °C, and minimal temperature for growth being at approximately 15 °C (Cato et al. 1986).

This group is heterogeneous according to some biochemical properties (fermentation of several sugars, production of indole and hydrogen sulfide). A subdivision into four subgroups have been proposed (Oguma et al. 1986).

C. argentinense (Also Referred as Group IV)

The strains of this species are proteolytic, hydrolyze gelatin and do not ferment any of the usual carbohydrates. Contrarily to the other strains of the *C. botulinum* groups, *C. argentinense* strains do not produce a lipase. They produce acetate, butyrate, isobutyrate, isovalerate, and phenyl acetate as end products of metabolism. The optimum temperature of growth is 30–37 °C. The spores have an intermediate heat resistance like group III *C. botulinum* strains. Phenotypic differences have been observed between toxic and nontoxic *C. argentinense* strains by using cellular fatty analysis and multilocus enzyme electrophoresis (Altwegg and Hatheway 1988; Ghanem et al. 1991). *C. argentinense* strains are phenotypically and genetically closely related to *C. subterminale* strains which are non-neurotoxigenic strains.

C. butyricum

Toxigenic *C. butyricum* strains are phenotypically and genetically related to the typical strains of this species. They are nonproteolytic, they do not hydrolyze gelatin and acidify strongly various carbohydrates including glucose, cellobiose, fructose, galactose, glycogen, lactose, maltose, mannose, melibiose, raffinose, ribose, salicin, starch, sucrose, trehalose, xylose, and pectin. They do not produce lipase neither lecithinase (Smith 1992). They are mesophilic bacteria with optimum growth temperature of 30–37 °C and minimum temperature for growth and toxin production of 10–12 °C. The lowest pH for growth and toxin synthesis was found to be 4.8 (Anniballi et al. 2002). Spore of neurotoxigenic *C. butyricum* are less resistant to heat ($D_{100°C} < 0.1$ min) than the nontoxigenic strains ($D_{100°C} = 4.7$ min) (Peck 2009).

C. butyricum strains producing type E neurotoxin have been isolated from infant botulism and young people in Italy (Aureli et al. 1986; Fenicia et al. 1999; Koepke et al. 2008; McCroskey et al. 1986), as well as from botulism associated with consumption of fermented soybean in China (Fu and Wang 2008; Meng et al. 1997, 1999; Wang et al. 2000) and also in India and Japan (Abe et al. 2008; Chaudhry et al. 1998). Based on toxin gene sequence, toxigenic *C. butyricum* strains from Italy and China are divided in two distinct subtypes, termed E4 and E5, respectively, indicating an independent evolution of *bont/E* gene after transfer in *C. butyricum* (Hill et al. 2007).

C. baratii

Toxigenic and nontoxigenic *C. baratii* strains display similar morphological and biochemical characteristics. *C. baratii* is phenotypically closely related to *C. perfringens*. Both species are nonmotile and appear as thick, straight rods (0.5–1.9 × 1.6–15 μm). Strains sporulate poorly in usual culture medium. They produce a lecithinase but not a lipase. *C. baratii* is readily differentiated from *C. perfringens* by its lack of hydrolysis of gelatin (Cato et al. 1986). Optimum growth temperature is around 30–45 °C and minimum growth temperature at 10–15 °C. *C. baratii* acidifies culture medium containing various carbohydrates.

Neurotoxigenic *C. baratii* strains are genetically related to nontoxigenic counterparts. Botulinum neurotoxin type F gene sequences from *C. baratii* form a different cluster of those from *C. botulinum* type F (Hill et al. 2007).

Neurotoxigenic *C. baratii* type F is involved in a limited number of human cases, mainly by intestinal colonization, in infants or adults (Barash et al. 2005; Gupta et al. 2005; Hall et al. 1985; Harvey et al. 2002; Koepke et al. 2008; McCroskey et al. 1986, 1991).

Sporulation and Germination

C. botulinum, as other Clostridia, forms spores which permit to survive for long periods in the environment under unfavorable conditions such as exposition to oxygen, dryness, high temperature, and deprivation of nutriments. Spores from *C. botulinum* of group I are among the most heat-resistant spores, and this constitutes an important problem in food industry. Germination is triggered by specific molecules, called germinants, and can occur in the presence of oxygen. However, further growth of *C. botulinum* vegetative cells requires a strict anaerobic environment.

In *C. botulinum* strain Hall, 111 genes have been assigned to germination/sporulation (Sebaihia et al. 2007). The gene cascade involved in sporulation has been well defined in *Bacillus subtilis*. In contrast, sporulation steps are not yet completely known in Clostridia. Spo0A is a key player of sporulation in *B. subtilis* as well as in *Clostridia*. Spo0A is a transcriptional regulator: when activated by phosphorylation at an aspartate residue, it binds to specific DNA motifs by its C-terminal part and thus activates and represses genes, thereby controlling the onset of sporulation. Spo0A is conserved in *C. botulinum*. However, the genes encoding the histidine kinases (kinA-E) and the phosphorelay system Spo0F-Spo0B responsible for Spo0A phosphorylation in response to nutrient limitation in *Bacillus* are not found in *C. botulinum* and other known clostridial genomes. In *B. subtilis*, the phosphorelay kinases of Spo0A are orphan kinases. *C. botulinum* A strain Hall encodes

five orphan kinases, which might potentially phosphorylate Spo0A, *C. acetobutylicum* contains six orphan kinase genes, *C. perfringens* 7, and *C. tetani* 3 (Paredes et al. 2005; Sebaihia et al. 2007). The CBO1120 orphan sensor histidine kinase was identified as being capable of phosphorylating Spo0A (Wörner et al. 2006). The environmental factors controlling sporulation in Clostridia are thereby different from those involved in *Bacillus*. The control of intracellular pH seems to be important to initiate sporulation of Clostridia. The organism prevents excessive acidification due to organic acids produced during the fermentation process. Thus, the intracellular pH in anaerobic bacteria is fluctuant and is generally one unit higher than the external pH. The terminal fermentation end products are exported through the cytoplasmic membrane in an undissociated form at low pH. But at higher pH, they dissociate into the cytoplasm decreasing the intracellular pH (Dürre 2005). In *C. acetobutylicum*, it has been suggested that phosphorylation of Spo0A might use butyryl phosphate as phosphate donor through a butyrate kinase (Paredes et al. 2005). The butyrate kinase gene is conserved in all *C. botulinum* genomes. Another difference to *B. subtilis* sporulation is that Clostridia require sufficient levels of carbon source and ATP.

Most of the genes downstream of Spo0A from *B. subtilis* including sporulation specific sigma factors such as the earliest sporulation sigma factor σ^H as well as σ^E and σ^K in the mother cell and σ^F and σ^G in the forespore are conserved in genomes of *C. botulinum* and other Clostridia (Paredes et al. 2005; Sebaihia et al. 2007).

Germination is the first step in the development of dormant spores into exponentially growing bacteria. This process is triggered by interaction of germinant molecules such as amino acids with specific receptors in the inner spore membrane. The most effective germinants consist in L-alanine or L-alanine/L-lactate for proteolytic *C. botulinum* strains and in L-lactate in combination with L-alanine, L-serine, or L-cysteine at neutral pH in 100 mM potassium phosphate for nonproteolytic *C. botulinum* strains. However, strain variations are observed (Peck 2009). The resulting loss of ions (K^+, Na^+, H^+, Ca^{++}) and dipicolinic acid as well as peptidoglycan hydrolysis in spore cortex leads to decreased resistance of spore coats and subsequent hydration and swelling of the spore core, thus permitting metabolism activity. Three tricistronic germinant receptor gene operons (*gerX/A, gerX/B, gerX/C*) and one orphan germinant receptor gene homologue have been identified in *C. botulinum* A strain Hall genome. One of the three operon is flanked by two additional *gerX/B* gene (Sebaihia et al. 2007). GerAA and GerAB are transmembrane proteins of the spore inner membrane, GerAB is a single component membrane receptor, and GerAC is predicted to be a lipoprotein. The three proteins from each operon are required to form a functional germinant receptor. The three operons and orphan gene of germinant receptors of strain Hall are conserved in the other proteolytic *C. botulinum* genomes including *C. botulinum* type A strains, *C. botulinum* type B Okra, and *C. botulinum* type L Langeland. But *C. botulinum* type B Okra and *C. botulinum* type L Langeland strains contain an additional tricistronic germinant receptor operon, possibly permitting to these strains to respond to different germinants. In proteolytic *C. botulinum* B NCTC7273 and *C. sporogenes*, the tricistronic operon (*gerA/A, gerA/B, gerA/C*) has been characterized to respond to L-alanine (Broussolle et al. 2002). In contrast, nonproteolytic *C. botulinum* genomes (C. *botulinum* type B Eklund17B and *C. botulinum* type E Alaska E43) only show one tricistronic germinant receptor operon which is related to that found in proteolytic *C. botulinum* strains Okra (type B) and Langeland (type F) but not to those found in the proteolytic type A strain genomes (Peck 2009). The *gerA* operon from *C. botulinum* B is highly conserved in *C. botulinum* A strain Hall (89–99% amino acid identity of encoded proteins), and the *gerA/A* and *gerA/B* genes from *C. sporogenes* are highly related to another *C. botulinum* A *ger* cluster. *ger* clusters are also present in multicopies in *C. acetobutylicum* (three copies), *C. tetani* (four copies), whereas *C. perfringens* contains only one bicistronic *ger* operon and *C. difficile* lacks genes similar to those from *Bacillus* or other Clostridia, indicating that the initial step of germination in *C. difficile* is different from that of other Clostridia (Sebaihia et al. 2006, 2007).

Clostridium tetani

Tetanus neurotoxin (TeNT) is produced by a uniform group of bacteria belonging to the *Clostridium tetani* species.

Morphological and Cultural Characteristics

The cells of *C. tetani* are usually 0.3–0.6 μm in width and may vary considerably in length between 3 and 12 μm. They are Gram-positive in young cultures, but they lose the Gram coloration upon prolonged incubations. *C. tetani* is usually highly motile by peritrichous flagella, this property being responsible of their swarming growth on agar medium. However, some strains are nonmotile and nonflagellated. These bacteria form spores, which appear as translucent terminal enlargements which give the typical appearance of drumsticks. The sporulation rate is variable according to the strains. At pH 7 or above and at temperature near 37 °C, the sporulation starts within 24 h of culture and continues for 4–12 days or more. Sporulation does not occur above 41 °C, and it is slow at pH < 6. The sporulation process depends on the nature of the culture medium (Bytchenko 1981). Spores generally survive moderate heating (75–80 °C for 10 min) but usually are destroyed within 1 h at 100 °C.

Germination of *C. tetani* spores occurs both under anaerobic and aerobic conditions, but the outgrowth of *C. tetani* which follows spore germination is strictly dependent upon a low oxidation-reduction potential (Smith 1975). In fact, *C. tetani* is an anaerobic bacterium and forms colonies on the surface of agar medium only under anaerobiosis. Motile strains swarm over the entire surface of the agar leading to a transparent film. Discrete colonies (2–5 mm) can be obtained with media containing 3–4% agar. On blood agar, colonies are slightly raised, semitranslucent, gray, with an irregular margin and

surrounded by a narrow zone of hemolysis. *C. tetani* grows fairly well on the usual media containing peptones or tissue extracts.

Most of the usual biochemical tests used for *Clostridium* identification are negative, as no carbohydrates are acidified, and there is no proteolysis nor production of lipase and lecithinase. Gelatin is liquefied slowly (2–7 days), the peptone used in the basal medium is of considerable importance to evaluate the ability of *C. tetani* to liquefy the gelatin. H_2S and indole are usually produced (Smith 1975).

C. tetani strains are sensitive to penicillin and metronidazole. However, intravenous administration of penicillin can be inefficient due to an impaired transport of the antibiotic in the wound, and wound debridement is a required step in the treatment of tetanus (Campbell et al. 2009).

Genetic Characteristics

The G+C content of *C. tetani* is 25–26% (Cato et al. 1986). This species has been classified in the *Clostridium* group II by using 23S rRNA homology (Johnson and Francis 1975). The *Clostridium* genus encompasses more than 100 species which display a wide range of phenotypes and genotypes (Hippe et al. 1992). Recently, phylogenetic analysis using 16S rRNA comparison indicate that the *Clostridium* genus should be restricted to the homology group I, as defined by Johnson and Francis (Johnson and Francis 1975; Lawson et al. 1993). According to these data, *C. tetani* should be classified in a different genus. However, a study based on restriction maps of 16S rRNA showed that *C. tetani* belongs to the same cluster as *C. perfringens, C. sporogenes*, and *C. botulinum* C and G, which are members of the homology group I of Johnson and Francis (Gurtler et al. 1991).

Ten neurotoxigenic and three nontoxigenic *C. tetani* strains studied by Nakamura et al. were homogeneous in DNA/DNA hybridization (85–93% similarity) (Nakamura et al. 1979). *C. tetani* is similar culturally and biochemically to *C. cochlearium* and *C. tetanomorphum*, but it can be distinguished from the two latter species by DNA comparison (Nakamura et al. 1979; Wilde et al. 1989). *C. cochlearium* and *C. tetanomorphum* are nontoxic, and they are difficult to differentiate from the nontoxic *C. tetani* strains according to the bacteriological characteristics, as they do not, or weakly, liquefy gelatin. Contrary to *C. tetani*, *C. tetanomorphum* acidifies glucose and maltose (Cato et al. 1986).

The complete genome sequence of a toxigenic *C. tetani* strain has been determined (Brüggemann et al. 2003). It consists of a 2,799,250 bp chromosome containing 2,372 putative genes and of a 74,082 bp plasmid containing 61 genes. *C. tetani* possesses many genes for peptidases, amino acid, and lipid degradation, whereas genes for sugar utilization are lacking. It contains numerous transport-related genes, in particular 35 genes, for sodium ion-dependent systems indicate that Na^+ gradient is a major driving force in membrane transport. The TeNT encoding gene and seven putative regulatory genes are localized on the plasmid, whereas the tetanolysin (a hemolysin) gene and putative adhesin genes are located on the chromosome. Many genes encoding for putative adhesins have been identified: 2 fibronectin-binding proteins, 11 related surface-layer proteins (SLP), 19 homologues to a *Clostridium difficile* adhesin, and 2 proteins with multiple leucine-rich repeat domains similar to the *Listeria monocytogenes* internalin A (Brüggemann et al. 2003). SLP-A shows important size variation from strain to strain (Qazi et al. 2007). Genome analysis has revealed different sets of surface-associated protein genes in pathogenic Clostridia, which probably mediate their interactions with the environment or host and account why some clostridial species such as *C. tetani* and to a lesser extent some *C. botulinum* strains of group I can develop in wound (Bruggemann and Gottschalk 2008).

Ecology of Neurotoxin-Producing *Clostridia*

C. tetani and BoNT-producing *Clostridia*, as the other *Clostridia*, are largely present in the environment mainly in the form of spores, which are able to survive for very long periods under extreme conditions (including heat, dryness, radiations, chemicals, and oxygen). Spore germination and cell division occur only under anaerobic conditions and in the presence of the appropriate nutritional requirements. This restricts the habitat of the *Clostridium* to anaerobic areas or areas with low oxygen tension and containing sufficient amounts of organic materials. Based on their physiological properties (tolerance or extreme sensitivity to oxygen, requirement of particular pH, temperature, and substrate for growth or spore germination), the distribution of the different *Clostridium* species in nature is not uniform (Hippe et al. 1992). Saccharolytic *Clostridia* such as *C. butyricum* are able to grow on carbohydrates and are mainly found in decomposing vegetables and fruits. Proteolytic and gelatinolytic *Clostridium* such as *C. botulinum* and *C. tetani* are preferentially associated with animal cadavers and soils or sediments rich in organic material. They can also be found in the digestive tract of healthy humans and animals, and, after death, they participate to the cadaver decomposition.

In the Environment

C. tetani

C. tetani is an ubiquitous organism which is commonly found in soil samples in all parts of the world. The frequency of its isolation is variable according to the different investigations. Surveys in Japan, Canada, Brazil, and the United States have yielded 30–42% positive samples (Smith and Williams 1984). Several factors influence the different frequencies of *C. tetani* isolation from soil sample, including pH, temperature, moisture, amount, and type of organic materials. Thus, germination and multiplication of *C. tetani* have been observed preferentially in neutral or alkaline soil, with temperatures >20 °C and humidity reaching 15% (Smith 1975).

Geographical distribution of *C. tetani* shows a higher presence in southern regions, and accordingly the incidence of tetanus is higher in warmer countries (West and Central Africa,

Southeast Asia, India, Pacific Islands, and south of the United States) than in the cooler parts of the world (Canada, Norway, England, Finland, Sweden) (Smith 1975). This bacterium can be found in the intestine of animals, but it does not represent a significant part of the normal digestive flora. Different surfaces and objects contaminated with soil particles, dust or feces may contain *C. tetani*. Toxigenic strains have also been isolated within hospitals from catgut, cotton wool, dust and air samples, human skin, and wounds (Bytchenko 1981).

BoNT-Producing *Clostridium*

C. botulinum is widespread in soils as well in lake and sea sediments in most parts of the world. However, the different toxinotypes of *C. botulinum* are not equally distributed, some of them being restricted to particular ecological areas. The factors responsible for the geographical distribution of the different toxinotypes are still poorly understood. In general, toxinotypes A, B, E, F, and G seem to have their principal habitat in soil and sea and freshwater sediments. The toxinotypes A and B occur more frequently in soil samples, and the regional distribution of these two toxinotypes are different. Toxinotype E is more predominant in sea or lake sediments and fish than in soil. The toxinotypes C and D appear to be obligate parasites of birds and of other animals. Cadavers of animals or birds died of botulism or from healthy carriers are the main source of these organisms. They are seldom encountered in soil samples, except in the areas where the incidence of animal botulism is high (Smith 1975). *C. botulinum* is not usually found in digestive tract of healthy humans, but it can be found in that of animals, particularly *C. botulinum* C and D, particularly in regions where botulism is frequent.

C. botulinum A and B

Numerous investigations have surveyed the prevalence of *C. botulinum*, particularly in the USA (reviewed in (Dodds 1993b; Hauschild 1989)). Types A and B were generally found from neutral to alkaline soil samples poor in organic matter and much more rarely from aquatic sediment. *C. botulinum* type A is predominant in the western part of the United States (west of the Missouri and Mississippi rivers), in soil that was neutral to alkaline (average pH 7.5) with a lower than average organic content. In contrast, type B prevails largely in the eastern part of the United States. This toxinotype was recovered in slightly more acidic soil samples (average pH 6.25) with a higher level of organic matter and mainly from cultivated soils (pasture, fields) (Smith 1978). Other investigations reported the prevalence of type B in cultivated samples, but noted that this type is rare in soils contaminated with manure or animal feces. The fertilization was not considered as a significant factor responsible of the *C. botulinum* type B frequent presence in cultivated soils (Dodds 1993b).

The incidence of *C. botulinum* A and B is very low from aquatic (sea or freshwater) sediments and soil samples in the northern part of America (Alaska, Canada). Fewer investigations have been performed in Central and South America. Surveys from soil samples in Argentina, Brazil, and Paraguay demonstrated the presence of *C. botulinum* A and B, with a prevalence of type A. *C. botulinum* type B is the most common type from soil and sediment samples in Central and South Europe (Great Britain, Ireland, Netherlands, France, Switzerland, and Italy) and from soil samples in Denmark and Ireland. In Great Britain and Ireland, *C. botulinum* is much more frequent in lake and loch sediments than in soil samples, and type B is predominant. *C. botulinum* A and B are also largely diffused in Asia including ex USRR, China, and Taiwan. Contamination level and type detection vary from region to region and depend from several factors: temperature, moisture and organic substance content, and other unidentified factors. Soil from south regions with warm climate or subtropical and tropical areas showed the higher incidence of *C. botulinum* A and B. Even higher populated regions are much more contaminated than desertic areas. Distribution of types A and B varies locally and does not correspond to large geographical areas like in Northern America.

Few reports concern the incidence of *C. botulinum* in Africa. In Kenya, the soil samples examined seemed to be heavily contaminated, with a predominance of type A. In contrast, the contamination in South Africa seemed to be low, *C. botulinum* B has been identified in 3 soil samples out of 102 (Knock 1952).

In Australia and New Zealand, *C. botulinum* A and B have been evidenced, but their presence in the environment is low reflecting a low incidence of human botulism in these areas.

