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Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses

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The clostridial neurotoxins responsible for tetanus and botulism are proteins consisting of three domains endowed with different functions: neurospecific binding, membrane translocation and proteolysis for specific components of the neuroexocytosis apparatus. Tetanus neurotoxin (TeNT) binds to the presynaptic membrane of the neuromuscular junction, is internalized and transported retroaxonally to the spinal cord. The spastic paralysis induced by the toxin is due to the blockade of neurotransmitter release from spinal inhibitory interneurons. In contrast, the seven serotypes of botulinum neurotoxins (BoNTs) act at the periphery by inducing a flaccid paralysis due to the inhibition of acetylcholine release at the neuromuscular junction. TeNT and BoNT serotypes B, D, F and G cleave specifically at single but different peptide bonds, of the vesicle associated membrane protein (VAMP) synaptobrevin, a membrane protein of small synaptic vesicles (SSVs). BoNT types A, C and E cleave SNAP-25 at different sites located within the carboxyl-terminus, while BoNT type C additionally cleaves syntaxin. The remarkable specificity of BoNTs is exploited in the treatment of human diseases characterized by a hyperfunction of cholinergic terminals.

Keywords: tetanus; botulism; neurotoxins; SNARE proteins

1. INTRODUCTION

Protein neurotoxins produced by *Clostridia* were identified as the cause of the paralytic syndromes of tetanus and botulism little over a century ago (Carle & Rattone 1884; Faber 1890; Tizzoni & Cattani 1890*a*,*b*; van Ermengem 1897). Studies carried out since have led to the possibility of preventing tetanus (Galazka & Gasse 1995) and to the use of botulinum neurotoxins (BoNTs) as therapeutic agents (Jankovic & Hallett 1994). The recent comprehension of the biochemical mechanism of action inside neuronal cells have established these toxins as useful tools in studying the processes of fusion of vesicles with target membranes within the cell (Rappuoli & Montecucco 1997). The seven BoNTs (serotypes are indicated using letters A to G) bind to and enter inside peripheral cholinergic terminals, from which they inhibit the release of acetylcholine, with ensuing flaccid paralysis. If the paralysis extends to respiratory muscles the patient dies of respiratory failure. At variance from BoNTs, tetanus neurotoxin (TeNT) blocks neurotransmitter release at the inhibitory interneurons of the spinal cord, which results in a spastic paralysis (van Heyningen 1968). Hence, despite the opposite clinical symptoms of tetanus and botulism, their causative agents intoxicate neuronal cells in the same way (Simpson 1989; Montecucco 1989). This has unified research on these neurotoxins, and led to the suggestion that they have a very similar structural organization due to their three-domain structure (Montecucco & Schiavo 1993), a conclusion now proven by the recently determined structure of the carboxyl-terminal domain of TeNT and of the structure of BoNT/A (Umland *et al*. 1997; Borden Lacy *et al*. 1998). The amino-terminal domain of TeNT and BoNTs is very similar and consists of a zinc-endopeptidase active specifically on protein components of the same cellular machine: the neuroexocytosis apparatus (Montecucco & Schiavo 1995).

2. STRUCTURE

The similar effect of the eight clostridial neurotoxins at nerve terminals is the result of their closely related protein structure. The toxins are synthesized in the bacterial cytosol without a leader sequence, and are released to the culture medium after bacterial lysis as a single polypeptide chain of 150 kDa. As such the toxin is inactive, but is activated by a specific proteolytic cleavage within a surface-exposed loop subtended by a highly conserved disulphide bridge. Several bacterial and tissue proteinases are able to generate the active di-chain neurotoxin (DasGupta 1989; Weller *et al*. 1989; Krieglstein *et al*. 1991; DasGupta 1994). The heavy chain (H, 100 kDa) and the light chain (L, 50 kDa) remain associated via both non-covalent protein–protein interactions and the conserved interchain S–S bond, the integrity of which is essential for neurotoxicity (Schiavo *et al*. 1990*b*; de Paiva *et al*. 1993). The structure of the carboxyl-terminal domain of TeNT and of BoNT/A heavy chain (Hc) has been recently determined (Umland *et al*. 1997; Borden Lacy *et al*. 1998). Hc consists of two distinct domains and has an overall elongated shape ($33 \times 42 \times 80$ Å). The N-terminal Hc domain is characterized by the presence