C. botulinum E

The distribution of type E is more regional than of other types. *C. botulinum* E is mainly found in north areas of the Northern Hemisphere: north part of America (Alaska, Canada, Northern United States), North Europe (Island, Greenland, Denmark, Norway, Sweden, coast of Baltic Sea), and North Asia (north part of ex USRR, Caspian Sea, North Japan Islands: Hokkaido, north part of Honshu) from soil that is in contact with water (Smith 1992). The ability of *C. botulinum* E to grow a very low temperature reflects its prevalence in areas with cold temperature. Its frequency decreases considerably in south areas with warm weather. This type is mainly found in aquatic environment (sediment, soil from the shore of lake, sea, and river). Other factors such as organic matter content, salinity, and other unidentified factors influence the distribution of *C. botulinum* E, which varies greatly from one region to other. Thus, this organism is commonly found in the Great Lakes in the north of the United States but more frequently in Lake Michigan than in any of the other lakes, and in Lake Michigan, it was found more frequently in Green Bay than elsewhere. *C. botulinum* E is also very common in the Pacific Northwest. Sediment samples of Lake Washington near Seattle contained 18–25 C. *botulinum* E per g. These particular areas seem to represent the principal habitat of *C. botulinum* E, where it can grow and multiply (Smith 1975). A particular high prevalence of *C. botulinum* E occurs in Baltic Sea. These bacteria are most frequent and spore counting more abundant in sea than in freshwater sediment samples. Low oxygen content, low salinity, presence of biomass, and depth seem to be more important factors than temperature controlling the propagation of *C. botulinum* E in aquatic environment

(Hielm et al. 1998c). The contamination of raw fish in Finland ranges from 10% to 40% depending on fish species (Hielm et al. 1996; Hyytia et al. 1998). *C. botulinum* E was also detected in fish roe (4–14% of the samples), vacuum-packed (5%), air-packed (3%) fishery products, and vacuum-packed hot-smoked whitefish (10%) (Hyytia et al. 1998). Investigations in Finnish trout farms evidenced *C. botulinum* E in farm sediment samples (68%), fish intestinal contents (15%), and fish skin samples (5%°) (Hielm et al. 1998a). Recent investigation in Northern France shows a prevalence of *C. botulinum* of 16.5% from 175 sea fish samples, but the predominant toxinotype was type B (72%) followed by type A (24%) and type E (4%). Only one sediment sample from 25 contained *C. botulinum* E (Fach et al. 2002).

C. botulinum C and D

C. botulinum types C and D are widespread throughout the world. *C. botulinum* C is mainly found in muds, sediments of marshes, ponds, and seashore where botulism in waterfowl is endemic. Outbreaks of botulism in birds and presence of *C. botulinum* C in their environment have been reported in the United States, Great Britain, Denmark, Netherlands, France, and Japan. Intestinal contents and cadavers of susceptible birds seem to be the principal habitat of *C. botulinum* C. This type has also be detected in soil from warm areas such as in Indonesia, Bangladesh, and Thailand (Dodds 1993b; Hauschild 1989; Smith and Sugiyama 1988).

C. botulinum D is more frequently associated with botulism in animals (ruminants, horses). Carcasses of these animals and also from small animals (rodents) constitute the most common source of this organism. *C. botulinum* D can also be identified in soil samples where animal botulism is common (South and Central Africa, Australia, America, Europe) (Smith and Sugiyama 1988).

C. botulinum F and G

The types F and G are much more less frequently encountered than other types. Since the first identification of *C. botulinum* type F from a homemade liver paste responsible of an human botulism outbreak in the Danish island of Langeland (Moller and Scheibel 1960), this type has been demonstrated in marine sediment of the west coast of the United States (Eklund and Poysky 1965) and in marine or freshwater sediments in Brazil, Venezuela, and Indonesia (reviewed in (Hauschild 1989)). *C. botulinum* G was first isolated from a soil sample in Argentina (Gimenez and Ciccarelli 1970), and was identified from necropsy specimens in cases of unexplained death in adults and infants (Sonnabend et al. 1981), and from 5 soil samples out of 41 in close association with cultivated land in Switzerland (Sonnabend et al. 1987b).

C. butyricum

C. butyricum is a very widespread bacteria in the environment including soil, freshwater and marine sediments, cheese, rumen of healthy calves, animal and human feces, and more rarely from clinical specimens (blood, urine, respiratory tract, pleural cavity, abdomen, wound, and abscesses) (Cato et al. 1986). *C. butyricum* has been involved in certain cases of necrotizing enterocolitis in newborns. Artificial feeding and intestinal stasis support abundant proliferation of *C. butyricum* in the intestinal content. The bacterial overgrowth is accompanied by large production of hydrogen and organic acids, mainly butyric acid, which have been recognized as the main virulence factors of *C. butyricum* in the neonatal necrotizing enterocolitis (Popoff 1990; Popoff et al. 1985).

The first two neurotoxigenic *C. butyricum* strains were isolated from infant botulism in 1985–1986 in Rome (Italy) (Aureli et al. 1986; McCroskey et al. 1986). Extensive studies have been carried out in the vicinity of Rome, and no strains producing BoNT/E were isolated; only *C. botulinum* A and B strains were found in 9.6% of 52 soil samples (Creti et al. 1990). Ten years later, two additional cases of toxi-infection with neurotoxigenic *C. butyricum* strains were described in young people in Italy. These isolates were genotypically and phenotypically identical to the former Italian strains (Fenicia et al. 1999). In 1994, several cases of foodborne botulism were reported in China. The implicated food consisted of salted and fermented paste made of soybeans and wax gourds (Meng et al. 1997). *C. butyricum* type E were isolated from soil samples around the patient's houses (Meng et al. 1999). The *bont*/E gene sequences from 11 Chinese *C. butyricum* strains were identical and differ from those of *C. botulinum* E (96.9% identity at the amino acid level) and the Italian *C. butyricum* strain BL6340 (95% identity). Pulsed field electrophoresis, Southern blot hybridization, and random amplified polymorphic DNA permitted to distinguish three clones among the neurotoxigenic *C. butyricum* strains: two corresponding to the Chinese isolates, one of which was responsible for the foodborne botulism outbreak, and one for the Italian strains. This indicates that the neurotoxigenic *C. butyricum* strains are clonally distributed in vast areas (Wang et al. 2000). In contrast, *C. botulinum* type E strains from fish and fish products showed a wide biodiversity (62 different subtypes among 92 isolates) (Hyytia et al. 1999b).

C. baratii

C. baratii is isolated from soil, sediments, normal and rat feces, and occasionally from war wounds, peritoneal fluid, infection of the eyes, ear, and prostate (Cato et al. 1986). The source of the toxigenic *C. baratii* producing a BoNT/F responsible of one case of infant botulism and two cases of adult botulism in the United States has not been elucidated (Hatheway 1993a).

In the Foods

This chapter concerns only *C. botulinum.*

In Fish and Seafood

In general, the contamination of fish and other aquatic animals by *C. botulinum* reflects that of the sediments of the respective areas. The incidence of *C. botulinum* depends on the areas and on the type of fish. In Northern America, the level of

contamination is high in fish from the Pacific Northwest (5% of ocean salmon, 17% of other fish, and 23% of salmon from rivers) (Dodds 1993a) and from the Great Lakes and adjacent bays (1–60% of fish) (Hauschild 1989). In the Pacific Northwest, 8% of coho salmon, 17% of sole and cod, 23% of steelhead trout, 24% of sockeye salmon, 31% of oysters, 23% of clams, and 18% of crabs were contaminated by toxigenic *C. botulinum* (Craig et al. 1968). Gills and intestines of fish are the most heavily contaminated parts. The presence of *C. botulinum* in gills seems to indicate an environmental contamination. *C. botulinum* E is predominant in fish and aquatic invertebrates from north part of America, Europe, and Asia. In general, *C. botulinum* E does not multiply in the intestine of living fish, but, after death, the bacterium can grow and produce BoNT. Dead fish in the sediments contribute to the persistence and multiplication of *C. botulinum* in the aquatic environment (Dodds 1993a) and to large outbreaks of botulism caused by the ingestion of dead fish containing high titer of BoNT causing a self-multiplicating chain reaction (Eklund et al. 1982). The incidence of *C. botulinum* is also high in Baltic Sea, Sweden and Denmark coastal waters, Caspian Sea, and Japan lakes, with a predominance of type E. In Great Britain, *C. botulinum* C was predominant in most fish farms, followed by types B, E, and F (Cann et al. 1975). Type C was also predominant in fish and aquatic animals from Indonesian waters (Dodds 1993a).

The contamination of prepared fish depends on several factors including the preparation method and the processing stages in addition to the possible infection of the harvested fish. The highest contamination was found in salted fish from Caspian Sea (Hauschild 1989) and dressed rockfish from California (Dodds 1993a). *C. botulinum* has been detected in brined, frozen, and vacuum-packed fish. Meat smoking reduces the incidence of *C. botulinum* contamination. In general, *C. botulinum* E is the predominant type in prepared fish, type A is most commonly found in fish from California, and type C followed by types D, A, B, and F in fish from Indonesia and tropical and subtropical waters (Dodds 1993a; Hauschild 1989).

In Meats

Meat contamination with *C. botulinum* is less frequent than that of fish, and it is lower in North America than in Europe. Animals may carry *C. botulinum* in their intestinal tract that may lead to meat contamination during its processing. Toxinotypes A and B are usually predominant, followed by C and more rarely by E. The incidence is variable according to the different investigations and to the samplings (Dodds 1993a). Thus, Robert and Smart reported that 2–6% of vacuum-packed bacon samples out of 140 in two investigations were contaminated by *C. botulinum*, and in one occasion, 20 of 138 samples were positive (65% contained type C and 15% type A, and the rest were untyped) (Roberts and Smart 1976).

In Fruit and Vegetables

Raw fruits and vegetables may be contaminated by *C. botulinum*, as a result of soil contamination. *C. botulinum* has been identified in fruits and vegetables but mainly in those harvested from the soil. Utilization of manure as fertilizer may affect the level of contamination. Cultivated mushrooms in Canada were found to be highly contaminated by *C. botulinum* B, while in other areas, this product was free or contained low level of *C. botulinum* (Dodds 1993a). Type A is predominant in fruits and vegetables from California; type B in vegetables from Italy, Germany, and other European countries; types A and B were detected in the former Soviet Union (Dodds 1993a).

In Other Foods

Honey from various areas (United States, Argentina, Canada, China, Hungary, Japan, Mexico, Spain) may contain *C. botulinum*. The level of contamination is usually low (1–10 spore per kg), but the honey samples associated with infant botulism contained 10^3–10^4 spores per kg. Type A was detected more frequently than type B, C, or D (Dodds 1993a). The presence of *C. botulinum* in other foods such as dairy products or prepared foods (boil-in-bag foods, vacuum-packed foods, pressurized foods, dehydrated and freeze-dried foods) is very rare (Dodds 1993a).

Genetics of Clostridial Neurotoxins

Neurotoxin Gene Organization

The Botulinum Locus

BoNTs are produced by neurotoxigenic strains of *Clostridium* together with several associated nontoxic proteins (ANTPs). BoNTs and ANTPs associate to form large complexes, also known as progenitor toxins. ANTPs encompass a nontoxic-nonhemagglutinin component (NTNH) and several hemagglutinin components (HAs) or OrfX proteins (Oguma et al. 1999; Popoff and Marvaud 1999; Sharma et al. 2003). In botulinum complex, the proteins are not covalently linked, but their association occurs in cultures and naturally contaminated food. The complex is stable at acidic pH but dissociates at alkaline pH ($\geq$ pH 7) (Eisele et al. 2011).

The genes encoding the neurotoxins and ANTPs, which associate with BoNT to form the botulinum complexes, have been cloned and sequenced in representative clostridial strains of each BoNT type. The neurotoxin and ANTP genes are clustered in close vicinity and constitute the botulinum locus. The organization of the botulinum locus is conserved in the 3′ part but differs slightly in the 5′ part in the different types of BoNT-producing Clostridia. The *bont* genes are at the 3′ part of the locus and are preceded by the genes of the NTNH component. *ntnh* and *bont* genes are transcribed in the same orientation (*Fig. 12.1*), whereas HA genes (*ha*33, *ha*17, and *ha*70), which are upstream to the *ntnh-bon*t genes, are transcribed in the opposite orientation. The *ha* genes are missing in the nonhemagglutinating toxinotypes A2, E, and F. The *ha* genes of *C. botulinum* G only comprise *ha*17 and *ha*70. In the toxinotype A2, E, and F, a gene (*p*47) encoding a 47-kDa protein is immediately upstream of the *ntnh* gene, and both genes are transcribed in the same orientation. In addition, two genes (*orfX1* and *orfX2*), which are not related to *ha* genes,

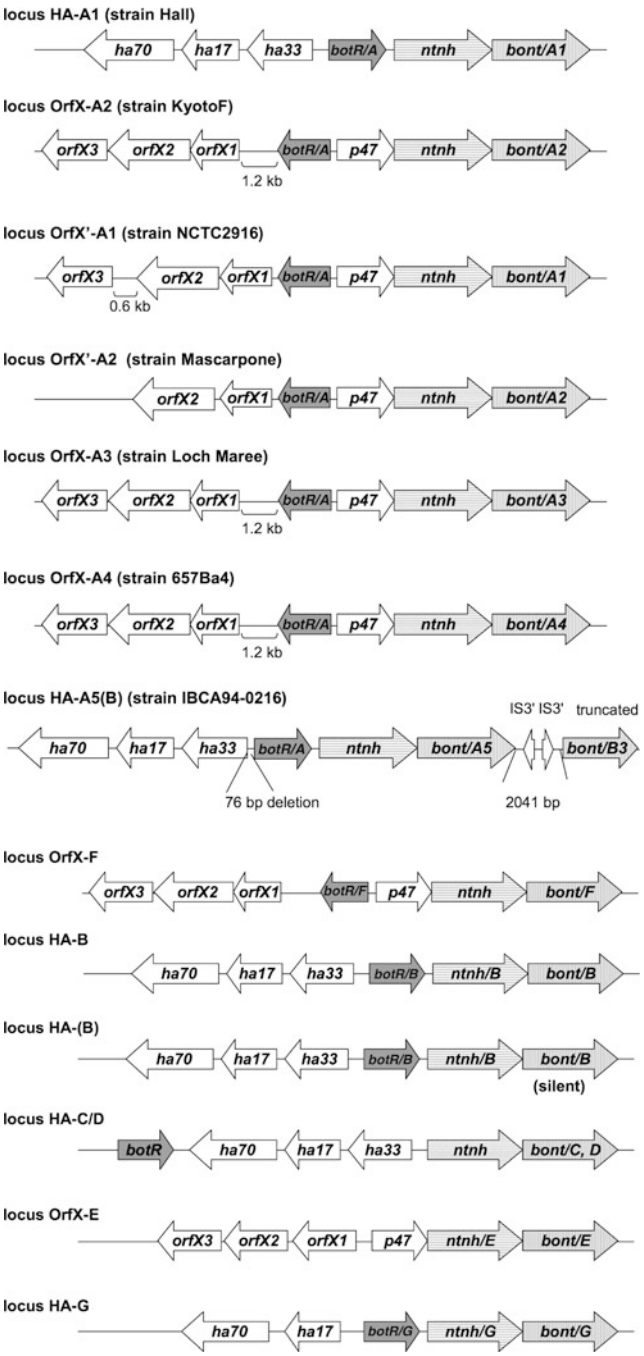

Fig. 12.1
Gene organization of botulinum loci in *C. botulinum* type A to G strains

lie upstream of *p47* in the opposite orientation. An additional gene, *orfX3*, was characterized in *C. botulinum* A2 strain Kyoto-F, downstream the gene *orfX2* and in the same orientation (Dineen et al. 2004). *p47* and the *orfX* operon are also associated with *bont* subtype A1, A3, or A4 (Jacobson et al. 2008a). Indeed, two main types of botulinum locus can be distinguished, the HA locus containing *ntnh* and *ha* genes and the OrfX locus containing *orfX*, *p47*, and *ntnh* genes in addition to *bont* gene (Fig. 12.1). The same *bont* gene can be inserted in a HA or OrfX locus. However, *bont/A1* is the only gene which has been found in either of the two types of botulinum locus.

A gene (*bot*R, previously called *orf*21 or *orf*22) encoding for a 21–22-kDa protein, which encodes for an alternative sigma factor involved in the regulation of botulinum locus gene expression (Raffestin et al. 2005), is present in different positions in different strains of *C. botulinum* (Fig. 12.1). In *C. tetani*, a gene (*tet*R) equivalent to *bot*R (Marvaud et al. 1998; Raffestin et al. 2005) is present upstream of the tetanus toxin (*tent*) gene. No *antp*-like genes have been identified in *C. tetani*

(Brüggemann et al. 2003). It is noteworthy that *bont*/R is not present in botulinum locus containing the *bont/E* gene, in *C. botulinum* or *C. butyricum* type E.

Usually, one clostridial strain produces only one type of neurotoxin and the botulinum locus is present in a single copy on the genome as suggested by Southern blotting of DNA fragments separated by pulsed field gel electrophoresis (Hutson et al. 1996; Lin and Johnson 1995). However, some rare strains synthesize two different BoNTs: BoNT/A-BoNT/B, BoNT/A-BoNT/F, and BoNT/B-BoNT/F producing strains have been isolated (Henderson et al. 1997; Hutson et al. 1996). The A-B strain contains two *bont* genes related to those of *C. botulinum* A2 and proteolytic *C. botulinum* B, respectively (Fujinaga et al. 1995). In such strains, the two neurotoxins are usually produced in different proportions. Thus, in Ba and Bf strains, BoNT/B is produced ten times more than BoNT/A and BoNT/F (Henderson et al. 1997). Some clostridial strains contain silent neurotoxin genes. Several *C. botulinum* A strains isolated from foodborne and infant botulism contain a silent *bont*/B gene. These strains are noted A(b). The characterization of strain NCTC2916 shows that it has two loci, A and B, which are 40 kbp distant within the chromosome. The botulinum B locus consists of *bont*/B, *ntnh*, *orf*21, *ha*33, and *ha*11 genes. The *bont*/A gene is identical to that of *C. botulinum* A1 strains, but the organization of the botulinum A locus is similar to that of *C. botulinum* A2 and F strains (*Fig. 12.1*). The BoNT/B nucleotide sequence is related to that of *C. botulinum* B strains (97% identity), but it has a stop mutation in position 128 and two base deletions (positions 2,839 and 2,944) resulting in reading frameshifts and multiple stop codons (Dineen et al. 2004; Hutson et al. 1996; Jovita et al. 1998). Silent *bont*/B has also been evidenced in nontoxigenic *C. subterminale* strains (Franciosa et al. 1994; Jovita et al. 1998). The strain *C. botulinum* 667 contains also two loci, A and B, 40 kbp distant within the chromosome. The genetic organization is the same than that in strain NCTC2916, and the *bont/B* gene is silenced by mutations and deletions (Hutson et al. 1996).

Genomic Localization of the Botulinum Locus

The genes encoding for the different types of BoNT are present on different genetic elements, including phages, plasmid, or chromosomes depending on the species and strain of Clostridia. In *C. tetani* and *C. argentinense*, the neurotoxin gene is present within a large plasmid (51 and 76 MDa, respectively). Plasmids of various sizes and bacteriophages have been found in *C. botulinum* A, B, E, and F, and previous works have shown that toxigenicity was not associated with the presence of these genetic elements (Eklund et al. 1989; Strom et al. 1984; Weickert et al. 1986). Therefore, the genes encoding for these neurotoxins were assumed to be located on the chromosome. However, it has been recently found that in some strains such as Loch Maree strain (subtype A3), 657Ba (type Ba and subtype A4), Okra (type B1), and Eklund 17B (type Bnp), the botulinum neurotoxin genes are harbored by large plasmids (47–270 kb) (Marshall et al. 2007; Smith et al. 2007). Plasmid location of neurotoxin genes seems common in *C. botulinum* type B strains, mainly in subtype B1, bivalent, and nonproteolytic strains (Franciosa et al. 2009). In the bivalent strain Ba657, the two botulinum locus, locus A and locus B, are located on the same plasmid and are separated by approximately 97 kbp. Similarly, the neurotoxin genes, *bont/B* and *bont/f*, from one Bf strain are located on a same plasmid (pBf),which is very related to pCLJ from 657Ba strain (Hill et al. 2009). Interestingly, none of botulinum plasmids show synteny to *C. tetani* plasmid pE88, which contains the *tent* gene (Hill et al. 2009). In *C. botulinum* type E and neurotoxigenic *C. butyricum* strains, the location of the BoNT/E-encoding gene is located on the chromosome. In *C. botulinum* C and D, it has been clearly evidenced that BoNT is encoded by bacteriophages (reviewed in (Poulain et al. 2006)).

The location of botulinum locus within chromosome or plasmid seems to occur not at random but at specific sites. Indeed, in strains from group I or II, whose genome sequencing is available, three specific sites of botulinum locus integration have been identified. OrfX-A2, orfX′-A1, and orfX-F locus are located in the *ars* operon, which contains 3–5 genes involved in arsenic reduction. OrfX′-A1 and orfX-F locus share a similar integration site at the 5′ end of the *ars* operon, whereas OrfX-A2 locus is inserted between two copies of *arsC* gene. HA-A1 and HA-B locus, which contain a recombinant *ntnh* gene type A and type B strains, are found in the *oppA/brnQ* operon encoding for extracellular solute-binding protein and branched chain amino acid transport proteins, respectively. This operon is lacking in nonproteolytic *C. botulinum* type B, *C. botulinum* type E, and *C. butyricum* type E strains. The third integration site is the *rarA* gene, which contains the OrfX-E locus in *C. botulinum* type E and *C. butyricum* type E strains. *rarA* encodes a resolvase protein involved in recombination or insertion events of transposons. Interestingly, the botulinum E locus is inserted in the same codon (102) of *rarA* gene in both *C. botulinum* type E and *C. butyricum* type E strains, and the inserted botulinum locus contains an additional intact *rarA* gene (Hill et al. 2009).

Two specific sites of botulinum locus location have been identified on plasmids from group I strains, one contains OrfX-A3, OrfX-A4 from Ba strain, or OrfX-F from Bf strain, and the second harbors the HA-B locus from *C. botulinum* B1 strain or bivalent Ba4 or Bf strains. The HA-npB locus is located on a plasmid different from those of group I strains. However, the downstream flanking region of the HA-npB locus contains an IS element, a transposon-associated resolvase, and a site-specific recombinase (Hill et al. 2009).

Genome Characteristics of *Clostridium botulinum*

The complete genome sequences of 10 C. *botulinum* strains from groups I and II have been solved so far. *C. botulinum* genomes consist of a circular DNA chromosome, the size of which (3,659,644–4,155,278 bp) is in the same range than that of other known *Clostridium* genomes (*C. perfringens* 3 031 430 bp; *C. difficile* 4,290,252 bp; *C. acetobutylicum* 3,940,252 bp) except that of *C. tetani* which is considerably smaller (2,799,250 bp). The G+C content (27–28%) is similar

to that of most clostridial species. The main features of the 10 C. *botulinum* genomes are listed in Tables 12.2–12.4. Most strains also possess plasmids which vary in size (16–270 kbp).

The *C. botulinum* A (strain Hall) genome (Sebaihia et al. 2007) shares 1,126 (31%) to 1,565 (43%) genes with the genomes of *C. acetobutylicum*, *C. perfringens*, *C. difficile*, and *C. tetani*, confirming the heterogeneity of the *Clostridium* genus. *C. botulinum* A shares a larger number of orthologous genes with *C. acetobutylicum* (43%), a nontoxigenic *Clostridium*, than with the other toxigenic Clostridia. Among the sequenced toxigenic Clostridia, the most related to *C. botulinum* A is *C. tetani* (40% orthologous genes) which also synthesizes a neurotoxin related to botulinum neurotoxin; the most unrelated is *C. perfringens* (31% orthologous genes).

The shared genes between *C. botulinum* and other Clostridia mainly encode basic functions such as cell division, macromolecule biosynthesis, central/intermediary metabolism, and germination/sporulation. The *C. botulinum* unique genes are spread all around the genome and are not clustered in specific regions. They seem to be involved in accessory functions including transport/binding proteins, energy metabolism, cell surface proteins, and regulation (Sebaihia et al. 2007).

In contrast to the *C. difficile* genome (strain 630) which shows a mosaic organization with multiple horizontal gene acquisitions mainly via mobile elements such as conjugative transposons (11% of the genome) (Sebaihia et al. 2006), *C. botulinum* genomes have a more homogeneous composition without evidence of recent gene acquisition. Only two prophages, two prophage remnants, one functional, and 11 nonfunctional transposase genes were found in the genome of *C. botulinum* A ATCC3502 (Sebaihia et al. 2007), and two prophages were evidenced in *C. botulinum* A ATCC19397. A relatively low proportion (0.8–6.8%) of genes are predicted (Colombo prediction) to be acquired horizontally in *C. botulinum* genomes. However, this portion is significantly higher in group I strains (4.5–6.8% of all genes) than in group II strains (0.8–1.2%).

An overall comparison between complete *C. botulinum* genomes revealed high similarity between genomes from group I strains and their strong distance to group II strains. Four to six large variable genomic regions exist which are scattered around the genomes. These are absent in some strains or show decreased similarity. Whereas most proteins (81–86%) of group I strains share a protein identity of over 90%, only 2–3% of proteins from group II genomes have orthologs encoded in group I genomes with such a high protein identity. Applying a less stringent cutoff (10% protein identity), group I and group II strains share only 48% of their proteome. In addition, there is almost no genome-wide synteny between group I and group II strains, except a region around the origin of replication. The fact that the genomes of six strains of group I (three *C. botulinum* A1, one *C. botulinum* A3, one proteolytic *C. botulinum* B1, and one proteolytic *C. botulinum* F) organisms are highly similar confirms previous genetic analysis, indicating that group I *C. botulinum* strains form a homogeneous bacterial species distinct from the other *C. botulinum* types (reviewed in (Popoff 1995)). This was further confirmed in a set of 5 C. *botulinum* A strains by comparative genomic hybridization microarrays and pulsed field gel electrophoresis (Raphael et al. 2008). However, these strains show limited genomic rearrangements in the botulinum locus (see below). A multiple-locus variable-number tandem-repeat analysis was designed in order to differentiate *C. botulinum* A strains within subtypes. Ten variable-number tandem-repeat (VNTR) regions dispersed throughout the *C. botulinum* A ATCC3502 genome, which are distant from the botulinum locus, permitted to differentiate the strains into subtypes A1-A4 and to distinguish 30 genotypes within 53 strains of subtype A1 (Macdonald et al. 2008). A multiple sequence typing (MLST) based on seven housekeeping genes has also been proposed for *C. botulinum* A subtyping and phylogenetic analysis. Thus, 24 lineages were identified from 73 C. *botulinum* A strains (Jacobson et al. 2008b). Chromosome organization of group I *C. botulinum* A, B, and F strains is also shared by *C. sporogenes* (Hill et al. 2009), confirming that this *Clostridium* species is very close from proteolytic *C. botulinum* strains except the absence of neurotoxin gene.

Two *C. botulinum* E strains (Beluga E1 and Alaska E3) and the nonproteolytic *C. botulinum* B Eklund17B share chromosome synteny, indicating that strains from group II contain a related chromosomal background, which is distinct from that of *C. butyricum* type E (Hill et al. 2009).

Plasmids are common in *C. botulinum* with sizes ranging from 16,344 to 270,346 bp and containing 19–329 genes. Plasmids from strains Loch Maree, Okra, and Ba657, although of different sizes, share large regions that are very similar to each other (Smith et al. 2007). However, they are poorly related to plasmids that do not contain neurotoxin genes. They can replicate independently, since they contain complete DNA polymerase III complex enzymes and DNA helicase II (Smith et al. 2007).

Genomic synteny of one representative type C strain and one representative type D strain indicates a related chromosome organization between these two strains from group III (Brüggemann et al. 2011). Chromosome organization is conserved between isolates, and it is closely related to that of *C. novyi* (Skarin et al. 2011). From group III, their genetic organization is distinct from that of group I and II strains. Botulinum C2 toxin, which is binary toxin involved in actin filament depolymerization (Barth et al. 2004), is encoded by genes located on a large plasmid in *C. botulinum* type C (Barth et al. 2004). Genomes sequencing of *C. botulinum* type D strain 1873 shows that this strain contains two plasmids, one pCLG1 harboring the genes for the enzymatic component and binding components of C2 toxin, and the other, pCLG2, containing genes for clostripain (a cysteine protease) and a thermolabile hemolysin. In addition, *C. botulinum* strains contain phages, which harbor the neurotoxin gene and the C3 exoenzyme gene.

Genetic Diversity of *C. botulinum* Strains and Botulinum Neurotoxin Gene Variation

Genetic analysis by 16s RNA gene sequence comparison or DNA/DNA homology have shown that *C. botulinum* strains form four

Table 12.2

Examples of foodborne botulism outbreaks. Additional botulism outbreaks are listed in (Lindstrom et al. 2006b; Peck 2006, 2009; Peck et al. 2011)

Country	Year	Type	Cases (deaths)	Food	References
Madagascar	1982	E	60 (30)	Commercial pork sausage	Viscens et al. (1985)
USA/Israel	1987	E	8 (1)	Commercial uneviscerated salted, air-dried fish (kapchunka)	Slater et al. (1989)
UK	1989	B p	27 (1)	Commercial hazelnut yogurt	O'Mahony et al. (1990)
Sweden	1991	E	2 (0)	Vacuum-packed hot-smoked rainbow trout	Korkeala et al. (1998)
Egypt	1991	E	>91 (18)	Commercial uneviscerated salted fish (faseikh)	Weber et al. (1993b)
Switzerland	1993–1994	B	12	Ham	Troillet and Praz (1995)
Sweden	1994	E		Vacuum-packed hot-smoked rainbow trout	Korkeala et al. (1998)
Italy	1996	A	7 (1)	Commercial dairy product (mascarpone)	Aureli et al. (2000); Franciosa et al. (1999)
Germany	1997	E	2	Commercial hot-smoked vacuum-packed fish (raucherfish)	Korkeala et al. (1998)
Iran	1997	A	27 (1)	Traditional cheese preserved in oil	Pourshafie et al. (1998)
Argentina	1998	A	9	Meat roll (matambre)	Villar et al. (1999)
Thailand	1998	A	13 (2)	Home-canned bamboo shoots	Control (1999)
Algeria	1998	A	340 (37)	Commercial sausage "halal"	Mesbah (2009)
Morocco	1999	B	78 (20)	Commercial sausage	Kissani et al. (2009); Ouagari et al. (2002)
USA	2001	A	15	Commercial frozen chili sauce	Kalluri et al. (2003)
South Africa	2002	A	2 (2)	Commercial tinned pilchards	Frean et al. (2004)
Georgia	1980–2002	B (85%) E (10%) A (1%)	879 (58)	Home-preserved vegetables (80%), smoked fish (12%), smoked meat (2%)	Gottlieb et al. (2007); Varma et al. (2004)
France	2003	B	10 cases 3 outbreaks	Commercial beef and poultry sausages "halal"	Carlier et al. (2007); Espié et al. (2003)
Germany	2004	E	1	Commercial vacuum-packed smoked salmon	Dressler, (2005)
Italy	2004	ß	28	Green olives (restaurant)	Cawthorne et al. (2005)
Finland	2006	E	1	Commercial vacuum-packed smoked whitefish	Lindstrom et al. (2006b)
Canada/USA	2006	A	6 (1)	Commercial refrigerated carrot juice	Sheth et al. (2008)
Thailand	2006	A	209	Home-canned bamboo shoots	Kongsaengdao et al. (2006); Ungchusak et al. (2007)
USA	2007	E	5	Home-salted uneviscerated fish	Sobel et al. (2007)
USA	2007	A		Commercial hot dog chili sauce	Control (2007)
France	2008	A	2	Commercial chicken enchiladas	King, (2008)
France	2008	A	3	Home pumpkin ham	Mazuet et al. (2011)
France	2009	E	3	Commercial vacuum-packed hot-smoked whitefish (Canadian origin purchased in Finland)	King et al. (2009)
France	2010	A	5 (1)	Home-canned string beans	Mazuet et al. (2011)
Brazil	2000–2008	A, B	27 cases 18 outbreaks	Home-canned meat, commercial canned soybean cheese, home confit of fish, chicken pie	Rowlands et al. (2010)
France	2011	A	8 cases 2 outbreaks	Commercial green olive paste	Pingeon et al. (2011)
Finland	2011	B	2	Commercial olives stuffed with almonds	Jalava et al. (2011)
Scotland	2011	A	3	Commercial "korma" sauce	Browning et al. (2011)

Table 12.3
Examples of indications benefiting from botulinum toxin

Indication	Botulinum toxin is an evidence-based treatment	References
Focal dystonia, spasticity, movement disorders, and related		
Strabismus		Rowe and Noonan, (2009); Scott (1980)
Blepharospasm		Costa et al. (2005a)
Hemifacial spasm	Yes	Costa et al. (2005b)
Cervical dystonia	Yes, type A and B	Albanese et al. (2006); Costa et al. (2005c)
Dysphonia	Yes	Watts et al. (2008)
Writer's cramp	Yes	Dashtipour and Pender, (2008)
Upper limb spasticity	Yes	Fehlings et al. (2010); Hoare et al. (2010); Sheean et al. (2010)
Lower limb spasticity	Yes	Love et al. (2010); Olver et al. (2010)
Masseter hypertrophy		Al-Muharraqi et al. (2009)
Autonomic disorders, Pain		
Sialorrhea (excessive saliva) in people with motoneuron disease/amyotrophic lateral sclerosis	Yes	Young et al. (2011)
Drooling	Yes, level B	Naumann et al. (2008)
Hyperhidrosis (palmar, axillary)	Yes, level B and A, respectively	Naumann et al. (2008)
Gustatory sweating		Naumann et al. (2008)
Neurogenic detrusor overactivity or idiopathic detrusor overactivity	Yes	Duthie et al. (2007); Naumann et al. (2008)
Benign prostatic enlargement		Chuang and Chancellor, (2006); Oeconomou et al. (2008)
Management of pain	Evidence-based conclusions are limited by the availability of data	Naumann et al. (2008); Qerama et al. (2010)
Secondary headaches and cranial neuralgias		Linde et al. (2011)
Others		
Facial aesthetics	Yes	Gadhia and Walmsley, (2009)

distinct clusters which correspond to the physiological groups I to IV (Collins and East 1997; Hutson et al. 1994). Amplified fragment length polymorphism (AFLP) and pulsed field gel electrophoresis (PFGE) analysis also confirms the classification of proteolytic types A, B, and F strains in group I and the nonproteolytic types B, E, and F strains in group II but can differentiate individual strains into each group (Hielm et al. 1998a, b; Hill et al. 2007; Hyytia et al. 1999a, b; Keto-Timonen et al. 2005; Nevas et al. 2005). These methods have been used in epidemiological studies and are useful tools to investigate relatedness between strains isolated from patients and food. For example, among proteolytic *C. botulinum* strains, PFGE analysis differentiates the toxinotypes A, B, and F at 83–86% similarity level and enables discrimination of most of individual strains. A greater diversity was observed between type A strains than in type B strains (Nevas et al. 2005). These studies also indicate that each *C. botulinum* group is heterogeneous at the genome level.

A high level of similarity was observed between strains from group I by using DNA hybridization with a DNA microarray including 94% of the coding sequences from strain Hall. Two type A strains share 95–96% of the strain Hall coding sequences, and seven other proteolytic strains have 87–91% common coding sequences (Sebaihia et al. 2007). A larger investigation reports that 58 C. *botulinum* strains from group I share 63% of coding sequences with those of strain ATCC3502 (Carter et al. 2009). Interestingly, two *C. sporogenes* strains (physiologically related to *C. botulinum* group I but nontoxigenic) are significantly similar to Hall strain and share 84–87% of the coding sequences (Sebaihia et al. 2007). In another microarray study, three *C. sporogenes* strains show approximately 63% common coding sequences with *C. botulinum* A ATCC3502 (Carter et al. 2009).

BoNT gene has been sequenced from a large number of strains, and sequence comparison has permitted to identify sequence variations in each toxinotype. Thereby, botulinum toxinotypes are divided into subtypes, which are defined as toxin sequences differing by at least 2.6% identity at the amino acid level (Smith et al. 2005). BoNT genes from type A strains show 92–95% nucleotide identities corresponding to 84–90% amino acid identities and are divided into four subtypes termed A1, A2, A3, A4, and A5 (*Table 12.1*). Subtypes A1 to A5 also differ in the botulinum locus composition. Type B genes differ from 2% to 4% at the nucleotide level and 3–6% at the amino acid level. They are classified in five subtypes, B1, B2, B3, bivalent B, and nonproteolytic B. BoNT genes from nonproteolytic type B strains form only one subtype, whereas those from proteolytic strains show a greater variation leading to a four subtype division (*Table 12.1*). Sequences of neurotoxin genes type B show an overall less variation than those of type A, but a more sequence variation is observed within members of each type B subtype compared to bont/A. BoNT/E sequences from *C. botulinum* type E (group II) fit in six subtypes (E1, E2, E3, E6, E7, E8), sharing 99% nucleotide identity and 97–99% amino acid identity, and are more distantly related to BoNT/E sequences from *C. butyricum* strains which are into two subtypes (E4, E5) with 97–98% nucleotide and 95–96% amino acid identities between sequences from both *Clostridium* species. Gene

Table 12.4
Examples of toxin products

Trade name	Manufacturing company	Form	Units/ pack	Toxin load /vial	Active substance	MW (kDa)	Main excipients
Botox®	Allergan Inc. (CA, USA)	Powder	100 Allergan units	~5 ng	Type A complex (neurotoxin + HAs + NTNH)	900	Human serum albumin (0.5 mg), NaCl
Vistabel® Botox Cosmetic®			50 Allergan units	2.5 ng			Human serum albumin (0.25 mg), NaCl
Dysport®	IPSEN Ltd. (GB)		500 Speywood units	4.35 ng		600–900	Human serum albumin (0,125 mg), lactose
Azzalure®	Galderma (manufacturer: IPSEN)		125 Speywood units	1.1 ng			Human serum albumin (0,125 mg), lactose
Neuronox®	Medytox (Rep. South Korea) / Q-Med Inc. (Sweden)		100	?		900	Human serum albumin, NaCl
CBTXA, BTXA Prosigne®	Lanzhou Inst. Biol. Products (Rep. of China)		100 (or 50)	~4–5 ng		?	Porcine gelatin, dextran, sucrose
Xeomin®(NT-201)	Merz Pharmaceuticals Gmbh (Germany)		100 units	0.6 ng	Type A neurotoxin (bi-chainal)	150	Human serum albumin (1 mg), sucrose
Bocouture®			50 units	0.3 ng			Human serum albumin, sucrose
Neurobloc® Myobloc®	Solstice Neurosci. Inc.	Solution	2,500 or more	25 ng or more	Type B	500/700	Human serum albumin, pH 5.6

diversity has also been evidenced in the other parts of the genome as tested by MLST and AFLP analysis, but most of *C. botulinum* E strains are conserved in a same clade. Subtype variation in *C. botulinum* E strains seems to result from recombination events rather than random mutations. High differences (up to 25%) have been found in nucleotide sequences of BoNT/F mainly in the region coding the light chain, and five subtypes have been identified in proteolytic *C. botulinum* F. The low number of strains type C, D, F, and G which were analyzed does not permit to significantly evaluate the neurotoxin gene diversity. In group III, mosaic genes between BoNT genes types C and D can be distinguished from classical types C and D strains. BoNT/F sequences from *C. botulinum* type F form a different cluster of those from *C. baratii* (Carter et al. 2009; Chen et al. 2007; Hill et al. 2007; Macdonald et al. 2011; Raphael et al. 2010).