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of 16 β-strands and four helices arranged in a jelly-roll motif, closely similar to that of legume lectins which are carbohydrate binding proteins. The amino acid sequence of this domain is highly conserved among clostridial neurotoxins, suggesting that it has a closely similar three-dimensional (3D) structure. The carboxyl-terminal region contains a motif present in several other proteins involved in recognition and binding functions, such as various trypsin inhibitors. Its sequence is poorly conserved among clostridial neurotoxins (CNTs). The removal from Hc of its N-terminal domain does not reduce membrane binding, whereas the deletion of only ten residues from the C-terminus abolishes binding of the toxin to spinal cord neurons (Halpern & Loftus 1993).

The 3D crystal structure of the 1296 amino acid BoNT/A has been recently determined (Borden Lacy *et al*. 1998). The toxin is subdivided into three *ca*. 50 kDa domains: (i) a catalytic domain containing a new metalloprotease active site; (ii) a translocation domain with features similar to colicin and influenza virus haemagglutinin with two 98-Å-long helices; and (iii) a binding domain composed of two unique sub-domains similar to the legume lectins and the Kunitz inhibitor. A motif included in the latter domain suggests that a dual receptor model may be used in cell surface recognition (Borden Lacy *et al*. 1998), as previously suggested on the basis of some theoretical considerations and of the analysis of peculiar features of TeNT intoxication (Montecucco 1986). These results are in complete agreement with the three-domain structural model of CNTs previously proposed to account for the available biochemical data (Montecucco & Schiavo 1993; Montecucco & Schiavo 1995).

Such structural organization meets nicely the properties of CNTs, in particular their ability to intoxicate neurons via a four-step mechanism consisting of (i) binding; (ii) internalization; (iii) membrane translocation; and (iv) enzymatic target cleavage. The L chain is responsible for the intracellular catalytic activity, whereas the amino-terminal 50 kDa domain of the H chain (H_N) is implicated in membrane translocation, and the carboxyl-terminal part (Hc) is mainly responsible for the neurospecific binding (Montecucco *et al*. 1994; Montecucco & Schiavo 1995).

The demonstration that CNTs are zinc-proteases first arose from the recognition that the 216–244 region contains the typical His-Glu-Xaa-Xaa-His binding motif (Montecucco & Schiavo 1995). Accordingly, their proteolytic activity is zinc dependent and heavy metal chelators, which remove bound zinc, generate inactive apo-neurotoxins (Schiavo *et al*. 1992*b*; Simpson *et al*. 1993; Hohne-Zell *et al*. 1994), without appreciable changes in L-chain secondary structure (De Filippis *et al*. 1995). The active site metal atom and the active holo-toxin can be reacquired upon incubation of the chelator-treated toxin in zinc-containing buffers (Schiavo *et al*. 1992*a*,*b*,*c*, 1993*b*,*c*, 1994, 1995, Simpson *et al*. 1993; Hohne-Zell *et al*. 1994). With the same procedure, the active site zinc atom can be exchanged with other divalent transition metal ions forming active metal-substituted toxins (Tonello *et al*. 1997).

In the active site of metalloproteases, the zinc atom is penta-coordinated by the imidazole rings of the two histidines of the motif and a water molecule bound to the glutamic acid of the motif. In addition, the crystallographic structure of BoNT/A shows the presence around the zinc atom of a glutamate and a tyrosine residue (Borden Lacy *et al*. 1998), a novel type of metal coordination, which accounts for the unique catalytic and spectroscopic properties of TeNT (Tonello *et al*. 1997). These data define clostridial neurotoxins as a distinct group of metalloproteases, whose origin cannot be at the present time traced in any of the known families of the enzymes (Rawlings & Barrett 1995).

Among the various CNTs the H_N portions are highly homologous (Minton 1995) and their predicted secondary structure is also highly similar (Lebeda & Olson 1995). The similarity of the CNTs extends to the N-terminal domain of Hc, whereas the C-terminal domain (residues 1140–1315 of TeNT) is the most divergent part (Minton 1995), particularly in the 150 residues-long region included between strands B19 and B29 (Umland *et al*. 1997). This is consistent with the notion that the Hc domain is involved in binding to the nerve terminals and that the different neurotoxins bind to different receptors (see below).