Signification of sequence diversity in each toxinotype is not yet well known but could be important in diagnostic tests and development of therapeutic agents such as those based on immunotherapy. Thereby, BoNT/A1 and BoNT/A2, which differs by 10% at the amino acid sequence level, show large differences in monoclonal antibody-binding affinity. Among six monoclonal antibodies, which bind to BoNT/A1 with high affinity, three show a marked decrease in binding affinity (500 to more than 1,000-fold) to BoNT/A2. Only combinations of monoclonal antibodies, which tightly bind to toxin subtype, potently neutralize the corresponding toxin in vivo. Association of the three monoclonal antibodies with high affinity binding to subtypes A1 and A2 completely neutralizes A1 or A2 toxin, while replacement of two from three monoclonal antibodies by two having a low binding affinity to BoNT/A2 induces a decrease in BoNT/A2 neutralization (50-fold less) (Smith et al. 2005). The impact of subtype variation in binding and neutralization potency of polyclonal antibodies remains to be determined. Thus, development of therapeutic polyclonal or monoclonal antibodies as well as vaccines based on single toxin subtype needs to be evaluated for their protection ability with the other related subtypes. Although two toxins show a low level of sequence difference, they can have marked difference in activity if amino acid variation occurs in strategic toxin sites. Subtypes A1, A2, A3, and A4 of BoNT/A have been analyzed by sequence comparison, as well as molecular modeling and structure comparison with the crystal structure of subtypes, the impact of which is not known. Ganglioside-binding site is conserved in all subtypes of BoNT/A. The greatest variability was found in the light (L) chain, mainly between subtypes A3 and A4 (76% identity). The enzymatic site of L chain is conserved, but nonconservative mutations are observed in domains involved

in substrate (SNAP-25) recognition. When compared to subtypes A1 and A2, subtypes A3 and A4 show sequence variation in α-exosite and S1′ subsite recognition, respectively, suggesting that these subtypes have a decreased affinity and catalytic efficiency for their substrate (Arndt et al. 2006). Indeed, L chains from subtypes A3 and A4 show different catalytic properties of the substrate SNAP25 compared to L chain from subtypes A1 and A2, which show the same catalytic activity, although all L chain isoforms bind SNAP25 with similar affinity. L chain from subtype A4 and to a lower extent from subtype A3 cleaves less efficiently SNAP25 than L chain subtype A1 (2- and 23-fold less, respectively) (Ahmed et al. 2001; Henkel et al. 2009). Another example of gene variation and toxin activity difference is given by neurotoxins type B. BoNT/B from strain 111 (subtype B2) isolated from infant botulism differs from strain Okra/NT associated with foodborne botulism in Japan by 56 amino acid changes (95.7% identity), from which most occur in the half C-terminal part of the toxin (Ihara et al. 2003). BoNT/B from strain 111 shows an about tenfold lower specific activity than that of strain Okra/NT, and most of monoclonal antibodies which recognize the C-terminus of Okra/NT BoNT/B do not react with BoNT/B of strain 111. Binding affinity of BoNT/B of strain 111 to the receptor synaptotagmin II in the presence of ganglioside GT1b is 4.2 lower than that of Okra/NT BoNT/B. Mutations of 23 residues in the C-terminus of BoNT/B of strain 111 have been attributed to the lower binding affinity of the toxin to its receptor and thus to the lower specific toxicity (Ihara et al. 2003; Kozaki et al. 1998).

Sequence comparison of *bont* genes suggests that they have evolved separately in different genomic backgrounds (Hill et al. 2007). BoNT genetic diversity could also reflect a different geographical distribution of strains or their involvement in different epidemiological situations. *C. botulinum* subtype A2 was first identified in infant botulism in Japan and was found to differ from strains involved in foodborne botulism in adults referred as subtype A1 (Tabita et al. 1991; Willems et al. 1993). However, no correlation was evidenced between strains subtype A1 and A2 isolated from the United States and UK and their clinical origin, foodborne or infant botulism (Cordoba et al. 1995; Johnson et al. 2005). But, strains A1 are more prevalent in the United States, whereas subtype A2 strains are commonly isolated in Europe. Indeed, all the strains from foodborne botulism in the United States which have been analyzed fall into subtype A1, and all 33 C. *botulinum* type A isolated from Italy belong to subtype A2 as well as two strains from infant botulism in United Kingdom (Franciosa et al. 2004; Hill et al. 2007; Johnson et al. 2005). But 18 C. *botulinum* strains type A isolated in France or Europe by Prevot during the period approximately 1950–1960 are of subtype A1 (Hill et al. 2007). Divergent strains of subtype A2 characterized by five amino acid differences in BoNT/A2 and a slightly different botulinum locus organization (locus A2-OrfX′) with a shorter intergenic region between *orfX1* and *botR/A* genes (77 vs. 1,228 nucleotides) when compared to strain A2 Kyoto-F have been identified in Italy such as strain associated with consumption of contaminated cheese (Mascarpone) (◉ *Fig. 12.1*) (Franciosa et al. 2006). Organization of botulinum locus of strain Mascarpone is closely related to that of locus containing *bont/A1* in strain type A(B) NCTC2916 (Dineen et al. 2003; Henderson et al. 1996). Strains Mascarpone and Kyoto-F have probably a common origin and then a distinct evolution including a gene rearrangement in strain Mascarpone with an ancestor of strain NCT2916. Four *C. botulinum* A5(B) strains were isolated from wound botulism in heroin users in UK and one from infant botulism in California (USA) supporting a *bont* gene evolution independent of the geographical location and epidemiological situation (Carter et al. 2009; Dover et al. 2009).

Genetic diversity is also observed in the two FGI regions. Six profiles have been evidenced in 58 proteolytic strains from group I by DNA microarray which correlate with the diversity of flagellin glycan composition as determined by mass spectrometry. The FGI genetic diversity does no match with that of the botulinum locus, indicating an independent evolution of FGI and botulinum locus genes in a relatively stable genomic background of group I *C. botulinum* strains (Carter et al. 2009). In addition to cell wall and surface structure variations, a marked difference in proteolytic strains of group I consists in resistance to toxic compounds. Thereby, group I *C. botulinum* type B strains representative of strains found in North Europe are divided in two clusters BI and BII which differ by 413 coding sequences but contain a same neurotoxin gene of B2 subtype in a HA locus. In contrast to cluster BI strains, cluster BII strains are more resistant to arsenic and more sensitive to cadmium. Moreover, strains from the two clusters show other differences in metabolism, such as cluster BII strains growing at lower temperature than cluster BI strains (Hinderink et al. 2009; Lindstrom et al. 2009). This suggests a differential evolution of these environmental Clostridia in response to adaptation to distinct ecological niches.

Another differential genetic evolution is illustrated by neurotoxigenic *C. butyricum* strains. *C. butyricum* strains producing type E neurotoxin have been isolated from infant botulism and young people in Italy (Fenicia et al. 1999; McCroskey et al. 1986), as well as from botulism associated with consumption of fermented soybean in China (Meng et al. 1997, 1999; Wang et al. 2000). Based on toxin gene sequence, toxigenic *C. butyricum* strains from Italy and China are divided in two distinct subtypes, termed E4 and E5, respectively, indicating an independent evolution of *bont/E* gene after transfer in *C. butyricum* (Hill et al. 2007).

The Cellular and Molecular Mechanisms Involved in Neuroexocytosis: An Overview

An Overview of Neurotransmission

Transfer of information or command between neurons, or neurons and target cells (muscle fibers, endocrine cells, etc.) is most often chemical in nature and occurs at highly specialized contact sites termed *synapses*. Here, the release of neurotransmitter molecules by the presynaptic elements enables activation of receptors localized on the postsynaptic target. Neurotransmitter

molecules are comprised of small organic molecules as acetylcholine (ACh), catecholamines like dopamine or noradrenaline, serotonin (5-HT), glutamate, gamma-aminobutyric acid (GABA), glycine, adenosine triphosphate (ATP), and numerous peptides such as vasointestinal peptide (VIP), substance P (SP), and calcitonin gene-related protein (CGRP). Released transmitter substance(s) can activate ligand-gated ionic channels or metabotropic receptors, thus mediating either transmembrane ionic fluxes or activation of intracellular signaling pathway(s). For example, in the central nervous system, depending on the ion species flowing through the channel, activation of ligand-gated ionic channels can cause depolarization (i.e., excitation) or hyperpolarization (i.e., inhibition) of the postsynaptic plasma membrane, respectively, and the excitation/inhibition net balance determines eventual initiation of action potentials propagated in the neuron until the next synapses. The evoked endplate potential at muscle fibers following stimulation of the motor nerve (and subsequent ACh release) is a depolarization that may reach the threshold for initiating muscle action potential, which itself propagates along muscle fiber ultimately triggering its contraction. In the enteric nervous system, release of neurotransmitter molecules (as VIP) by mucosal nerve endings directly contacting the enterocytes, or indirectly via the activation of enterochrommaffin cells releasing 5-HT, can lead to activation of metabotropic receptors, intracellular activation of the adenylate cyclase and downstream cAMP-dependent pathways, resulting in an active cotransport of ions species (Na^+, K^+, Cl^-) and water efflux for osmotic compensation in the intestine lumen (viz. the molecular mechanisms of diarrhea (Burleigh and Banks 2007)).

In nerve terminals, transmitter molecules are stored into at least two different classes of secretory organelles: the small lucent synaptic vesicles and large dense core vesicles or granules. The small lucent vesicles, approximately 50 nm in diameter, contain small organic molecules and are formed by either budding from the early endosome or recycling of empty vesicles (Murthy and De Camilli 2003; Takamori et al. 2006). The large dense core vesicles are analogous to the secretory granules present in endocrine and exocrine cells and have a biogenesis different from that of the synaptic vesicles. Similar to many cargo vesicles, they are formed by vesicle budding from the trans-Golgi system, followed by homotypic fusion to form larger vesicles. Usually, they mediate release of peptide transmitter or certain catecholamines whose synthesis depends on the presence in the granule lumen of enzymes (as the dopamine beta-hydroxylase). Small synaptic vesicles and certain large dense core vesicles are equipped with a vacuolar-type ATPase that creates a proton gradient which drives specific vesicular transporters, thereby allowing active uptake and storage of neurotransmitter molecules inside these vesicles. Regardless the secretory organelle size, large or small, the release of its content into the extracellular space implicates a fusion of its membrane with the plasmalemma, which then allows passive diffusion of its content into the surrounding medium. This exocytotic process is triggered by a rise in the cytosolic concentration of Ca^{++}, in response either to the arrival of a propagated action potential (i.e., at the neuron nerve endings) or following activation of ionotropic or/and metabotropic receptors located on the plasma membrane of secretory cells (reviewed by (Kasai 1999)).

Mechanisms of Exocytosis and SNAREs

More than 100 proteins are implicated in Ca^{++}-dependent exocytosis. However, only a dozen of them participate in the core machinery required for transmitter release, while the others serve regulatory roles (Jahn and Scheller 2006; Rizo and Rosenmund 2008). Interestingly, several proteins of the release machinery are targeted and disabled by various bacterial toxins. A large body of evidence indicates that the synaptic vesicles do not move freely within the nerve ending cytosol, and regulation of their interaction with actin-based cytoskeleton allows fine-tuning of their movements (Doussau and Augustine 2000; Garner et al. 2000). At the fusion site, they bind to the scaffold proteins associated with a special presynaptic matrix termed "active zone" (Garner et al. 2000). When tethered to the fusion site, the synaptic vesicles are not yet fusogenic and must acquire fusion competence. A key step in this process is the pairing and assembly of a fusion particle comprised of VAMP (vesicle-associated membrane protein, also termed synaptobrevin), SNAP-25, and syntaxin, on the inner face of the plasma membrane (reviewed by (Jahn and Scheller 2006; Rizo and Rosenmund 2008)). These three proteins are also designed as the SNARE proteins (*s*oluble *N*-ethylmaleimide-sensitive factor *a*ttachment protein *re*ceptors). Munc-18 protein is the fourth essential protein partner; it participates in SNARE complex formation and binds to it (reviewed by (Jahn and Scheller 2006; Rizo and Rosenmund 2008)). The SNARE proteins or closely related isoforms are also involved in the fusion of large dense core vesicle or secretory granules. Assembly of the three SNAREs, and possibly munc-18, triggers hemifusion of vesicles and plasma membrane. Termination of fusion is triggered by a rise in intracellular Ca^{++} concentration, which may result from either Ca^{++} influx through voltage-gated channels (e.g., at nerve terminals), Ca^{++} release from intracellular stores, or both (e.g., at many neuroendocrine cells) (Petersen 2003; Rettig and Neher 2002). The principal Ca^{++} sensor for triggering fusion is synaptotagmin, which is an integral synaptic vesicle protein interacting with SNAREs (Bhalla et al. 2006; Chapman 2002; Koh and Bellen 2003; Rizo and Rosenmund 2008). Synaptotagmin is equipped with two "C2 domains," which acquire high affinity for membrane lipids upon binding to Ca^{++} ions but in a pretty high intracellular concentration (10–100 μM). Thus, synaptotagmin is likely sensing the Ca^{++} changes due to activation of voltage-dependent calcium channels. Its Ca^{++}-dependent interaction with plasma membrane leads to changes in membrane curvature and destabilization of the lipids permitting the fusion (Martens et al. 2007). Several SNARE/synaptotagmin complexes (possibly 4–6) must act synergistically to allow fusion of a synaptic vesicle (reviewed by (Jahn and Scheller 2006; Rizo and Rosenmund 2008)). Recently, a role similar to that of synaptotagmin has been assigned to the

Doc2 protein, given the high affinity of Doc2 for Ca^{++}. Thus, Doc2 is likely intervening in spontaneous exocytosis at nerve endings and secretory cells, which is triggered/regulated by minute changes in the resting intracellular Ca^{++} concentration (Groffen et al. 2006).

Actin Cytoskeleton and Small GTPases in Exocytotic Mechanisms

Many other proteins play key roles in synaptic vesicle trafficking and priming of tethered synaptic vesicles. Inside nerve terminals, vesicles traffic along actin filaments, and this implicates molecular motors like myosins II or V, and small GTPases of the Rab family (Rab3 and others) (Augustine 2001; Segev 2001). Reorganization of the actin cytoskeleton is coupled to Ca^{2+}-regulated exocytosis in endocrine cells. However, this is far from being clear in neurons (Bader et al. 2004; Doussau and Augustine 2000; Eitzen 2003). The role of actin in exocytosis seems to consist in governing the vesicle-granule trafficking toward release site (Bader et al. 2004; Eitzen 2003). Organization of the actin-based cytoskeleton is controlled by several proteins, including several small GTPases like Rho proteins (Hall 1998) and ADP ribosylation factor (ARF). Like most small GTPases, ARF and Rho proteins cycle between GDP-bound (inactive) and GTP-bound (active) states, thereby acting as signal transducers that respond to upstream signals. Thus, they activate downstream effector molecules which carry out their biological functions. Rho proteins (Rho, Rac, Cdc42) are widely expressed monomeric GTPases. Their translocation to specific membrane domains enables intervention of distinct biological functions, including: (1) regulation of actin cytoskeletal dynamics, (2) cell cycle progression, (3) gene transcription, (4) membrane transport, and (5) exo/endocytosis (Bader et al. 2004; Hall 1998). In chromaffin and PC12 cells, RhoA associates with secretory granules, whereas Rac1 and Cdc42 are found in the subplasmalemmal region (Bader et al. 2004). Activation of phosphatidylinositol 4-kinase by RhoA promotes the formation of granule-associated actin filaments and/or stabilize the subplasmalemmal actin barrier (Bader et al. 2004). In chromaffin cells, Cdc42 and Rac1 control actin polymerization and secretion (Gasman et al. 2004; Li et al. 2003). In neurons, Rac1 is associated with synaptic vesicles and plasma membrane (Doussau et al. 2000). Rac1 is involved in a post-docking step of neuronal exocytosis during which it controls in an all-or-none manner the functionality of release sites (Doussau et al. 2000; Humeau et al. 2002), possibly via regulation of phospholipase D (PLD) activity (Momboisse et al. 2009). Additional pathways converging on PLD1 implicates ARF6 GTPases (Vitale et al. 2002). Ral-GTPase is abundant in nerve terminals and associates with synaptic vesicles (Bielinski et al. 1993). This molecule apparently plays a key role in neurotransmitter release by regulating the pool size of readily releasable synaptic vesicles (Polzin et al. 2002). Ral has been implicated in regulating PLD activity too (Luo et al. 1998). Downstream from ARF6, Ral, Rho, Rac, and Cdc42, PLD produces PA. PLD is possibly activated by these GTPases upon docking of synaptic vesicles, or secretory granules, at the release sites. PLD activation is an important event for exocytosis in neurons and many secretory cell types (Choi et al. 2002; Humeau et al. 2001b, 2007; Vitale et al. 2001). PLD production of phosphatidic acid (PA) may either signal attachment of some proteins of the fusion machinery to the fusion site or play a role in vesicle fusion. Indeed, PA is a cone-shaped lipid whose local accumulation and possibly destabilization of the lipids at the fusion site (Humeau et al. 2001b, 2007; Vitale et al. 2001) may promote negative curvation of the inner (cytoplasmic) plasma membrane leaflet (Chernomordik and Kozlov 2003).

Structure and Mode of Action of Clostridial Neurotoxins

Structure

BoNTs and TeNT share a common structure. They are synthesized as a precursor protein (about 150 kDa), which is inactive or weakly active. The precursor which does not contain signal peptide is released from the bacteria possibly by a yet misunderstood cell wall exfoliation mechanism (Call et al. 1995). The precursor is proteolytically activated in the extra-bacterial medium either by *Clostridium* proteases or by exogenous proteases such as digestive proteases in the intestinal content. The active neurotoxin consists of a light chain (L, about 50 kDa) and a heavy chain (H, about 100 kDa), which remain linked by a disulfide bridge. The structure of BoNTs shows three distinct domains: L chain containing α-helices and β-strands and including the catalytic zinc-binding motif, the N-terminal part of the H chain forming two unusually long and twisted α-helices, and the C-terminal part of the H chain consisting of two distinct subdomains (H_{CN} and H_{CC}) involved in the recognition of the receptor (◉ *Fig. 12.2*). While the three domains are arranged in a linear manner in BoNT/A and BoNT/B, both the catalytic domain and the binding domain are on the same side of the translocation domain in BoNT/E. This domain organization in BoNT/E might facilitate a rapid translocation process (Breidenbach and Brunger 2005; Emsley et al. 2000; Fotinou et al. 2001; Fu et al. 2006; Kumaran et al. 2009; Lacy and Stevens 1999; Lacy et al. 1998; Stenmark et al. 2008; Swaminathan 2011; Swaminathan and Eswaramoorthy 2000; Umland et al. 1997).

The overall sequence identity at the amino acid level between BoNTs and TeNT ranges from 34% to 97%. Several domains are highly conserved which account for the common mode of action of these toxins. Thereby, the central domains of L chains are related in all the clostridial neurotoxins and contain the consensus sequence (His-Glu-X-X-His) characteristic of zinc metalloprotease active site. The half N-terminal domain of the H chains is also highly conserved, and it is involved in the translocation of the L chain into the cytosol. Thus, a similar mechanism of internalization of the intracellular active domain into target cells is shared by all the clostridial neurotoxins. In contrast, the half C-terminal parts of H chain, mainly the H_{CC}

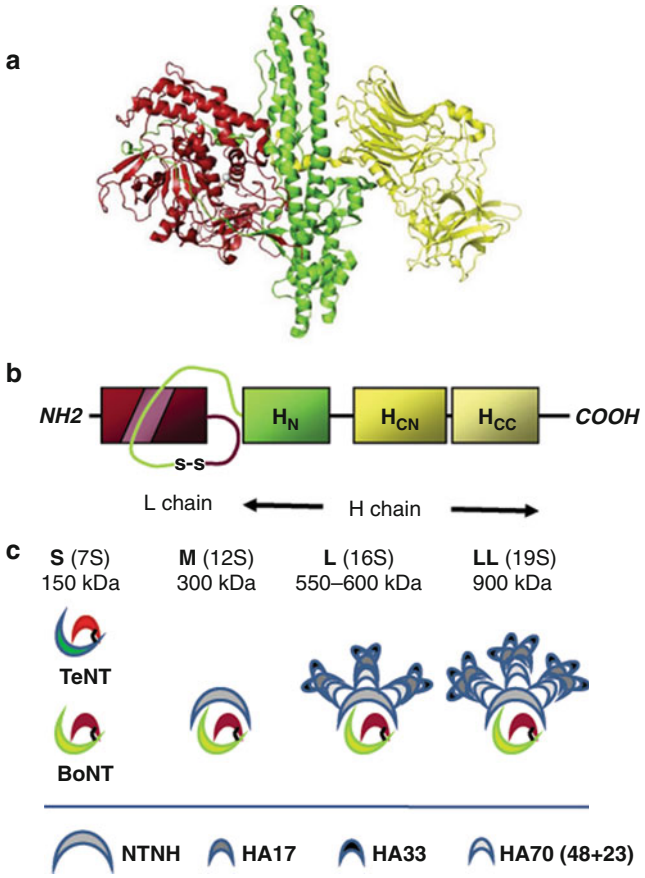

Fig. 12.2

Structure of BoNT or TeNT and conceptual scheme of the botulinum toxin complexes (a) 3D structure of BoNT type A, with (b) the corresponding organization in functional domains. L and H denote light and heavy chains, respectively, H_N and H_c denotes the N- and C-terminus halves of H chain and H_{CN} and H_{CC} N- and C-terminal portions of H_C. (c) The BoNTs associate with ANTP proteins (NTNH and several HA of various MW) to forms complexes of various size (M, L, and LL). Intermediates forms are omitted. TeNT does not make complex with companion proteins and exists only under the S form

subdomains, are the most divergent (Popoff and Marvaud 1999; Poulain et al. 2008). This accounts for the different receptors recognized by the clostridial neurotoxins (see below).

Mode of Action

Although BoNTs and TeNT use different routes to enter their final neuronal targets, they display a similar intracellular mechanism of action. BoNTs enter by oral route or are produced directly in the intestine subsequently to a *C. botulinum* intestinal colonization and then undergo a transcytosis across the digestive mucosa (Ahsan et al. 2005; Couesnon et al. 2008; Jin et al. 2009; Maksymowych and Simpson 2004, 1998; Matsumura et al. 2008). After diffusion into the extracellular fluid and bloodstream circulation, BoNTs target motoneuron endings. In contrast, TeNT is formed in wounds colonized by *C. tetani*. TeNT diffuses in the extracellular fluid and can target all types of nervous endings (sensory, adrenergic neurons and motoneurons), but it is mainly retrogradely transported through the motoneurons (see below) (Manning et al. 1990; Rossetto et al. 2001; Wellhöner 1989) (*Fig. 12.3*).

Each type of BoNT and TeNT recognizes specific receptors on demyelinated terminal nerve endings, mainly through the H_{CC} subdomain. BoNT/A, /C, /E, /F exploit the three isoforms of the vesicle protein SV2 as specific receptors, while BoNT/B and /G bind to synaptotagmin I or II (Dong et al. 2006, 2007, 2008; Mahrhold et al. 2006; Nishiki et al. 1994; Rummel et al. 2004a, 2009). The GPI-anchored membrane protein Thy-1 has been proposed to act as a TeNT receptor (Herreros et al. 2001; Munro et al. 2001), but this has not been confirmed. Albeit SV2 has not been defined as a receptor for TeNT, SV2A and SV2B are involved in the uptake of TeNT into central neurons (Yeh et al. 2011). Ganglioside-binding sites have been characterized in the H_{CC} subdomain. Interestingly, TeNT and BoNT/D exhibit two carbohydrate-binding sites, whereas BoNT/A and BoNT/B show only one (Rummel et al. 2003, 2004b, 2007; Strotmeier et al. 2010; Swaminathan and Eswaramoorthy 2000). Accordingly, TeNT can bind simultaneously to two gangliosides (Chen et al. 2009). BoNT/C and BoNT/D interact with gangliosides (GD_{1b}, GT_{1b}) and phosphatidylethanolamine, respectively, by their H_{CC} subdomain (Tsukamoto et al. 2008). Gangliosides (GD_{1b}, GT_{1b}, and GD_2) and SV2A/B/C also mediate the entry of BoNT/D into neurons but by a different mechanism than that used by BoNT/A and BoNT/E (Kroken et al. 2011; Peng et al. 2011). The role of H_{CN} subdomain, which may interact with phosphatidylinositol phosphates (Muraro et al. 2009), is still unclear. Overall, whatever the considered clostridial neurotoxin, the identified protein receptors are not neurospecific and are expressed on several cell types including intestinal crypt epithelial cells in the intestine (Couesnon et al. 2008). Distribution of the gangliosides recognized by BoNTs differs from that of the protein receptors. Thus, the high affinity of BoNTs and TeNT for presynaptic membranes probably results from multiple and synergistic interactions with the ganglioside and protein parts of receptor, and binding to gangliosides which induces conformational changes in the Hc domain probably facilitates subsequent binding to protein receptor (Chen et al. 2008; Yowler and Schengrund 2004). Copresence of the *ad hoc* ganglioside(s) and protein receptors likely facilitates the identification of cell subset targeted by TeNT or BoNTs at very low concentrations encountered in the physiological medium during the disease. At higher concentrations, binding to the protein receptor is likely sufficient for mediating toxin binding. Indeed the number of cell types affected by these toxins expands with increasing toxin concentrations. Therefore, BoNTs can target numerous neurons but not all, as well as nonneuronal cells at high concentrations, inhibiting the release of various compounds.

Neurotoxin bound to its receptor is internalized by receptor-mediated endocytosis. An essential difference between both types of neurotoxins is that BoNTs are directly endocytosed in recycling synaptic vesicles or clathrin-coated vesicles, which, when acidified, trigger the translocation of the L chain into the

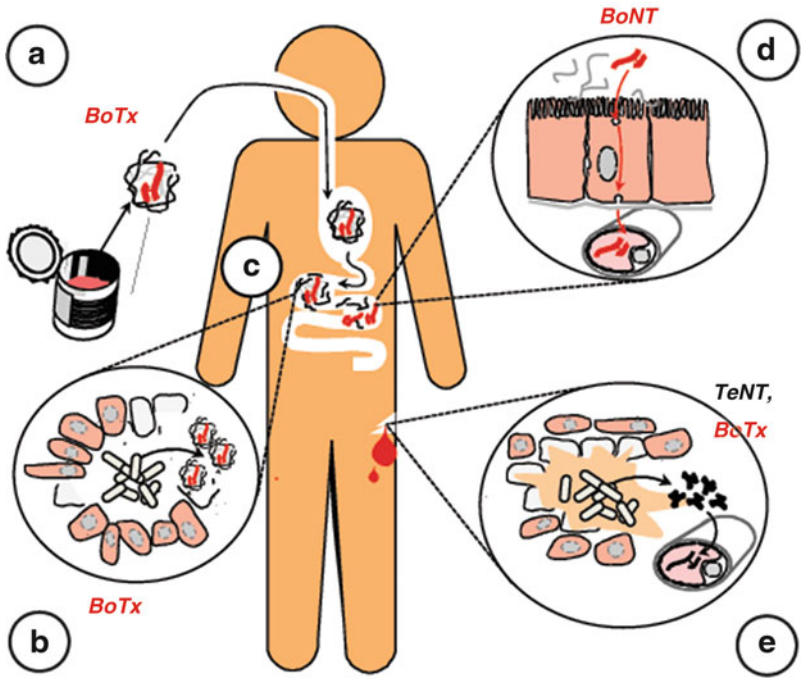

Fig. 12.3

Dissemination of tetanus and botulinum neurotoxin (a) Botulinum toxin (BoTx complex) produced in contaminated food is ingested (foodborne botulism). (b) Under certain conditions, *C. botulinum* spores can grow within the intestine, possibly favored by presence of lesions; this leads to production of BoTx in situ (infant and intestinal forms of botulism). (c) When the BoTx complex arrives in intestinal fluids with near neutral pH (i.e., duodenum, small intestine, and lower part), BoTx complex dissociates and releases the neurotoxin (BoNT). (d) BoNT undergoes transcytosis (passage) in the intestinal epithelial cells and is release in circulatory compartments (lymphatic system and bloodstream), making its dissemination in the whole body. (e) Colonization of anaerobic, necrotic wound by *C. tetani* (tetanus) or, rarely, by *C. botulinum* (wound botulism) leads to production of tetanus neurotoxin (TeNT) or BoTx complex, in situ, followed by dissemination of TeNT or BoNT

cytosol. Therefore, BoNT L chain is delivered in the peripheral nervous system to neuromuscular junctions where it blocks the release of acetylcholine leading to a flaccid paralysis. In contrast, TeNT enters different endocytic vesicles, which are not acidified. The vesicles retrogradely transport the toxin in a microtubule-dependent manner to the cell body of neurons in the spinal cord. Like nerve growth factors, TeNT is transported by tubulovesicular organelles characterized by the presence of neurotrophin receptor such as $p75^{NTR}$ (Bohnert et al. 2006; Bohnert and Schiavo 2005; Deinhardt et al. 2006a; b; Lalli et al. 2003; Lalli and Schiavo 2002). The C-terminal fragment of TeNT drives the retrograde transport of the toxin and can be used to transport heterologous protein in the same way (Li et al. 2001; Maskos et al. 2002). When released in the extracellular space, TeNT carries out a transsynaptic migration and reaches the target neurons, which are inhibitory interneurons involved in the regulation of the motoneurons. TeNT enters target inhibitory interneurons via vesicles that are acidified, thus permitting the delivery of the L chain into the cytosol, where it inhibits the regulated release of glycine and GABA. Overall, the mechanism of translocation is not completely understood for BoNT and TeNT. Acidification of the vesicle lumen triggers a conformational change of the neurotoxin and subsequent translocation of the L chain into the cytosol. H chains form tetramers and insert into lipid membranes, thus forming cation selective channels permeable to small molecules (<700 Da). It remains unclear whether a single H chain or a tetramer of it forming a transmembrane channel mediates the passage of defolded L chain. The N-terminal part of H chain mediates the translocation of L chain into the cytosol at acidic endosomal pH by modifying the electrostatic interactions with the phospholipids without detectable conformational changes. In addition, the disulfide bond between the two chains has a crucial role in the translocation process (Fischer and Montal 2007; Fischer et al. 2008; Galloux et al. 2008; Koriazova and Montal 2003). Then, the L chain refolds in the neutral pH of the cytosol. Cytosolic translocation factors such as β-COPI are possibly involved in this mechanism, as it has been found for diphtheria toxin (Humeau et al. 2000; Meunier et al. 2002b; Ratts et al. 2005; Schiavo et al. 2000).

L chains of all clostridial neurotoxins are zinc metalloproteases that cleave one of the three members of the SNARE proteins. TeNT and BoNT/B, D, F, and G attack synaptobrevin (or VAMP), BoNT/A and E cleaves SNAP25, and BoNT/C1 cut both SNAP25 and syntaxin. The cleavage sites are different for each neurotoxin except BoNT/B and TeNT, which

proteolyze synaptobrevin at the same site. Cleavage of SNARE proteins occurs only when disassembled. Since VAMP, SNAP25, and syntaxin play a major role in the regulated fusion of synaptic vesicles with the plasma membrane at the release sites, their cleavage induces a blockade of the neurotransmitter exocytosis.

SNAP25 cleavage by BoNT/A or BoNT/E deeply decreases both SNAP25 and Ca^{++} binding to synaptotagmin and subsequently the fusion process of exocytosis (◉ *Fig. 12.2*) (Gerona et al. 2000; Lynch et al. 2008; Sakaba et al. 2005; Tucker et al. 2004). Removal of the nine C-terminal amino acids of SNAP-25 by BoNT/A deeply disrupts the coupling between Ca^{2+} sensing and the final step in exocytosis (Sakaba et al. 2005). Truncated SNAP-25 can behave as a dominant negative mutant upon the exocytotic process suggesting that after BoNT/A treatment, the block of release is due to both functional elimination of SNAP-25 and accumulation of the cleavage product which competitively inhibits exocytosis (Apland et al. 2003; Gutierrez et al. 1997; Keller and Neale 2001). In contrast, blockade of exocytosis by BoNT/E is only due to cleavage of SNAP-25, not to the production of competitive antagonists of SNARE complex formation. Indeed, inhibition of exocytosis by BoNT/E can be rescued by supplementing the C-terminal portion of SNAP-25 removed by the toxin (Chen et al. 1999, 2001; Schuette et al. 2004). Truncation of SNAP-25 by BoNT/E destabilizes the four-helix bundle of the SNARE complex (Chen et al. 1999, 2001), and SNAP-25 truncated by BoNT/E is not retained by syntaxin (Bajohrs et al. 2004).

VAMP cleavage abolishes the interaction of VAMP with the adaptor protein AP3 and affect synaptic vesicle recycling via early endosomes (Salem et al. 1998). The SNARE cleavage products have also the potential to interfere with fusion processes (Cornille et al. 1995; Tucker et al. 2004). Consistent with synaptophysin-1 controlling specifically the targeting of VAMP2 but not VAMP1 to synaptic vesicles is the observation that the cytosolic cleavage product of VAMP2 but not VAMP1, released upon TeNT or BoNT/B activity, blocks the neurotransmitter release (Cornille et al. 1995). This result suggests an alteration of the exocytosis due to a disturbance of synaptophysin-1/VAMP2 interaction and of coupling between detecting Ca^{++} and synaptic vesicle triggering (Sakaba et al. 2005). Since the synaptic vesicles docked with unproductive complexes cannot fuse or undock, they stay at the fusion sites (with slightly increased numbers) irreversibly plugging the fusion sites that would normally accommodate intact vesicles. This progressively reduces the number of release sites to which exocytosis can occur as recently demonstrated for TeNT at identified *Aplysia* cholinergic synapses (Humeau et al. 2007). When VAMP is cleaved by TeNT and BoNT/B or /G, the VAMP portion (~20 amino acids) remaining in the synaptic vesicle membrane does not contain interaction sites for the other SNAREs. Therefore, the synaptic vesicle membrane is no longer linked to a SNARE complex, and fusion with the plasma membrane cannot occur. When VAMP is cleaved by BoNT/D or /F, the C-terminal fragment remaining in the vesicle membrane is long enough to anchor the synaptic vesicle to the SNARE complex, but fusion cannot occur because the SNARE complex cannot transit into the thermally stable four-helix bundle.

BoNT/C cleaves both syntaxin-1 and SNAP-25, but in vitro cleavage of SNAP-25 by BoNT/C occurs with low efficiency (~1,000-fold difference) versus cleavage by BoNT/A or /E (Foran et al. 1996) (Vaidyanathan et al. 1999). This raises the question which of the two targets is involved in BoNT/C neuroexocytosis blockade? In squid giant synapses, BoNT/C cleaves syntaxin-1, but not SNAP25 (O'Connor et al. 1997), whereas in cultured hippocampal slices or spinal neurons from mammal, BoNT/C efficiently removes nearly all SNAP25 (Capogna et al. 1997; Williamson et al. 1996). Thus, depending on the cell type, the secretory blockade is likely due to syntaxin and/or SNAP-25 cleavage. In addition, BoNT/C mutant, which only retains cleavage activity against syntaxin, blocks the neurotransmitter release further supporting the role of syntaxin in Ca^{++} triggered neuroexocytosis (Wang et al. 2011a). Upon syntaxin cleavage, SNARE complexes are formed but loosely docked to plasma membrane, thus synaptic vesicles remain tethered to plasma membrane and cannot fuse (discussed by (Poulain et al. 2008)) (◉ *Figs. 12.3*, *12.4*, *12.5*).

Although the physiological properties induced by the cleavage of either VAMP, SNAP25, or syntaxin are not equivalent at the neuromuscular junctions, all the clostridial neurotoxins cause a blockade of the regulated neurotransmission, which varies in intensity and duration according to each neurotoxin type. TeNT and BoNT/B share the same molecular mechanism. They are translocated in different subset of neurons (excitatory neuron: BoNTs >> TeNT; inhibitory neurons: TeNT >> BoNTs) which produce strongly different symptoms. This induces different clinical signs (TeNT: spastic paralysis; BoNTs: flaccid paralysis, ◉ *Fig. 12.5*). Indeed the peripheral dysautonomia and flaccid paralysis caused by BoNTs result from preferential inhibition of acetylcholine release. In the spinal cord or facial motor nuclei, TeNT-mediated blockade of glycine or GABA release disrupts the negative controls exerted by the inhibitory interneurons onto the motoneurons turning on excessive firing of the motoneurons and ensuing muscle contraction (review in (Meunier et al. 2002a; Poulain et al. 2006, 2008; Schiavo et al. 2000)).

Duration of Intoxication

The main factor governing the duration of intoxication is the BoNT toxinotype. The half-lives of exocytosis blockade in rat cerebellum neurons are more than 31 days for BoNT/A, more than 25 days for BoNT/C1, about 10 days for BoNT/B, about 2 days for BoNT/F, and less than 1 day for BoNT/E, and these durations correlate with the paralysis duration (Foran et al. 2003; Keller et al. 1999; O'Sullivan et al. 1999). The factors governing the toxin longevity within the nerve terminals are not fully unraveled, yet presence of a N-terminal sequence and a C-terminal dileucine motif in BoNT/A L chain, and not in the other toxinotypes, may explain the retention of BoNT/A L chain to the plasma membrane (Fernandez-Salas et al. 2004). Other key aminoacids have been identified (Wang et al. 2011) allowing engineering of novel forms of botulinum neurotoxins with modified duration effects (Dolly et al. 2011). Other factors

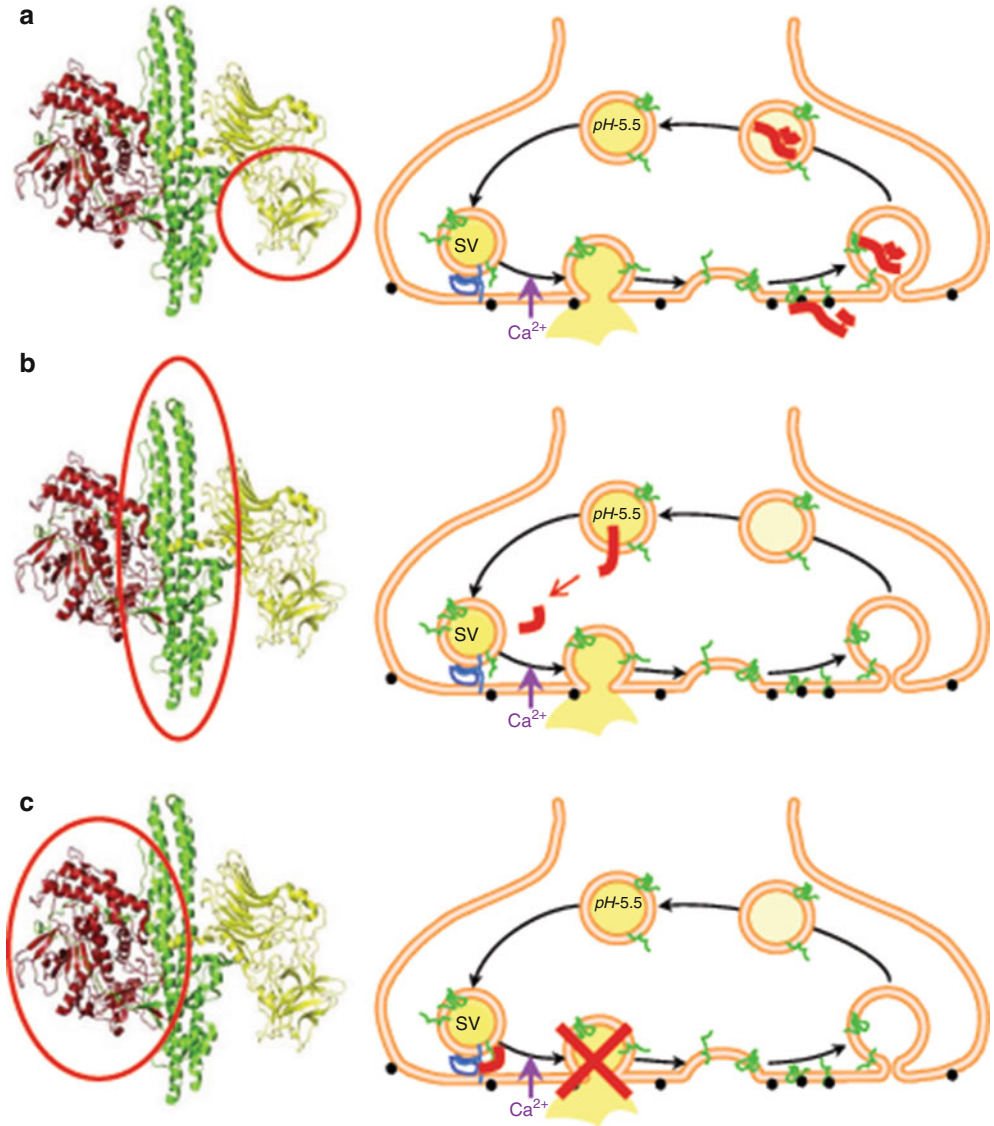

Fig. 12.4

Synaptic vesicles act as Trojan horse in promoting BoNT internalization Left: 3D structure of BoNT. *Right*: schematic representation of a nerve terminal. (a) Upon fusion of synaptic vesicle (SV) with plasma membrane, the luminal domain of their integral proteins (*green*) is exposed at the surface of the neurons nerve ending. Thus, the H_{CC} domain of BoNT molecules (circled in *red*) binds both to gangliosides (*black dot*) and their protein receptor (a SV protein as SV2 or synaptotagmin). Then, BoNT molecules (bound to their receptor) are trapped into nascent recycling vesicles: they are internalized. (b) Refilling of recycled SV with neurotransmitter is mediated by specific transporters which driven force is given by accumulation of protons in the SV lumen. This acidification triggers unfolding of endocytosed BoNT, insertion of H_N chain (circled in *red*) in SV membrane. This allows translocation of L chain into the cytosol. (c). Here the L chain (circled in *red*), which is a Zn-metalloendopeptidase, cleaves its SNARE target, preventing fusion of SV with plasma membrane

should be considered: in contrast to BoNT/E truncation, SNAP25 lacking 9 C-terminal residues, released by BoNT/A proteolysis, remains localized at the membrane in association with syntaxin. Therefore, unproductive SNARE complex at the active zone induces long-term impeding of synaptic vesicle fusion, whereas SNAP25 cleaved by BoNT/E cannot bind to syntaxin and is released into the cytosol (Bajohrs et al. 2004; Fernandez-Salas et al. 2004).