3. NEUROSPECIFIC BINDING

From the site of production or adsorption, BoNTs and TeNT diffuse in the body fluids, up to the presynaptic membrane of cholinergic terminals where they bind. TeNT may also bind to sympathetic adrenergic fibres (reviewed by Wellhoner (1992), and by Habermann & Dreyer (1986)). *In vitro*, CNTs are capable of binding to a variety of non-neuronal cells, however only at concentrations far exceeding those of clinical significance. Under the latter conditions, the binding sites for BoNT/A and /B at the rat neuromuscular junction are several hundreds per μm^2 (Black & Dolly 1986*b*), whereas the number of TeNT receptors in a neuroblastoma-glioma cell line is around 450 per cell (Wellhoner & Neville 1987). Careful experiments have revealed the binding of these neurotoxins to the presynaptic membrane to be heterogeneous, with both sub-nanomolar and nanomolar binding affinities (Bakry *et al*. 1991; Halpern & Neale 1995). Available evidence indicates that the Hc domain plays a major role in neurospecific binding (Bizzini *et al*. 1977; Helting *et al*. 1977; Morris *et al*. 1980; Shone *et al*. 1985; Weller *et al*. 1986; Kozaki *et al*. 1989; Coen *et al*. 1997). However, it appears that additional regions of CNTs are also involved in binding inasmuch as Hc provides only a partial protection from intoxication with the intact CNT molecule, and the Hc fragment of TeNT does not prevent the retroaxonal transport of the holotoxin (Bizzini *et al*. 1977; Mochida *et al*. 1989; Poulain *et al*. 1989*a*,*b*, 1991; Takano *et al*. 1989; Weller *et al*. 1991; Francis *et al*. 1995).

Identification of the presynaptic receptor(s) of CNTs has been attempted by several investigators. Polysialogangliosides are certainly involved (Halpern & Neale 1995), however, it is unlikely that they are the sole receptors of these neurotoxins. Some evidence indicates that proteins of the cell surface have a part in the process (Pierce *et al*. 1986; Yavin & Nathan 1986; Parton *et al*. 1988; Schiavo *et al*. 1991*b*). The presence of both lectin-like and protein binding sub-domains in the Hc domain of TeNT

(Umland *et al*. 1997) supports the suggestion that CNTs bind strongly and specifically to the presynaptic membrane because they display multiple interactions with sugar and protein binding sites, as suggested by a double-receptor model (Montecucco 1986). Recent experiments provided strong evidence in favour of such a model. In fact BoNT/B was shown to bind strongly to the synaptic vesicle protein synaptotagmin II, however only in the presence of polysialogangliosides. Moreover, CHO cells transfected with the synaptotagmin II cDNA bind the toxin with a low affinity which is converted to high affinity after the incorporation of gangliosides GT1b into their membrane (Nishiki *et al*. 1994, 1996*a*,*b*).

Electrophysiological studies clearly showed that BoNTs block neuroexocytosis at peripheral terminals, whereas TeNT causes the same effect at CNS synapses of the spinal cord (Wellhoner 1992). These different final destinations must be determined by specific receptors of TeNT and BoNTs which drive them to different intracellular routes. Identification of the peripheral motor neuron TeNT receptor(s) will uncover an entry gateway leading from the peripheral to the central nervous system (CNS). This is expected to help in devising novel routes to deliver biological agents, including analgesic and anaesthetic drugs, to the spinal cord. The knowledge of the receptors for the various BoNTs will also contribute to improve present therapeutic protocols, explaining in particular why there are patients who do not benefit from the current BoNT/A treatments.