To summarize, despite the scenario that the blocking actions of the various BoNTs differ at the molecular level, they all share several commonalities. Indeed, in all cases following toxin action, the formation or stability of the SNARE complex is compromised (Hayashi et al. 1994; Pellegrini et al. 1995), and it is no longer fusogenic. Thus, synaptic vesicles remain docked at the fusion sites with slightly increased numbers, suggesting

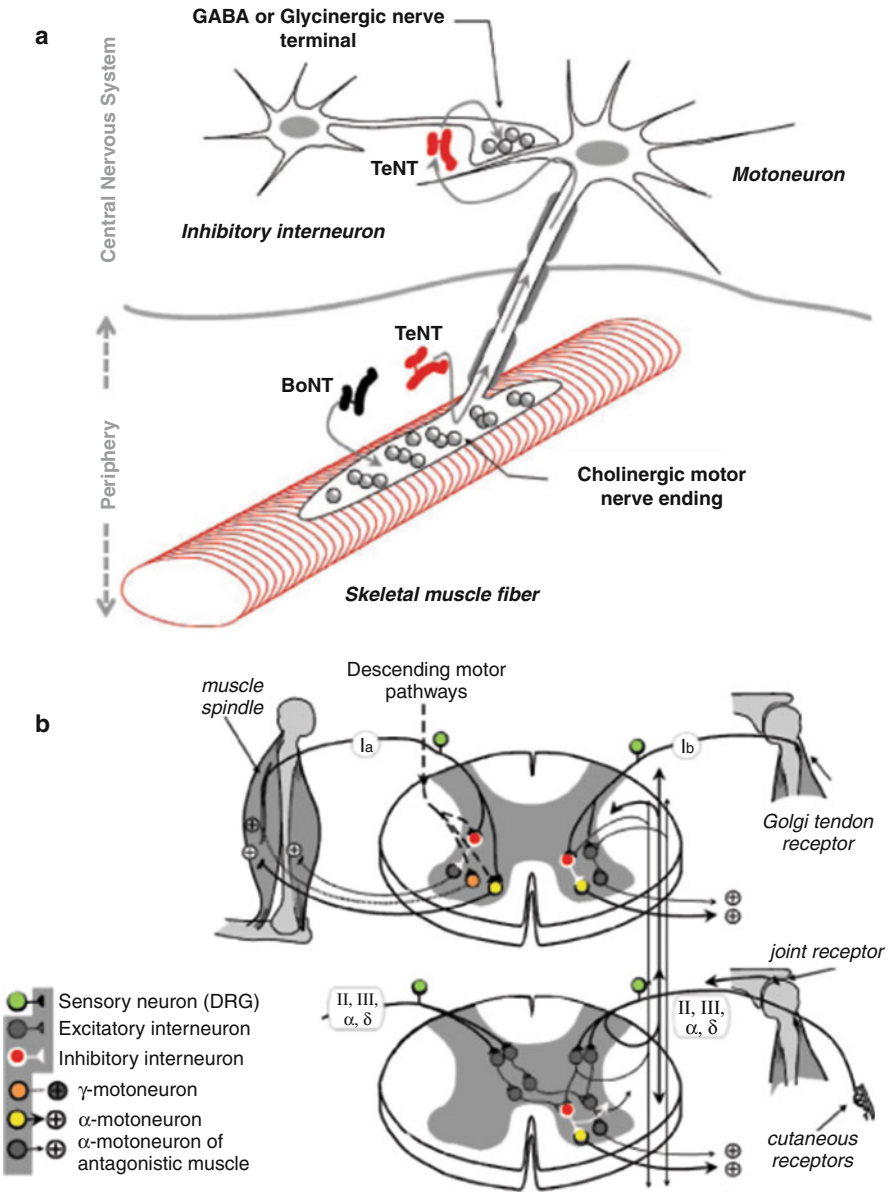

Fig. 12.5

Clostridial neurotoxins and their neuronal targets in periphery and central nervous system (a) *Different sorting of endocytosed BoNT or TeNT into motoneurons*. Neurotoxin molecules taken up in the periphery by the motor nerve endings are sorted in different vesicles. BoNT (*red*), which is into acidic vesicles, is quickly translocated in the cytosolic compartment of motor terminals where it blocks ACh release. TeNT (*black*) is sorted in neutral vesicular cargos undergoing retroaxonal ascent. Then, TENT is exocytosed in the extracellular space surrounding the motor cell body and recaptured by inhibitory (GABAergic, glycinergic) neurons. Here, TeNT is sorted in acidic vesicles allowing is translocation into cytosol and ensuing attack of the release machinery. (b) *The neuronal circuit in spinal cord and clostridial neurotoxins*. Descending pathways, originating from cortical motor areas, project on both motoneurons and interneurons. The neuronal circuit for the myotatic reflex is comprised of the sensory Ia neurons, which cell body (*green*) lies in dorsal root ganglion (DRG) and T-shape axons convey information from the muscle spindle toward (excitatory connections) α- (*yellow*) or γ- (*orange*) motoneurons innervating the homonymous (or synergistic) muscles, and inhibitory interneurons (*white*) neurons downregulating activity of motoneurons (*dark gray*) innervating the antagonistic muscles. Other sensory neurons, which cell somata lies in the DRG (*green*), convey sensory information from tendon, joint and cutaneous receptors and are implicated in more complicated circuits, activating inhibitory or excitatory interneurons. Collateral branches of the interneurons allow activation of interneurons in the neighboring, upper or lower, spinal segments. BoNT action is prominent on nerve endings of the α-motoneurons (*yellow*), while terminals of γ-motoneurons (*orange*) are not exposed due to their insulation by spindle capsule. TeNT can undergo retroaxonal ascent in both α-motoneurons (*yellow*) and sensory neurons (DRG, *green*). In the latter, it stops at the DRG level. Following it transcytosis in motoneurons, it can attack spinal inhibitory neurons (*white* with *red dot*), thus disinhibiting the motoneurons. This results in periphery in cocontraction of muscles and their antagonists

that when tethered to the plasma membrane or docked in an unproductive complex, not only exocytosis but also undocking cannot proceed. Therefore, it is likely that synaptic vesicles docked with unproductive complexes can irreversibly plug the fusion sites that would normally accommodate intact vesicles.

Nonproteolytic Molecular Actions of BoNTs and TeNT

Yet a direct cause-effect relationship exists between the cleavage of the SNAREs and the blockade of neuroexocytosis by BoNTs or TeNT (Humeau et al. 2000; Poulain et al. 2008; Rossetto et al. 2006), a controversial possibility is that BoNTs and TeNT may interfere with exocytosis and other cell functions *via* molecular actions unrelated to their proteolytic activity. Indeed, when mutated in the catalytic site at positions crucial for either Zn^{++} binding (His233 and His237) or cleavage of the Gln-Phe bond in VAMP-2 (Glu234), TeNT L chain cannot cleave VAMP-2 in vitro (Li et al. 1994; Yamasaki et al. 1994). However, several of point-mutated TeNT L chain constructs are able to produce inhibition of neurotransmitter release (His233->Ala233, Leu233 or Val233; Glu234->Ala234; His237->Ala237; Asp237, Gly237 or Val237) albeit with reduced potency as compared to wild-type TeNT L chain (Ashton et al. 1995; Niemann et al. 1994). Such a nonproteolytic mechanism may explain why endopeptidase blockers, which abolish VAMP-2 cleavage in vitro, counteract only partially the inhibitory action of TeNT on neurotransmitter release (Ashton et al. 1995; De Paiva et al. 1993). Moreover, the observation that antagonism of the intracellular action of BoNT/A can be relieved fast by the means of injecting monoclonal antibodies directed against the BoNT/A L chain (Cenci Di Bello et al. 1994) is difficult to conciliate with the proteolytic activity of the neurotoxin protein cleavage being in essence irreversible. The observation that TeNT binds with high affinity to, and strongly activates the GTP-binding protein transglutaminase type II (TGase II) in vitro, suggests that TGase II may participate in the intracellular action of TeNT (Facchiano et al. 1993; Facchiano and Luini 1992). However, the precise contribution of TGase II to the blockade of neurotransmission by TeNT has never been clarified and conflicting data exist in the literature (Ashton et al. 1995; Coffield et al. 1994; Gobbi et al. 1996). TGase II belongs to a large family of bifunctional and Ca^{++}-dependent cross-linking enzymes (Fesus and Piacentini 2002; Lorand and Graham 2003) abundant in neurons and nerve endings (Facchiano and Luini 1992; Maggio et al. 2001) which has been implicated in secretory mechanisms (Driscoll et al. 1997; Pastuszko et al. 1986; Walther et al. 2003). The identification of the vesicular protein synapsin I as one of the two main substrates cross-linked by TGase II needs to be considered for explaining part of the nonproteolytic TeNT-induced decrease in neurotransmitter release. Indeed, synapsin I regulates synaptic vesicle trafficking via interactions with the actin cytoskeleton and participates in post-docking steps of exocytosis (Baldelli et al. 2007; Humeau et al. 2001a). Possibly, TeNT stimulation of TGase II leads to reduced synaptic vesicle availability for release. This view is supported by several observations: (1) the depolarization-stimulated phosphorylation and redistribution of synapsin I are altered after the action of TeNT (Presek et al. 1992); (2) the blocking action of TeNT is diminished after disassembly of microfilaments (Ashton and Dolly 1997); and (3) the amplitude of post-tetanic potentiation, a plasticity paradigm which involves synapsin I in *Aplysia* synapses, is highly reduced after TeNT treatment (Humeau et al. 2001a, 2007). As TeNT can access VAMP-2 only during a defined "physiological window" (Humeau et al. 2000; Poulain et al. 2008), TGase II activation may modulate this access via the modification of proteins involved in regulation of the synaptic vesicle cycle. TGase II substrates include RhoA GTPase, several cytoskeletal proteins (Fesus and Piacentini 2002), SNAP-25 and Synapsin I (Facchiano et al. 1993; 2010). When endogenous TG2 is stimulated by TeNT, Synapsin I and SNAP-25 are covalently modified. TeNT-TG2-induced SNAP-25 modification may explain why TeNT mutants devoid of proteolytic activity against VAMP-synaptobrevin are still able to inhibit neurotransmitter release at Aplysia synapses (Facchiano et al. 2010). TeNT-TGase II-induced synapsin modification may explain why the amplitude of Post-Tetanic Potentiation at Aplysia synapses declines faster than basal neurotransmitter release upon TeNT treatment (Humeau et al. 2007). However, the importance of the proteolytic and nonproteolytic mechanisms of TeNT may be variable from one model systems to other and may depend on differential expression of endogenous TGase II. Contrasting with the observations made using brain synaptosomes or *Aplysia* preparations (Ashton et al. 1995; Niemann et al. 1994), nonproteolytic TeNT mutants have been found ineffective at the mouse hemidiaphragm (Li et al. 1994) or neurohypophysial nerve endings (Dayanithi et al. 1994), and participation of TGase II activation in the blockade of secretion by TeNT has been ruled out at the mouse neuromuscular junction and in NG108 cells (Coffield et al. 1994). In vitro, BoNT/E light chain has been reported cleaving actin, and all the 11 cleavages sites identified involved Arg or Lys residues in *P*'1 position exactly as in SNAP-25 (DasGupta and Tepp 1993). Thus, another unexpected intracellular effect of TeNT is the modification of actin cytoskeleton. This is supported by several observations: TeNT inhibits the rearrangements of subcortical microfilaments that accompany secretion in chromaffin cells (Marxen and Bigalke 1991). Actin cytoskeleton network is altered when TeNT L chain is expressed in Sertoli cells in mice (Eisel et al. 1993). Consistent with the well-documented implication of small GTPases Rho in the dynamics and organization of actin-based cytoskeleton (Hall 1998), BoNT/A has been reported to target RhoB to the proteasome, causing both blockade of exocytosis and actin cytoskeleton disorganization (Ishida et al. 2004). This may relate to a crosstalk between actin cytoskeleton remodeling and SNARE- and Rho-GTPase-dependent mechanisms of exocytosis, as illustrated for Cdc42 and VAMP-2 during insulin secretion (Nevins and Thurmond 2005).

TeNT shows unconventional cellular action time before its classical proteolytic effects became evident. Aguliera and Yavin first reported the in vivo activation and translocation

of protein kinase C in rat brain (Aguilera and Yavin 1990). Concomitantly, an increase of phosphoinositide hydrolysis was observed (Gil et al. 1998). The intracellular pathway activates phospholipase C-1 and other kinases (Gil et al. 2000). Among the different targets these enzymes can attain, the best characterized is the 5-HT transporter which is phosphorylated and has its activity modulated at low toxin concentration (10^{-12} M) in less than 30 min (Inserte et al. 1999; Najib et al. 2000; Pelliccioni et al. 2001). These effects are carried out by the half C-terminal part of the H chain, the portion that carries the binding domain of the toxin to the receptor (Chaib-Oukadour et al. 2004; Gil et al. 2003). It is of interest to note that this portion of the H chain is able to protect from death neuronal cells, in vitro as well as in vivo (Chaib-Oukadour et al. 2004, 2009; Mendieta et al. 2009). Indeed, TeNT Hc activates phosphatidylinositol 3-kinase (PI-3 K)/Akt (a serine/threonine kinase) as well as extracellular signal-regulated kinases 1 and 2 (ERK-1/2) pathways through phosphorylation of tyrosine kinase receptor leading to protection of apoptosis by preventing the proteolytic activation of procaspase-3, cytochrome c release from mitochondria, and chromatin condensation (Chaib-Oukadour et al. 2004, 2009; Gil et al. 2003). Interestingly, TeNT Hc prevents apoptosis induced by 1-methyl-4-phenylpyridinium (MPP+), which is a mitochondrial poison used to reproduce a Parkinson-like disease (Chaib-Oukadour et al. 2009). Since TeNT Hc is retrogradely transported to the central nervous system, it could be used in the prevention/treatment of the Parkinson's disease. In vivo in a rat model of Parkinson's disease, TeNT HC has been found to improve the dopaminergic system and to enhance the survival rate (Mendieta et al. 2009).

Botulism

Main Clinical Forms of Botulism

Three main forms of human botulism are recognized according to the mode of acquisition (◉ *Fig. 12.3*).

Intoxication or Foodborne Botulism. Foodborne botulism is due to ingestion of food in which *C. botulinum* has developed and produced sufficient amounts of BoNT. This is the most common form of disease in adults. Usually, several persons are intoxicated at the same time by sharing contaminated food. In the large majority of outbreaks of botulism, BoNT/A, /B, and /E are involved. Only few cases of human botulism due to type F, type C, or type D have been reported (Hatheway 1993b). It is questionable why types C and D botulism occur so rarely in humans. *C. botulinum* C and D seem to be unable to grow and produce toxin in foods for humans (Hatheway 1993b). Type G has been isolated from autopsy specimens, but its implication in human botulism has not been confirmed (Sonnabend et al. 1981, 1987b).

The incidence and the types of botulism depend on the occurrence of *C. botulinum* in the environment and subsequently in foods and on the cooking practices. The geographical distribution of the different types of botulism corresponds to the distribution of the different toxinotypes of *C. botulinum* in the environment. Type E botulism is mainly found in the colder regions of the Northern Hemisphere, and food based on fish and aquatic animals is usually involved (Hauschild 1989, 1993). Types A and B botulism occur generally in the temperate countries. Type A is predominant in the Western United States, Argentina, and China, and the most frequently implicated foods are fruits and vegetables.

In central and southern Europe, type B is prevalent, and meat has been recognized as the main source of these outbreaks except in Italy and Spain where vegetables are more frequently incriminated. In Italy, 412 cases of botulism have been recorded between 1988 and 1998, 65% were originated from home-canned vegetables (mushrooms, aubergine), 7% from ham and sausage, and 14% from industrial foods (tuna in oil, milk products) (There 1999). An outbreak of type A botulism was due to contamination of a milk product (mascarpone), which is a very unusual type of food substrate (Aureli et al. 1999). In Germany, 177 cases of botulism were reported from 1988 to 1998. Types A and B occurred from ingestion of home-canned vegetables or meat, and several type E cases were related to the consumption of smoked fish (There 1999). The incidence of botulism in France ranges between 10 and 45 annual cases with type B being most frequently involved, but with recent severe type A outbreaks (King 2008; Mazuet et al. 2011). More frequent was type B botulism in Poland with 317 annual cases between 1960 and 1998 (There 1999).

In the majority of outbreaks, botulism is due to home-canned, home-fermented products or home-processed slightly acidic vegetables. Preservation of fish and marine mammal meat by fermentation is the most common source of *C. botulinum* E. Home-canned or home-preserved fruit and vegetables are responsible of the majority of outbreaks in the United States, Spain, Italy, and China. Most of the implicated foods are vegetables home preserved in oil in Italy and fermented vegetables in China. Home-cured smoked ham is the major cause of botulism in several European countries in particular in France, Belgium, Germany, and Portugal (Hauschild 1989, 1993). However, in the recent period, commercial products are more and more involved in botulism outbreaks. Minimally heated, chilled foods such as ready-to-eat foods, ready meals, cook-chill foods, sous-vide foods, and refrigerated processed foods, which correspond to an increased demand of the consumers for high-quality convenience, are at risk for the development of *C. botulinum* notably nonproteolytic strains (Peck 2006). Examples of foodborne botulism throughout the world are shown in ◉ *Table 12.2*.

Botulism by Intestinal Colonization

Infant Botulism. Infant botulism results from ingestion of *C. botulinum* spores that germinate, multiply, and produce BoNT in the infant's gastrointestinal tract. The minimum infective dose for human infant has been estimated to 10–100 C. *botulinum* spores (Arnon 1989). Moderate to high toxin level (10–10^5 mouse lethal dose per gram) and viable *C. botulinum* are recovered from stools of affected infants for

a long period after the onset of the symptoms, as long as 158 days. The seriousness of these diseases varies greatly from a sudden death type of syndrome to a minor flaccid paralysis. The affected infants present constipation, difficulty of feeding, and hypotonia. In the more severe cases, the patient becomes lethargic and loses head control; ptosis and ophthalmoplegia and weak sucking may also be present. The disease progresses to a flaccid paralysis which may extend to respiratory muscles with arrest. Again, type A is generally more dangerous than type B or E, and the recovery time is accordingly longer (Arnon et al. 2001).

Infant botulism has been identified in many developed countries, but most cases were recorded in USA, particularly in California. It is presumed that contamination occurs with *C. botulinum* spores from the environment, and honey feeding has been implicated so far (Arnon 1989; Domingo et al. 2008; Koepke et al. 2008). However, the occurrence of infant botulism is low in Europe (Fenicia and Anniballi 2009; King et al. 2010; Wolters 2000).

Most of the *C. botulinum* strains involved in infant botulism belong to the group I (*C. botulinum* A and proteolytic *C. botulinum* B strains). One of the *C. botulinum* B strain produces also minor amount of BoNT/F. The other implicated *C. botulinum* strains include one *C. botulinum* type C, two BoNT/E producing *C. butyricum*, and one BoNT/F producing *C. baratii* strains (Hatheway 1993b). This could indicate that *C. botulinum* strains from group I are more able to colonize the infant's digestive tract. However, although belonging to the same group, the *C. botulinum* strains from infant botulism and from foodborne botulism could be different. The strains isolated from infant botulism in Japan fall into the group A2; the strains from foodborne and infant botulism from the USA and United Kingdom fall into the groups A1 and A2 (Cordoba et al. 1995). Host susceptibility factors are also involved in the colonization of the digestive tract by *C. botulinum*. In adult mice, the normal intestinal microflora prevents the growth of *C. botulinum* in the digestive tract, while infant mice are susceptible to colonization with *C. botulinum* within the first days of life (7–13 days) (Moberg and Sugiyama 1979). In human infants, most botulism cases have been recorded in newborn between 1 and 6 months of age at onset (Arnon 1989). Moreover, nutritional factors are also involved such as breast-feeding or formula-feeding and subsequent intestinal content pH and composition of the digestive microflora (formula fed infants have less acidic feces (pH 5.9–8) and lower numbers of *Bifidobacterium* than infants fed human milk (pH 5.1–5.4)). Infant botulism with more chronic symptoms seems to be correlated with breast-feeding, and infant botulism form associated to sudden infant death with formula-feeding (Arnon 1989).

Intestinal Toxin Infection in Adults. Colonization by *C. botulinum* and BoNT production in the intestine has been recognized as the botulism origin in certain adults. Predisposing factors such as intestinal surgery, antimicrobial agents, chronic inflammation, and necrotic lesions of the intestinal mucosa could support the growth of *C. botulinum*. *C. botulinum* A and B from group I are generally involved.