To reach its final site of action, TeNT has to enter two different neurons: a peripheral motor neuron and an inhibitory interneuron of the spinal cord. Its binding to peripheral and central presynaptic terminals is different, as indicated by the following pieces of evidence: (i) cats and dogs, which are highly resistant to TeNT administered peripherally, in contrast become very sensitive when the toxin is injected directly in the spinal cord (Shumaker *et al*. 1939); (ii) the L-HN fragment of TeNT injected in the cat leg is non toxic, while it causes a spastic paralysis upon direct injection into the spinal cord (Takano *et al*. 1989). It is possible that the concentration of TeNT in the limited space of the synaptic cleft between the peripheral motor neuron and inhibitory interneuron is significantly higher than that at the periphery. Hence, even a low affinity receptor could mediate the entry of TeNT into the latter cells. Lipid monolayer studies have clearly documented the ability of 10^{-8} M TeNT to interact with acidic lipids (Schiavo *et al*. 1990*a*). Similar concentrations are routinely used with cells in culture, with *in vivo* injections into the hippocampus (Mellanby *et al*. 1984) or in experiments of flaccid paralysis in mice treated with one thousand times the mouse LD50 (Matsuda *et al*. 1982). On the other hand, in clinical tetanus and botulism, the concentrations of TeNT and BoNTs at the periphery are sub-picomolar. A possible scenario that reconciles the data presently available can be summarized as follows. Glycoprotein and glycolipid binding sites are implicated in the peripheral binding of CNTs, characterized by high affinity and high specificity. The protein receptor of TeNT would be responsible for the inclusion of the toxin in endocytic vesicles that move in a retrograde direction along all of the axon, whereas BoNT protein receptors would guide them inside vesicles that acidify within the neuromuscular junction. When the TeNT-carrying vesicles reach the cell body they move to dendritic terminals to release the toxin in the intersynaptic space. Once equilibrated between presynaptic and postsynaptic membranes, TeNT binds and enters the inhibitory interneurons, again via synaptic vesicle endocytosis.

4. INTERNALIZATION INSIDE NEURONS

Since the L chains of CNTs block neuroexocytosis by acting in the cytosol, at least this part of the toxin must reach the cell cytosol. All available evidence indicates that CNTs do not enter the cell directly from the plasma membrane. Rather, they are endocytosed inside acidic cellular compartments. Electron microscope studies have shown that, after binding, CNTs enter the lumen of vesicular structures by a temperature and energy dependent process (Dolly *et al*. 1984; Critchley *et al*. 1985; Black & Dolly 1986*a*,*b*; Staub *et al*. 1986; Parton *et al*. 1987; Matteoli *et al*. 1996). Internalization of gold-labelled TeNT was examined by Parton *et al*. (1987) in dissociated spinal cord neurons. The toxin was found inside a variety of vesicular structures, with only a minority within the lumen of small synaptic vesicles (SSVs). At variance, Matteoli *et al*. (1996), found that, following a 5 min membrane depolarization, TeNT was almost exclusively inside SSVs of hippocampal neurons. It has long been known that nerve stimulation facilitates intoxication (Ponomarev 1928; Kryzhanovsky 1958; Hughes & Whaler 1962; Wellhoner *et al*. 1973; Habermann *et al*. 1980). The simplest way to account for the shorter onset of the paralysis induced under conditions of nerve stimulation is that the neurotoxins enter the synaptic terminal via endocytosis inside the lumen of SSVs. This explanation can account for the results in hippocampal neurons (Matteoli *et al*. 1996) and in granular cells of the cerebellum (O. Rossetto, G. Schiavo and C. Montecucco, unpublished data). In conclusion, TeNT uses SSVs as Trojan horses to enter the CNS neurons and BoNTs may enter peripheral cholinergic terminals by the same way. In contrast, no hypothesis can be presently made on the nature of the vesicles which internalize TeNT at the neuromuscular junction.

5. TRANSLOCATION TO THE NEURONAL CYTOSOL

Once the neurotoxins have reached the vesicle lumen, their L chain needs to cross the hydrophobic barrier of the vesicle membrane to reach the cytosol where it can display its proteolytic activity. The different trafficking of TeNT and BoNTs at the neuromuscular junction clearly indicates that internalization and membrane translocation are distinct steps of the process of cell intoxication (Menestrina *et al*. 1994; Montecucco *et al*. 1994). Indirect, but compelling evidence indicates that TeNT and BoNTs have to be exposed to a low pH step for nerve intoxication to occur (Simpson 1982, 1983, 1994; Adler *et al*. 1994; Williamson & Neale 1994; Matteoli *et al*. 1996). Acidic pH does not induce a direct activation of the toxin via a structural change. Rather, it is required in the process of transmembrane translocation of the L chain. In this respect, TeNT and BoNTs appear to behave similarly to the other bacterial protein toxins characterized by a structure composed of three distinct parts (Montecucco *et*

al. 1994). Studies with model membrane systems have shown that TeNT and BoNTs undergo a low pH driven conformational change from a water soluble 'neutral' structure to an 'acid' structure characterized by the surface exposure of hydrophobic patches. This hydrophobicity enables the penetration of both the H and L chains in the hydrocarbon core of the lipid bilayer (Boquet & Duflot 1982; Boquet *et al*. 1984; Cabiaux *et al*. 1985; Roa & Boquet 1985; Montecucco *et al*. 1986; Menestrina *et al*. 1989; Montecucco *et al*. 1989; Schiavo *et al*. 1990*a*). Following the low pH-induced membrane insertion, TeNT and BoNTs form ion channels in planar lipid bilayers (Boquet & Duflot 1982; Hoch *et al*. 1985; Donovan & Middlebrook 1986; Blaustein *et al*. 1987; Shone *et al*. 1987; Gambale & Montal 1988; Menestrina *et al*. 1989; Rauch *et al*. 1990; Schmid *et al*. 1993) and, in the case of TeNT, also in spinal cord neurons. The channels, which open with high frequency at pH 5.0 but not at neutral pH, are non selective for Na^+, K^+, Ba^{2+} and Cl^-, and have single conductance of 45 pS (Beise *et al*. 1994).

There is a consensus that the toxin channels participate in the process of transmembrane translocation of the L domain, from the vesicle membrane to the nerve terminal cytosol. However, there is no agreement on how this process may take place. In particular, one hypothesis proposes that the L chain unfolds at low pH and permeates through the transmembrane pore formed by H chain(s). Following exposure to the neutral pH of the cytosol, the L chain refolds and is released from the vesicle by reduction of the interchain disulphide bond. A second model (advanced by Beise *et al*. (1994)) envisages that, as the vesicle internal pH decreases following the operation of the vacuolar-type ATPase proton pump, CNTs insert into the lipid bilayer, forming ion channels that grossly alter the electrochemical gradients. Eventually, such permeability changes cause an osmotic lysis of the toxin-containing acidic vesicle, sustained also by the toxin-induced destabilization of the lipid bilayer (Cabiaux *et al*. 1985). An alternative view envisages an H channel opened laterally, with the L chain crossing the membrane in contact with both the H chain and the membrane lipids, rather than inside a wholly proteinaceous pore (Montecucco *et al*. 1991; Montecucco *et al*. 1994). Since the two toxin polypeptide chains are supposed to change conformation in a concerted fashion at low pH, both of them can expose hydrophobic surfaces and enter into contact with the hydrophobic core of the lipid bilayer. The cytosolic neutral pH induces the L chain to refold and to regain its water-soluble neutral conformation, after reduction of the interchain disulphide. It is possible that in the processes of threadmilling out of the vesicle membrane and cytosolic refolding, the L chain is assisted by chaperones. As yet, however, no supporting evidence is available. As the L chain is released from the vesicle membrane, the transmembrane hydrophilic cleft of the H chain tightens up to reduce the amount of hydrophilic protein surface exposed to the membrane hydrophobic core, leaving across the membrane a peculiarly shaped channel with two rigid protein walls and a flexible lipid seal on one side. This is proposed to be the structure responsible for the ion-conducting properties of TeNT and BoNTs. In this cleft model, the ion channel is a consequence of membrane translocation, rather than a prerequisite, such as in the case of the tunnel model.

6. ZINC-ENDOPEPTIDASE ACTIVITY

The catalytic activity of CNTs was discovered when the determination of their primary structure (Minton 1995) revealed the presence of the His-Glu-Xaa-Xaa-His zinc-binding zinc-endopeptidases motif (Kurazono *et al*. 1992; Schiavo *et al*. 1992*b*,*c*; Wright *et al*. 1992). Building upon this observation, it was soon demonstrated that TeNT was blocking ACh release at synapses of the buccal ganglion of *Aplysia californica* via a zinc-dependent protease activity (Schiavo *et al*. 1992*a*,*b*). Identification of the cytosolic substrates of such enzymatic activity followed assays of proteolysis performed on proteins of SSVs and on other synaptic proteins that had been suggested as candidates for the neuroexocytosis apparatus (Söllner *et al*. 1993).