C. botulinum types A and B from group I are generally involved. One case with *C. botulinum* type F and two cases with BoNT/F producing *C. baratii* have been documented (Hatheway 1993b; Sonnabend et al. 1987a). Surgical modification of the bowel, achlorhydria, and antimicrobial use has been proposed as predisposing factors. Moreover, chronic inflammation and necrotic lesions of the intestinal mucosa due to underlying disease could support the growth of *C. botulinum* (Tacket and Rogawski 1989).

Wound Botulism. Wound botulism, like tetanus, results from colonization of a wound with *C. botulinum* and subsequent local production of BoNT. However, wound botulism is much rarer than tetanus, despite the fact that the general population is not immunized against BoNT, indicating that *C. botulinum* strains have a low ability to grow in wounds compared to *C. tetani*. A total of 47 cases of wound botulism have been recorded by the Centers for Disease Control (Weber et al. 1993a), caused by toxinotypes A or B from group I. Among the *C. botulinum* strains, those from group I seem to be the most capable of multiplication within the human host (Hatheway 1993b).

Other Forms of Botulism

Rare cases of inhalational botulism have been described, mainly in laboratory workers (Sobel et al. 2004). A few cases have been reported in patients who inhaled cocaine (Roblot et al. 2006). The 50% lethal dose by inhalation has been estimated to 550 mouse lethal intraperitoneal doses (MLIPD) per kg (0.017 μg/kg) for BoNT/A1 and 21,600 MLIPD/kg for BoNT/B in primates (Sanford et al. 2010), and to 0.010–0.013 μg/kg of BoNT/A in human (Arnon et al. 2001). Thereby, BoNT is considered as a potential biological weapon, which could be disseminated by aerosol (Smith 2006: Arnon 2001 #1264). In addition, iatrogenic botulism is a new form of this disease and can result from a hematological spread of therapeutic doses of toxin or from toxin overdoses (Chertow et al. 2006; Coban et al. 2010; Sobel 2005).

Animal Botulism. Botulism is rather common among domestic and wild animals, and types C and D are involved in addition to A, B, and E. Botulism of domestic animals causes economic losses, but it is also a source of a possible risk factor of transmission to human. Animal botulism can be contracted by eating carcass or chewing on bones contaminated with BoNT, by drinking water from contaminated ponds, or by ingestion of forage poisoned by diffusion of BoNT from carcass decomposition of small animals or birds. Among fish and birds, botulism outbreak can involve up to millions of individuals, and the diseases may take the appearance of an epidemic because insect larvae grow in the decomposing cadaver becoming full of BoNT, which is innocuous to insects. Healthy birds and fish are eager of larvae; they become intoxicated and die providing a rich anaerobic medium for the growth of Clostridia and for the deposition of eggs by insects. A self-perpetuating cycle is thus obtained, and rapidly many individuals can die particularly when the animal population is dense as in farms. Where botulism is common, animals often carry in their digestive tract *C. botulinum* which rapidly the carcass becomes highly toxic. Poultry litter often containing chicken carcass are capable of transmitting botulism to cattle (Kennedy and Ball 2011; Payne et al. 2011; Smart et al. 1987; Smith and Sugiyama 1988). In the Netherlands, an outbreak of

type B botulism has been observed in cattles contaminated by eating brewer's grains containing *C. botulinum* B and BoNT/B (Breulink et al. 1978; Notermans et al. 1981). *C. botulinum* B has been recovered in the rumen and feces of the affected animals and also in pasture the cattle were feeding on (Notermans et al. 1981). Silage which is wrapped in plastic and non-acidified can be also a source of botulism for cattle. Although rare, raw milk contamination with *C. botulinum* spores may be responsible for transmission of botulism to human through the dairy chain (Lindstrom et al. 2010).

In France, type D botulism was responsible of important losses in cattle. The animals were contaminated by grazing pasture containing poultry litter which was fed with meat derived from dead animals, including some suspected of botulism. Hen are relatively resistant to BoNT/D (Smith and Sugiyama 1977), and no outbreaks of botulism were observed in the hen farms. *C. botulinum* type D was identified in meat meal, feedstuff for hen, poultry litter, carcass of hen died for unknown reason, and cattle grazing pasture containing poultry litter who died with paralytic symptoms (Popoff 1989; Popoff et al. 1986).

Botulism is also frequent in domestic and wild birds throughout the world. Almost all species of birds are susceptible to botulism, usually type C is involved (Eklund and Dowell 1987). Type C botulism outbreaks have been observed in battery-reared turkeys and chickens in the United States, the United Kingdom (Smart 1983; Smart and Roberts 1977), and in France (unpublished), and in water birds in England (Dorland et al. 1977; Smith and Oliphant 1983), Netherlands, America, Australia, Japan (Eklund and Dowell 1987), and in wild ducks in France (Gourreau et al. 1986). Equine botulism occurs sporadically throughout the world and is commonly due to types C and D, but some type B outbreaks have been reported (Haagsma et al. 1990; Smith and Sugiyama 1988). But animal botulism has also been identified in many other animal species including minks, sheep, monkeys, dogs, sea lions, and lions (Dodds 1993; Smith and Sugiyama 1988).

Clinical Symptoms and Physical Findings During Botulism Pinpoint a Peripheral Action of the Botulinum Toxins

The clinical picture of botulism often initiates as a dysautonomia, followed by motor paralysis. There is no marked distinction in symptoms and physical findings displayed by the various forms of botulism. However, consistent with higher lethality of type A versus type B botulism, the motor findings appear more severe during type A botulism than during type B (Hughes et al. 1981). Initial symptoms of dysautonomia include visual symptoms (blurring, diplopia, and presbytia), followed by dry mouth and constipation. Dysautonomia induced by botulinum toxin can sometimes be the only manifestation of botulism and likely corresponds to a benign form of the disease associated with very low amounts of toxin. The nausea, vomiting, and diarrhea often reported during food botulism may be due to ill-defined enterotoxins unrelated to botulinum toxin. Indeed, foodborne botulism can be considered as the result of the ingestion of a cocktail of several toxins produced by several Clostridia or other bacteria growing together with *C. botulinum* into the contaminated food.

The many symptoms of autonomic dysfunction during botulism are consistent with botulinum toxin acting upon both cholinergic and catecholaminergic neurotransmission but restricted to the peripheral nerve system. Several of the cardiovascular reflexes can be abnormal during botulism. Loss of vagal cardiac control, hypothermia, and urinary retention pinpoint an action of botulinum toxin upon the parasympathetic system. Hypotension without reflex tachycardia, and depressed vasomotor response to postural changes, indicate an action upon the sympathetic nervous system. Effects of BoNTs are exerted at pre- and postganglionic levels, since ganglions are not insulated by the blood–brain barrier. Gastrointestinal troubles observed during foodborne and infant, but not wound, botulism suggest that BoNTs can directly (without hematogenous dissemination) affect neurons of the enteric nervous system.

The second series of manifestations during botulism consists of symmetric weakness or descending paralysis. Observation of this symptom prompts the clinician to diagnose botulism, but, confusion is possible in making diagnosis (Arnon et al. 2001). By contrast with the Guillain-Barre and certain myasthenic syndrome, botulism is characterized by an absence of sensory symptoms. Flaccid paralysis clearly indicates that BoNT effects on the motor system which initially affects striated muscles innervated by cranial nerves producing ptosis, followed by a depressed gag reflex, dysphagia, dysarthria, facial paralysis, tongue weakness, and several neuro-ophthalmological signs. After these early symptoms, general paralysis extends from proximal, to distal, muscles of the neck and limbs. The deep tendon reflex is strongly depressed. When the diaphragm is attacked, respiration is depressed, and the need for mechanical ventilation becomes imperative (reviewed by Hughes 1981; Low 2002; Tacket and Rogawski 1989). Overall, the clinical picture of botulism indicates that flaccid neuromuscular paralysis is due to selective inhibition of neurotransmission, ensuing from the blockade of ACh release, between α-motoneurons and striated muscle fibers. BoNT also blocks transmission between the alpha motoneurons and the muscle spindle, albeit with different kinetics as compared to the motor endplate (Filippi et al. 1993; Rosales et al. 1996). This latter action of the neurotoxin alters the spinal reflexes initiating from the muscle spindle via the sensory Ia fibers.

The large spectrum of application of botulinum toxins in therapy fits very well – at the exception of the potential use of the toxin in pain – with the various manifestations observed during botulisms. Thus, injecting of botulinum toxin produces a local form of botulism, limited in extent to the tissues close to the injection site(s). Both components – dysautonomia and paralysis – of botulism are exploited. Dysautonomic symptoms, as per side effects observed following therapeutic injection of toxin, correspond to hematogenous dissemination of minute amounts of BoNT from the injection site (reviewed by

(Dressler and Benecke 2003)). These latter undesirable manifestations are more frequent with the therapeutic use of type B toxin rather than with type A. Perhaps, this is related to the fact that during type B botulism, the motors symptoms are less marked as compared to type A, whereas dysautonomia manifestations look similar (Hughes et al. 1981), and higher amounts of type B than type A toxin need to be used to produce similar paralytic effect.

Tetanus

Tetanus is a major infectious disease caused by a wound contaminated with *C. tetani*, and it is characterized by persistent tonic muscle spasms with episodes of exacerbation. Several clinical forms of tetanus have been described: local, generalized, cephalic, and neonatal (Bleck 1989). Each form has a clinical relevance because of its prognostic value; however, these clinical entities share common cellular and molecular mechanisms. In all these forms, TeNT is the only etiological cause of neurological symptoms. Other secreted proteins like tetanolysin do not act on nerve cells, but their cytotoxicity may favor abscess formation and growth of *C. tetani* in an anaerobic environment. Although the gut may sometimes offer physiological conditions that promote spore germination of various Clostridia, including *C. tetani*, until now there has been no evidence for an intestinal form of tetanus. It has been hypothesized that the lack of associated protective proteins facilitates toxin degradation in the digestive tract. It is also possible that TeNT cannot cross the gut barrier due to a paucity of receptors that might otherwise enable transepithelial movement (Maksymowych and Simpson 1998).

Dissemination of TeNT and Physical Findings During Tetanus and Poisoning Experiments

Local tetanus consists of persistent muscle spasms in close proximity to the infected site. A rare variant is cephalic tetanus, which is caused by *C. tetani* growth in a wound on the head or neck. The spastic paralysis (i.e., a peripheral physical finding) observed during local tetanus has a central origin with disinhibited motoneurons. TeNT cannot cross the blood–brain barrier composed of tightly sealed endothelial cells that form the spinal cord and brain capillaries. Compelling evidence (Wellhöner 1992) has shown that the entry port of TeNT into the central nervous system is the motoneuron, which essentially acts like a Trojan horse. Indeed, the TeNT molecules first bind to motor cholinergic nerve endings responsible for innervating striated muscles, followed by endocytosis and transport toward the spinal cord (*Fig. 12.5*). Transport studies with 125I-labeled TeNT reveal a retrograde axonal ascent of TeNT along the motor axons. Indeed, 125I-TeNT was found in the ventral roots (i.e., containing motor axons) and ventral horn of the spinal cord (i.e., the area containing motoneuron cell bodies). Apparently, α-motoneurons that innervate striated muscle fibers and γ-motoneurons that innervate the muscle spindle can both take up, as well as transport, TeNT. Toxin labeling of the dorsal root, which contains sensory fibers, has also been reported. However, this toxin transport does not seem relevant for the pathophysiology of tetanus because ligature of the dorsal (sensory) root does not prevent labeling of the spinal cord. Moreover, recent studies with a recombinant, fluor-labeled TeNT-fragment reveal binding, uptake, and transport of TeNT in motoneurons, but only binding without transport in dorsal root neurons (Bigalke and Shoer 2000; Halpern 1995; Wellhöner 1992). Identification of receptors at motor nerve endings that enable internalization of TeNT, as well as the identity of any facilitating molecule(s) implicated in retroaxonal transport, still remain unresolved. Although not presented here, these issues are fully discussed in TeNT molecules, when retroaxonally transported, arrive at the motoneuron soma and dendrites where they are released into the extracellular space. This transcytosis through motoneurons allows the toxin to bypass the blood–brain barrier and reach the vicinity of nerve terminals that are afferent onto the motoneurons. High TeNT concentrations can be found in particular compartments of the central nervous system during tetanus: for instance, release of one TeNT molecule into a small volume like a synaptic cleft ($\sim$ 1 µm $\times$ 1 µm $\times$ 50 nm) leads to a "concentration" of $\sim$30 nM! According to in vitro experiments, TeNT molecules are recaptured by different types of nerve endings, independent of the type of neurotransmitter molecules they release (Bigalke and Shoer 2000; Wellhöner 1992). Consistent with the preferential blocking action of TeNT on GABA and glycine release observed in vitro, TeNT suppresses the inhibitory inputs afferent to motoneurons, while the excitatory inputs, which constantly fire motoneurons, are not modified. Therefore, motoneurons are disinhibited, and their firing rate increases, inducing sustained muscle contractions that lead to spasms and rigidity. Both the inhibitory descending controls and spinal reflexes which converge onto motoneurons are deeply altered. Spindle sensory inputs conveyed by Ia fibers during physiological conditions positively activate the synergistic motoneurons afferent to the same (homonymous) muscle and simultaneously inhibit motoneurons to antagonize muscles through inhibitory interneurons. Therefore, upon TeNT-induced blockade, or when TeNT blocks the inhibition pathway mediated by Ia interneurons, the myotatic reflex is deeply altered muscles working the same joint. TeNT also depresses recurrent inhibition, implicating Renshaw cells. Inverse myotatic reflex involves Ib fibers coming from the Golgi tendon organ and participates in a negative feedback system preventing further development of muscle tension upon reaching the desired point. Alteration of this reflex leads to an uncontrolled increase in muscle tension, causing muscle and tendon damage. In a similar way, the many reflexes based on polysynaptic circuits converging on inhibitory interneurons are disorganized. Therefore, all local stimuli causing an increase in excitatory input (e.g., sensorial or noxious stimuli) leads to local exacerbation of muscle contractions (Bleck 1989; Wellhöner 1992). The situation is not too different in generalized tetanus. TeNT molecules primarily taken up by motor endings can

undergo other transcytotic cycles throughout neurons of second, third, etc. order (Manning et al. 1990). Thereby, TeNT entering a single peripheral site and released into the spinal cord disseminates vertically to proximal spinal segments and horizontally to the contralateral side of the spinal cord, thus resulting in progressive muscle rigidity in all muscles. Retroaxonal transport of TeNT along a chain of functionally connected neurons has been exploited to trace the projection pathways between distant brain structures (Cabot et al. 1991). Furthermore, to study trafficking pathways and connectivity in sensorimotor circuits, several fusion proteins have been engineered using the atoxic TeNT C-subunit (TTC) fused to a reporter gene such as *LacZ*, GFP (green fluorescent protein), or EGFP (enhanced green fluorescent protein) after direct injection of the hybrid proteins (Coen et al. 1997; Miana-Mena et al. 2002), or expression as a transgene in mice (Maskos et al. 2002). When there is a hematogenous portal of entry for TeNT, it enters all motor nerve terminals and reaches the spinal cord at a number of sites. The most dramatic form of the disease, called *generalized tetanus*, then occurs and is characterized by an overall increase in muscle tone, rigidity, and trismus (Bleck 1989) Almost all spinal reflexes based on GABAergic and glycinergic transmission are abolished, leading to horizontal and vertical spreading of the response from any stimulus. Therefore, a local stimulus leads to a general response. For example, application of a cutaneous stimulus on a limb can induce profuse contraction of the face, neck, trunk, arms, and legs. When the spasm affects the diaphragm, respiratory failure and death can ensue. Although TeNT injected directly into the brain can induce epilepsy (Benke and Swann 2004), no central disorder like seizure is observed during tetanus (Bleck 1989). Therefore, the intrathecal dissemination of TeNT remains largely confined to the spinal cord. Several other symptoms are detected during generalized tetanus and indicate that TeNT acts also on the autonomous nervous system (e.g., hypertension due to vasomotor spasm, tachycardia). This hypersympathetic condition is due to disinhibition of the sympathetic reflexes, analogous to that for motoneurons. This is fully consistent with a report that TeNT is also taken up and retrogradely transported in adrenergic neurons. This is followed by toxin delivery to the inhibitory interneurons, whose terminals are afferent to the cell body of adrenergic neurons. Alteration of functions linked to the parasympathetic system has been reported too (Bleck 1989; Wellhöner 1992). All of the above mentioned physical findings observed during local tetanus have a unique origin: TeNT blockade of inhibitory neurotransmitter (GABA and glycine) release.

When the local concentration of TeNT is high, it can also block the release of other neurotransmitters. A high dose of TeNT injected into the lateral rectus muscle of the cat blocks both inhibitory and excitatory synapses simultaneously in abducens neurons (Gonzalez-Forero et al. 2003). At the neuromuscular junction (NMJ), TeNT inhibits ACh release, thereby inducing flaccid muscle paralysis in vitro (Wellhöner 1992) and during disease (e.g., cephalic tetanus in humans) (Bleck 1989). In certain animal species, flaccid motor paralysis may be the only action of the toxin. For instance, application of TeNT into the periphery of goldfish does not produce central actions but it inhibits ACh release from motor nerve endings. This is consistent with motor terminals being the primary binding targets for TeNT. When captured by motoneuron endings, TeNT is preferentially routed to a transport compartment whose neutral pH prevents translocation into the cytosol of motor nerve terminals. This preferential routing is not absolute, and TeNT appears far less potent than BoNTs in inhibiting ACh release by a factor of 100–1,000. This trafficking seems to depend strongly upon the TeNT receptor. Indeed, when the active moiety of TeNT is recombined with the binding domain of BoNT, it acquires a very high efficacy in cholinergic nerve terminals (Wellhöner 1992).

To summarize, the clinical and experimental findings of tetanus reveal a prominent inhibitory action of TeNT on the release of GABA and glycine. Complexity of the neuronal network and an inherent difficulty in dissociating direct, from indirect, effects has not allowed identification of other neuronal targets for TeNT. However, the in vitro experiments clearly indicate that TeNT blocks release of many, if not all, neurotransmitters.

Botulinum Neurotoxin Therapy

Many Potential Indications

Consistent with the well-established action of botulinum neurotoxin on ACh exocytosis, injection of small doses of botulinum toxin into muscles or glands innervated by cholinergic nerve endings is currently used a pharmacological tool to disrupt motor or secretion command in a localized and long-lasting manner. Since the pioneer use of botulinum toxin type A in strabismus (Scott 1980), thousands of papers have addressed an amazing large number of indications potentially benefiting of botulinum toxin. This includes the syndromes related to focal overactivity in skeletal, sphincter, and other muscles (e.g., blepharospasm, hemifacial spasm, cervical dystonia, other dystonia, dysphonia, writer's cramp, limb spasticity secondary to cerebral palsy or stroke, anal fissure, overactive bladder), hypersecretory activity of glands (hyperhidrosis, hypersialorrhea, gustatory sweating as Frey syndrome), drooling associated with Parkinson's disease, and muscle or gland hypertrophy (masseter hypertrophy, benign prostatic enlargement). Consistent with the inhibitory action of botulinum toxin on glutamate and peptides (e.g., CGRP) release, botulinum toxin type A has been assessed in animal to reduce neurogenic inflammation by blocking the release of glutamate from nociceptive fibers (Aδ and C) and ensuing pain (Cui et al. 2004; Favre-Guilmard et al. 2009; Meng et al. 2009). Toxin is tentatively used in the case of numerous pain syndromes in man. Use of botulinum toxin in facial aesthetics is in essence closely related to the above mentioned therapeutic indications: erasing wrinkles is obtained by toxin-induced relaxation of the muscle(s), whose contraction is responsible for skin folding.

Despite the extensive literature produced on many indications (reviewed by (Jankovic 2009; Truong et al. 2009), the consensus assessment studies published in the last decade have

established that botulinum toxin-based therapy is proved to benefit to the patients in a restricted number of indications (Naumann et al. 2008; Simpson et al. 2008); several examples are mentioned in *Table 12.3*). This does not mean that botulinum toxin therapy is unsafe or useless in the case of the other indications: this just indicates that the quality of scientific evidence provided by the clinical studies is not sufficient (too small number of case; presence of bias, marginal statistical validity) to recommend botulinum toxin as an evidence-based treatment.

Toxin Products for Clinical Use

Overall, toxin products available on the market are made from purified toxin or neurotoxin, to which is added several excipients. Although the formulation of the few products approved by the United State and European authorities is known, there exists on the world market non-approved products, the formulation of which remains unclear.