The eight CNTs are remarkably specific proteases: among the many proteins and synthetic substrates assayed so far, only three targets, the so called SNARE proteins, have been identified (table 1). TeNT and BoNT/B, /D, /F and /G cleave VAMP, each at a single site (Schiavo *et al*. 1992*a*; Schiavo *et al*. 1993*a*,*c*; Schiavo *et al*. 1994; Yamasaki *et al*. 1994*a*,*b*,*c*); BoNT/A and /E cleave SNAP-25, each at a single site while BoNT/C cleaves both syntaxin and SNAP-25 (Blasi *et al*. 1993*a*,*b*; Schiavo *et al*. 1993*a*,*b*; Binz *et al*. 1994; Schiavo *et al*. 1995; Foran *et al*. 1996; Osen Sand *et al*. 1996; Williamson *et al*. 1996). Strikingly, TeNT and BoNT/B cleave VAMP at the same peptide bond (Gln76–Phe77) and yet when injected into an animal they cause the opposite symptoms of tetanus and botulism, respectively (Schiavo *et al*. 1992*a*). This observation is particularly relevant because it has clearly demonstrated that the different symptoms derive from different sites of intoxication rather than from a different molecular mechanism of action.

Recombinant VAMP, SNAP-25 and syntaxin are cleaved at the same peptide bonds, and at the same rate, as the corresponding cellular proteins, indicating that no additional endogenous factors are involved in the proteolytic activity of the CNTs. During the last few years numerous isoforms of SNARE proteins have been identified in different species and tissues, and surely more remain to be discovered. Only some of them are susceptible to proteolysis by the CNTs. In general, a SNARE protein is resistant to a neurotoxin because of mutations at the cleavage site or in other regions involved in neurotoxin binding (Patarnello *et al*. 1993). An inspection of the nature and sequence of the amino acid residues at and around the cleavage sites of the three SNARE proteins (table 1) reveals no conserved pattern that could account for the specificity of these metalloproteases. Analyses of their primary and secondary structure (Lebeda & Olson 1995; Minton 1995) suggest that these neurotoxins are structurally very similar, however their cleavage sites and flanking regions are very different and cannot account for the high specificity of the CNTs for the three SNARE proteins. These considerations suggested that the SNARE targets could have a common structural element that would serve as recognition motifs for the neurotoxins. Comparison of the sequence of the

Table 1. *Tetanus and botulinum neurotoxins target and peptide bond specificities*

toxin type	intracellular target	peptide bond cleaved P4-P3-P2-P1—P1′-P2′-P3′-P4′
TeNT	VAMP	Gly-Ala-Ser-Gln—Phe-Glu-Thr-Ser
BoNT/A	SNAP-25	Glu-Ala-Asn-Gln—Arg-Ala-Thr-Lys
BoNT/B	VAMP	Gly-Ala-Ser-Gln—Phe-Glu-Thr-Ser
BoNT/C	syntaxin	Asp-Thr-Lys-Lys—Ala-Val-Lys-Phe
BoNT/C	SNAP-25	Ala-Asn-Gln-Arg—Ala-Thr-Lys-Met
BoNT/D	VAMP	Arg-Asp-Gln-Lys—Leu-Ser-Glu-Leu
BoNT/E	SNAP-25	Gln-Ile-Asp-Arg—Ile-Met-Glu-Lys
BoNT/F	VAMP	Glu-Arg-Asp-Gln—Lys-Leu-Ser-Glu
BoNT/G	VAMP	Glu-Thr-Ser-Ala—Ala-Lys-Leu-Lys

(sequences refer to human SNAREs)

neuroexocytosis-specific SNARE proteins of different species has revealed the unique presence of a nine-residue-long motif, characterized by three carboxylate residues alternated with hydrophobic and hydrophilic residues, termed thereafter the SNARE motif (Rossetto *et al*. 1994). The motif, two copies in VAMP and syntaxin and four copies in SNAP-25, is always contained within regions predicted to adopt a helical conformation and, if plotted as an alpha-helix, the three negatively charged residues cluster on one face, adjacent to a hydrophobic face of the helix. Several pieces of experimental evidence support the hypothesis of a key role of the SNARE motif: (i) only protein segments including at least one SNARE motif are cleaved by neurotoxins (Shone *et al*. 1993; Cornille *et al*. 1994, 1997; Foran *et al*. 1994); (ii) the motif is exposed at the protein surface, as shown by the binding of anti-SNARE motif antibodies. These antibodies cross-react among the three SNAREs and inhibit the proteolytic activity of the neurotoxins (Pellizzari *et al*. 1996); (iii) the various neurotoxins cross-inhibit each other (Pellizzari *et al*. 1996); (iv) proteolysis performed on site-directed mutated VAMP or VAMP fragments indicates that the three carboxylate residues of the V2 copy of the motif are very important for the recognition by BoNT/B and /G, whereas those of the V1 copy are implicated in the recognition of BoNT/F and TeNT (Shone & Roberts 1994; Pellizzari *et al*. 1996; Wictome *et al*. 1996; Pellizzari *et al*. 1997). BoNT/D shows a particular requirement for the Met46 present in V1 (Yamasaki *et al*. 1994*a*; Pellizzari *et al*. 1997). In view of the fact that V1 is more amino-terminal with respect to V2, these results explain why the minimal length of VAMP segments cleaved by TeNT is longer than that required by BoNT/B (Shone *et al*. 1993; Foran *et al*. 1994). Due to the similarity between TeNT and BoNT/B, these results also suggest the possibility that the two copies of the SNARE motif of VAMP are paired in such a way that they adopt the same spatial orientation with respect to the Gln76–Phe77 bond (Pellizzari *et al*. 1996). In addition, a basic region located after the cleavage site of TeNT and BoNT/B is important for their binding and optimal cleavage of VAMP (Shone *et al*. 1993; Yamasaki *et al*. 1994*a*; Cornille *et al*. 1995, 1997); (v) the SNARE motif is also important for binding and proteolysis of SNAP-25 by BoNT/A and /E. The analysis of the rate of proteolysis of several SNAP-25 fragments deleted of SNARE motif(s) shows that the latter are hydrolysed, provided however that at least one the four copies of the motif is retained. In other words, the four copies of the SNARE motif present in SNAP-25 can largely substitute for one another for the recognition and proteolysis by BoNT/A and /E (Washbourne *et al*. 1997). This result indicates a large flexibility of SNAP-25, not surprising for a molecule that has to interact in a reversible way with its partners in the neuroexocytosis apparatus (Chapman *et al*. 1994; Hayashi *et al*. 1994; Hayashi *et al*. 1995; Pellegrini *et al*. 1995).

7. CLOSTRIDIAL NEUROTOXINS IN THE STUDY OF EXOCYTOSIS

The peptide bonds hydrolysed by each neurotoxin have been identified (table 1). Apart from TeNT and BoNT/B, each one of the different CNTs catalyses the hydrolysis of a different peptide bond. Thus, CNTs are well defined tools to identify the role of their targets in different cellular processes. Moreover, finer dissections of SNARE activities can be performed based on the different peptide bond hydrolysed by different CNTs on the same SNARE protein. BoNT/A removes only nine residues from the SNAP-25 C-terminus and yet this is sufficient to impair neuroexocytosis, indicating that this part of the molecule plays a relevant role in the function of the exocytosis apparatus. The fact that neuroexocytosis can be rescued in the neuromuscular junctions poisoned with BoNT/A by the application of α-LT and calcium, while BoNT/E poisoning cannot, indicates that the SNAP-25 region included between the two cleavage sites (16 residues) is involved in a late stage of exocytosis taking place after ATP priming when the calcium dependence is conferred to the complex (Banerjee *et al*. 1996).