Most of the approved formulations are based on toxin type A and each of them approved for a restricted number of indications in neurology, rehabilitation medicine, ophthalmology, or aesthetic. The formulations with a marketing authorization in the European Union or United States of America contain toxin or neurotoxin type A produced by strains closely related to the strain Hall of *C. botulinum* (Botox®: (Zhang et al. 2003); Xeomin®: (Bigalke 2009), Dysport®: (Panjwani et al. 2008)). Thus, these formulations should be comprised of neurotoxin subtype A1. All the formulations derived from these products (Vistabel® and Botox Cosmetic® derived from Botox®, Azzalure® derived from Dysport®, and Bocouture® from Xeomin®) contain also of the subtype A1. Note that in the early literature, bivalent strain NCTC 2916 A2/B1 has been often incorrectly mentioned for Dysport®. Overall, the other A subtypes and associated complex proteins remain insufficiently characterized, and studies remain at preclinical stage (for recent studies on A2 subtype see (Akaike et al. 2010)).

Botulinum toxin type B has similar clinical gross action as type A but needs to be injected at much higher doses than type A to produce similar myorelaxing effects in man (Sloop et al. 1997). Only a formulation of toxin type B (Myobloc®/NeuroBloc®) is approved, it is based on a toxin produced by the strain Bean of *C. botulinum* and is most probably of subtype B1.

Toxin type C has been tested in the humans (Eleopra et al. 2002, 2006) and found producing similar effects as type A (similar efficacy and duration of paralysis). Although toxin type C has been reported to cause neurodegeneration when applied to cultured spinal cells (Williamson and Neale 1998), no such effect has been reported following its intramuscular injection (Eleopra et al. 2002; Morbiato et al. 2007). To date, too little is known about toxin complex type C to determine whether the cytotoxic associated proteins present in its complex (Jin et al. 2009) make the action of the toxin (complex) type C significantly distinct from that of purified neurotoxin type C.

Lack of epidemiological form of human botulism type D together with the report that botulinum toxin type D fails to induced paralysis of surgically excised human muscles (Coffield et al. 1997) indicates that this serotype is useless in human therapy.

Toxins of types E and F have been tested in humans (Eleopra et al. 1998; Greene and Fahn 1993). As compared to type A toxin, their effect is short lasting (Eleopra et al. 1998; Mezaki et al. 1999).

To this list should be added new products under development that are based on reengineered neurotoxins. They are based on the idea that BoNT has a modular structure, and each module can be either modified or exchanged to produce neurotoxins with new properties. A derivative from type A neurotoxin has been produced in which the C-terminal half of heavy chain has been replaced by lectin from *Erythrina cristagalli* has been produced (Duggan et al. 2002). Since this lectin specifically binds to galactose, this allows retargeting of the neurotoxin biological activity against nerve terminals expressing this sugar on its plasma membrane. This is the case of neurons from the dorsal root ganglion (DRG), whose peripheral axon is specialized in the transfer of noxious information (nonmyelinated C fibers) and central branch makes synapse in the upper lamina of spinal cord. This construct inhibits GCRP release with high efficacy. Its intrathecal administration in rodent induces potent resistance to pain tests (Chaddock et al. 2004). Identification of the key amino acids that determine the intraneuronal life duration of light chain (Fernandez-Salas et al. 2004; Wang et al. 2011b) is exploited to engineer novel forms of botulinum neurotoxins (e.g., derivative of BoNT/E with longer duration effects) to improve and/or extend therapeutic applications (Dolly et al. 2011).

Depending on the manufacturers, formulations include toxin (i.e., complex comprising a neurotoxin, a NTNH, and several HAS) or purified bichain neurotoxin. The complex toxins used may differ in size or by accessory proteins. The different sizes of the complex are indicated in *Table 12.4* (column 7). According to Allergan, Botox®, and derived products Botox Cosmetic® and Vistabel®, should contain only the ~900 kDa (i.e., LL) complex (Zhang et al. 2003). It is suggested that Dysport® and its derived product Azzalure® contain a mixture of the 600 and 900 kDa (i.e., L and LL) complexes (Panjwani et al. 2008). Given the published stoichiometry in B-type complex, Myobloc®/NeuroBloc® is likely containing only complexes of intermediate size L; however the proportion of bichain neurotoxin versus unnicked single chain is unknown. Xeomin® and derived product Bocouture® are based on neurotoxin produced by purification to homogeneity, after dissociation of the toxin (complex) (Jost et al. 2007).

All the formulations available on the market include several excipients, some of which vary from one commercial product to another. The presence of human serum albumin is designed to minimize nonspecific adsorption of the toxin or neurotoxin on the walls of syringes and vials. It is well tolerated by patients. Its amount per vial varies with the formulations, even from the same manufacturer. For example, vials containing 500 units Dysport® and 125 units Azzalure® differ from a ratio of 1–4 for the amount of toxin A but contain the same amount of

Table 12.5
New toxin names according to the USAN COUNCIL

Manufacturer	New name	Product(s)
Allergan Inc. (CA, USA)	OnabotulinumtoxinA	Botox®, Botox Cosmetic®
IPSEN Ltd. (GB)	AbobotulinumtoxinA	Dysport®
Merz Pharmaceuticals Gmbh. (Allemagne)	IncobotulinumtoxinA	Xeomin®, Bocouture®
Solstice Neurosci. Inc.	RimabotulinumtoxinB	Myobloc®

human serum albumin (*Table 12.4*, column 8). For the same purpose, animal gelatin is present in some products instead of human serum albumin. Given the potential allergic effect of animal gelatin, this raises safety questions.

For several years, there has been an increasing diversity of trade names under which the toxin products are distributed, although sometimes based on the same manufactured "toxin." Indeed, a same formulation can receive different trade names in different regions of the world (e.g., Vistabel® and Botox Cosmetic®). Moreover, the biological activity of the various formulations is expressed with systems of units that are not equivalent (see below), thus making the products not interchangeable. To minimize the risk of errors, the FDA USAN Council has introduced in the United States of America a new name for the toxin produced by a given manufacturer, independent of the technical trade names (*Table 12.5*) (FDA-alert-289 2009). This new name is not used abroad.

Toxin Units in Human Therapy

The amount of toxin that is recommended to inject to a patient for a given indication is defined as a number of units of biological activity to be injected in a single or several sites. Depending on the indications, the number of units may range from few tens to several hundreds. The assays used by the manufacturers to measure the biological activity of commercial preparations of toxin/neurotoxin are based on the mouse LD50 assay, with 1 unit of biological activity defined as 1 toxin mouse LD50 (~10 pg purified neurotoxin type A). None of the commercial products contains human serum albumin enough to fully prevent nonspecific absorption of toxin/neurotoxin on the vial and syringe walls (Bigalke et al. 2001), therefore adding or not gelatin to the toxin sample to be assessed by the mouse LD50 assay impacts on the measurement of its biological activity and leads to the situation that a same quantity (amount by weight) of active toxin is associated with a different number of units. For example, Hambleton and Pickett (Hambleton and Pickett 1994) have reported that 100 units of Botox® evaluated with the Speywood's test (i.e., with gelatin (Hambleton et al. 1981)); this test is used to evaluate toxin products from the manufacturer (IPSEN) ended in an assessment of 270–360 units, while 500 Dysport® units evaluated with the assay used by the manufacturer Allergan (i.e., without gelatin) resulted in 267 units. This two to threefold difference corresponds to the conversion factor determined empirically in clinic. Allergan's assay (without gelatin) gives an assessment of the toxin biological activity that can be actually mobilized from the vial in the conditions of clinical practice, whereas the IPSEN-Speywood's assay (with gelatin) measures the amount of biological activity actually present in the vial. The other manufacturers also determine the biological activity of the toxin products by modified mouse LD50 assays. Overall, the units are different, and this makes the products not interchangeable. Misunderstanding these differences may lead to overdosage of botulinum toxin, with the risk of causing iatrogenic botulism.

Spreading and Half-Life of Botulinum Toxin Injected in Therapy

A major difference with generalized forms of botulism and botulinum toxin-based therapy is that the latter remains mostly localized. However, spreading of neurotoxin molecules beyond the injection site can cause adverse manifestations (see below). Muscles and all tissues susceptible to be injected with toxin for therapeutic purpose are comprised of defined compartments (e.g., the different fascia in large muscles) that are insulated each other by tunica or sera. Therefore, the most important pathway for the spreading of toxin from its injection site is the blood capillary network and vascular tree that permit its hematogenous dissemination.

Since the toxin complexes rapidly dissociate (half-time of less than 1 min) at plasma pH (i.e., pH 7.4) (Eisele et al. 2011), the neurotoxin is the only entity responsible for the spreading of the toxin effects beyond the site of injection, whatever is the initial formulation of toxin (purified neurotoxin or complexes of different sizes, see *Table 12.4*). This is supported by observation of no significant difference in the spread of 125-iodine-labeled toxin A and neurotoxin A (Tang-Liu et al. 2003), or Dysport®, Botox®, or Xeomin® in the *tibialis* muscle (as assessed by measuring changes in NCAM expression (Carli et al. 2009)). Also, when neurotoxin (Xeomin®) or toxin complex (Botox®) is injected into the *extensor digitorum longus* muscle, there is similar "diffusion" of the paralytic effects to adjacent muscles (Wohlfarth et al. 2007).

Apart from the small fraction of neurotoxin that is captured by the endings of motoneurons, most of the neurotoxin disappears from the injection site by its dilution in the circulatory system. In the rat, only ~70% of the injected neurotoxin is detectable 30 min after injection (evaluated in the rat gastrocnemius muscle). After 6 h, only 30% remains and only 5% after 24 h (Tang-Liu et al. 2003). In the blood compartment, the neurotoxin half-life is of ~10 h (Al-Saleem et al. 2008; Ravichandran et al. 2006). These values contrast with presence of circulating neurotoxin for several days, or longer than a week, after the onset of botulism. The short life span of extracellular neurotoxin needs to be compared to that reported once captured by the nerve endings. By extrapolating to man the murine data, the long duration of paralysis observed in human therapy (order of several months with types A and C,

several weeks for types B and F, and that of type E even shorter). Montecucco and Molgó (Montecucco and Molgo 2005) suggest an intracellular half-life of several months, and even more: indeed, duration of toxin effect when injected into glands may reach 1 year or more in man.

Botulism Manifestations as Side Events of Botulinum Toxin-Based Therapy

In mammals, the lethal dose of botulinum toxin (900 kDa complex) type A is the order of 1 ng/kg: 1.2 ng/kg ip in mice, 0.5–0.7 ng/kg in guinea pigs, rabbits, or monkeys (Gill 1982). By extrapolation, the lethal dose for humans should be ~90–150 ng of toxin A (complex) injected intravenously or intramuscularly (Arnon et al. 2001; Smith 2006). From experiments carried out in monkeys, and on the basis that 1 unit of biological activity = 1 mouse LD50, Scott and Suzuki (Scott and Suzuki 1988) have suggested that the lowest dose of toxin type A producing a systemic botulism (with respiratory distress) in humans is in the range of 33 unit/kg and the LD50 for humans ranges 38–42 unit/kg (~ 2,800 unit for an adult of ~70 kg). Overall, these evaluations remain above the suggested maximum doses of type A toxin/neurotoxin recommended by the pharmaceutical companies for the approved indications. However, in clinical practice, the dosage of botulinum toxin is determined by the practitioner to adapt on individual patient response, and there is the possibility to inject toxin for not approved indications. The actual injected doses can be very high, for example, in the case of limb spasticity, 800 units (type A toxin) or more are used by certain practitioners.

The adverse effects due to spreading of toxin from the injection site are numerous (Dressler and Benecke 2007), albeit severe case is scarce. The more frequent effects are an extension of muscle paralysis to noninjected adjacent muscles and/or action on the neighboring glands (e.g., dry mouth after injection of toxin in the neck muscles), symptoms of systemic dysautonomia. When large amount of toxin is used (i.e., 800 units), more frequent are the cases of fatigue and long-lasting muscle weakness in numerous noninjected muscles. This is evocative of attenuated forms of iatrogenic botulism. Importantly, however, observation of distant effects of the toxin does not necessarily mean that toxin had spread and exerted its action elsewhere! This will discussed below.

The use of type B toxin in human therapy has shown that this toxin is much less powerful than that of type A to induce myorelaxation in humans (Sloop et al. 1997). Therefore, the dosage recommended for type B toxin to relax overactive muscles is much higher than that of products of type A. This produces a larger spreading of type B toxin beyond the injected muscle. Consistent with observation that botulism type B displays more pronounced autonomic manifestations than motor dysfunction (Hughes et al. 1981; Tacket and Rogawski 1989; Woodruff et al. 1992), there is a higher occurrence of unwanted dysautonomic events associated with the use of toxin B from toxin A (Dressler and Benecke 2007).

Indirect Effects of Botulinum Neurotoxin Observed in Humans

The local administration of botulinum toxin for therapeutic purpose in humans, or in the course of in vivo animal experiments, has been the occasion of revealing several indirect effects of the toxins.

Atrophic Response

Following their paralysis, muscle fibers develop very quickly atrophy (Morbiato et al. 2007; Rosales et al. 1996). This is an indirect effect caused by any situation inducing synaptic silencing. Muscle atrophy is reversible and correlates in intensity to that of the paralytic effect of the neurotoxin (Borodic and Ferrante 1992; Chhetri et al. 2003; Horn et al. 1993; Morbiato et al. 2007; Rosales et al. 1996, 2006). This indirect effect of neurotoxin has been exploited as such to reduce muscle hypertrophy (Al-Muharraqi et al. 2009). Not all muscles are susceptible to atrophy after toxin injection. For example, it is not observed after paralyzing by botulinum toxin the extraocular eye muscles in monkeys (Porter et al. 1991) or rabbits (Ugalde et al. 2005). One reason postulated for this resistance to atrophy is the activation of satellite cells and their rapid fusion with the paralyzed muscle fiber.

Muscle fiber atrophy is associated with significant increases in the sarcolemmal electrical resistance that makes it better responding to motor synaptic inputs. Therefore, although botulinum toxin inhibits ACh release, the better responsiveness of atrophic fibers may compensate for a while the former, delaying the onset of paralysis.

Decrease of synaptic transmission results also in atrophy of the glandular tissue (Oeconomou et al. 2008; Swartling et al. 2004; Teymoortash et al. 2007). This effect participates to reducing the volume of secretion induced by the toxin. Consistent with normal cell proliferation in prostate is under control of cholinergic innervation; several recent studies have explored the possibility of using the toxin to reduce benign prostatic enlargement (Chuang and Chancellor 2006; Oeconomou et al. 2008).

Sprouting

In mammals, muscle paralysis following a single injection of botulinum toxin A elicits within 3–5 days a nerve outgrowth or sprouting that occurs along intramuscular axons at the nodes of Ranvier (nodal sprouting), and at motor nerve terminals of the NMJs (terminal sprouting). The induction of sprouting is not specific of the BoNTs and is also produced by other agents that render the muscle inactive via prolonged blockade of either nerve conduction or muscle nAChRs. Terminal sprouts predominate on nodal sprouts in mature muscles treated with BoNT/A.

Sprouting is greater in younger animals. Generally, it increases with time after BoNT injection and depends upon the contractile properties of the muscle as defined by its innervation pattern. Thus, sprouting is more prominent in slow (e.g., *soleus*), than in fast (e.g., *extensor digitorum longus*), contracting muscles. In addition, sprouting is more abundant in muscles innervated by short axons (reviewed in (Meunier et al. 2002a)). Nerve terminal sprouts evoked by BoNT/A in mouse *levator*

auris longus muscle appear as thin, unmyelinated filaments usually oriented parallel to the longitudinal axis of the muscle extending beyond the original nerve terminus (Angaut-Petit et al. 1990; de Paiva et al. 1999; Molgo et al. 1990). After a BoNT/A injection, the sprouts usually increase in length as well as complexity for about 40–50 days and continue to grow for 30–40 days, despite the recovery of nerve-evoked muscle twitches (Juzans et al. 1996), as confirmed by time-lapse imaging of the same mouse NMJ (de Paiva et al. 1999). The persistence of nerve terminal sprouts has also been reported in the human orbicularis muscle after repeated injections of BoNT/A (Holds et al. 1990). Nerve terminal sprouting was also detected in mouse *levator auris longus* muscle injected with BoNT/D (Comella et al. 1993) and BoNT/C (Morbiato et al. 2007), as well as in mouse sternomastoid muscle injected with BoNT/F (Meunier et al. 2003). Interestingly, no sprouts were visible in the mouse sternomastoid muscle following BoNT/E injection (Meunier et al. 2003), which is probably due to short-lived muscle inactivity since recovery from paralysis starts within 2–3 days after neurotoxin administration.

Distant Effects Unrelated to Hematogenous Dissemination of Toxin

In the course of therapeutic use of the toxin, change in reflexes and central effects of botulinum neurotoxins have been reported (Caleo et al. 2009; Gracies 2004; Gracies et al. 2009; Lim and Seet 2008; Priori et al. 1995). They are consistent with similar observations in animals (Moreno-Lopez et al. 1994; Pastor et al. 1997; Wellhöner 1992). Since the botulinum neurotoxins molecules do not cross the blood–brain barrier, the reported central actions may refer to (1) axonal retrograde ascent of botulinum neurotoxin molecules followed by transsynaptic passage or (2) change in proprioceptive peripheral information and central plasticity.

When applied at very high concentrations, a fraction of the botulinum neurotoxin molecules endocytosed by the motor and sensory nerve endings is routed in a vesicular retroaxonal pathway, leading to its transport up to the cell body of the motoneurons (i.e., located in the ventral horn of the spinal cord), or of the sensory neurons (i.e., in the dorsal root ganglions; DRG) (Antonucci et al. 2008; Habermann 1974, 1989; Wiegand et al. 1976). Experiments carried out in animal have shown that retrogradely transported neurotoxin can also undergo transsynaptic transport (Antonucci et al. 2008; Caleo et al. 2009; Caleo and Schiavo 2009). So, potentially, the neurotoxin injected in the periphery could act directly in the central nervous system. However, after more than 25 years of clinical use of botulinum toxins, no clinical manifestation has been reported demonstrating a direct central action of botulinum toxin when injected for therapeutic purpose. In fact, most of central effects mentioned above may relate to changes in muscular proprioception. The musculoskeletal proprioceptive information conveyed to central nervous system originates from distinct peripheral receptors (muscle spindle, tendon, and joint receptors). Muscle spindles are comprised of sensory fibers type IA and modified muscle fibers that are innervated by cholinergic fibers (i.e., intrafusal fibers) from the γ-motoneurons. The latter are co-activated with the α-motoneurons to adjust the spindle length with that of skeletal muscle. Spindle stretching (e.g., when contraction of skeletal muscles fibers is late as compared to that of spindle organ) leads to activation of IA sensory fibers that in turn reinforces direct activation of homonymous α-motoneurons and inhibitory neurons projecting onto the motoneurons of antagonistic muscles and other inhibitory interneurons (as the Renshaw cells). Although intrafusal cholinergic transmission can be blocked by botulinum neurotoxin (Dressler et al. 2005; Filippi et al. 1993; Rosales et al. 1996; Trompetto et al. 2006) and intrafusal muscle fibers can undergo atrophy (Rosales et al. 1996), such effects have never been demonstrated occurring in the context of therapeutic use of botulinum toxin (Gracies 2004). Indeed, the spindle capsule insulates intrafusal motor nerve fiber from toxin molecules injected in muscle tissue. Thus, in the clinical practice, intramuscular injection of botulinum toxin/neurotoxin effects on the skeletal neuromuscular junctions (i.e., those innervated by α-motoneurons) but the intrafusal ones. The resulting stretching of the spindle leads to increased activity of sensory IA neurons and activation of the inhibitory interneurons projecting onto motoneurons of the antagonistic muscles or other spinal interneurons; therefore, reports on changes in the inhibition by Renshaw cells (Caleo and Schiavo 2009; Gracies 2004) and paralysis of noninjected muscles (Gracies et al. 2009; Wohlfarth et al. 2001) can be explained as the result of a change in the spinal reflex neuronal network induced by myorelaxation of toxin-injected muscles.

The change of proprioceptive information can quickly lead to functional alterations of the upper levels of the motor system. In rats, the position in space of the whiskers is controlled by motoneurons located in the facial nucleus, and their position is detected by sensory fibers. Following injection of botulinum toxin into the whisker pads, whiskers are immobile and proprioceptive inputs dramatically disturbed, indirectly causing quick functional reorganization of primary motor cortex (M1) by synaptic plasticity (Franchi and Veronesi 2004). Functional alterations of the cortical neural network have been also observed in humans in the context of the clinical use of botulinum toxin (Curra et al. 2004). These alterations of central neural network are indirect consequences of the action of the neurotoxin and, in turn, can be manifested in the periphery by change in motor behavior of noninjected muscles.

To summarize, distant manifestations of botulinum toxin deserve careful analysis to distinguish between those issuing from spreading of botulinum neurotoxin molecules from injected sites from the indirect ones mediated by the spinal reflex neuronal network.

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