Cleavage of VAMP and syntaxin by CNTs leads to the release to the cytosol of a large part of their cytosolic portions. Based on their respective proposed roles as vesicular and target SNAREs, vesicle docking should be impaired in CNT intoxicated synapses. On the contrary, it appears that poisoned and electrically silent synapses show an increased number of docked vesicles, as judged from electron microscopy (Mellanby *et al*. 1988; Neale *et al*. 1989; Hunt *et al*. 1994; Osen Sand *et al*. 1996). These results suggest that VAMP and syntaxin play additional role(s) in exocytosis, and are possibly involved in vesicle re-uptake as well.

Given the general role of SNAREs in vescicular trafficking, the use of CNTs is not limited to neuronal cells possessing CNT receptors. Detailed protocols for the use of CNTs on different cell type and organelles preparations are now available (Schiavo & Montecucco 1995; Blasi *et al*. 1997; Lang *et al*. 1997) In addition, incubation with very high doses of CNTs can be sufficient to elicit effects in cells characterized by a large fluid phase endocytosis (Pitzurra *et al*. 1989), and the same occurs in cells transfected with the gene encoding for the L chain of one neurotoxin (Eisel *et al*. 1993; Sweeney *et al*. 1995).

8. THERAPEUTIC USES OF BOTULINUM NEUROTOXINS

Since the publication of the first study of the effect of BoNT/A injection on strabismus (Scott 1989), papers

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reporting clinical uses of BoNTs largely outnumber those dedicated to botulism or to the cellular biochemistry of BoNT. Nowadays, BoNTs are used in the therapeutic management of several focal and segmental dystonias, of strabismus and in any situation where a reversible depression of a cholinergic nerve terminal activity is desired.

Injection of BoNT into a mammalian striated muscle causes a variety of histological changes (Borodic *et al*. 1994). The first sign is the accumulation of SSVs on the cytosolic face of the plasma membrane. This is the immediate consequence of the proteolytic cleavages discussed above: vesicles are no longer able to fuse and discharge their neurotransmitter content and hence cluster in direct contact with the plasma membrane. Contrary to what happens in denervation obtained by means such as nerve ligation, anatomical contacts between nerve and muscle are maintained and there is no apparent loss of motor axons. Under the effect of growth factors released by muscles, the motor end plate rather enlarges and sprouts develop from the end plate itself, from the terminal part of the axon and from adjacent nodes of Ranvier, ultimately growing into the muscle. This leads, on the one hand, to the increase of the number of motor end plates on single muscle fibres; on the other hand to the increase of the number of fibres innervated by a single motor axon, and of single muscle fibres innervated by more than one motor axon. In the muscle, the alterations induced by BoNT parallel those documented in other forms of denervation. Fibres undergo progressive (4–6 weeks) atrophy with a reduction of their mean diameter, already appreciable in the first two weeks after BoNT injection. Acetylcholinesterase and acetylcholine receptors spread from the neuromuscular junction to the whole muscle plasma membrane. Following axonal sprouting and reformation of functional nerve–muscle junctions, the muscle eventually regains its normal size and both acetylcholinesterase and acetylcholine receptors reconcentrate exclusively at the junctions. The muscle atrophy induced by BoNT in animal models and in humans is therefore largely reversible, even after repeated BoNT injections (Borodic *et al*. 1994).

The use of BoNT in human therapy is rapidly expanding. So far, BoNT/A has been by far the most used serotype. We have a programme of testing all BoNT serotypes with the aim of overcoming the problem of immunization against BoNT/A and to find the best serotype for any particular disease. BoNT/B, BoNT/F and BoNT/E are very effective because they cause a strong paralysing effect (Ludlow *et al*. 1992; Eleopra *et al*. 1998). However, their effect is short lasting and hence they are not a valid alternative to BoNT/A, as was found to be the case for BoNT/C (Eleopra *et al*. 1997, 1998). The short lasting effect of BoNT/E came as a surprise because that toxin cleaves the same substrate as BoNT/A. It is possible that the removal of a long segment from the carboxyl-terminal of SNAP-25 (25 residues by BoNT/E as compared to the nine residues removed by BoNT/A) leads to a different impairment of the tSNARE functions, leading to a more rapid removal of the fragment and a consequent more rapid remodelling of the end plate. Alternatively, it is possible that SNAP-23, which is cleaved by BoNT/E, but not by BoNT/A (Washbourne *et al*. 1997), is implicated in the control of synaptic terminal plasticity and remodelling.

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