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Central effects of tetanus and botulinum neurotoxins

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ABSTRACT

Tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNTs; from A to G) are metalloproteases that act on nerve terminals to prevent exocytosis. They are extensively exploited for the study of cellular physiology. Moreover, BoNTs are also employed in clinical neurology for the treatment of several disorders characterized by hyperexcitability of peripheral nerve terminals. This review summarizes recent studies that have provided a deeper understanding of the mode of action of TeNT and BoNTs. TeNT and BoNTs bind with extreme specificity and are internalized at the neuromuscular junction. We first examine the retrograde transport mechanisms by which TeNT gains access to the central nervous system. We also discuss recent findings indicating that, besides their well known local actions at the neuromuscular junction, BoNTs can also affect central circuits.

The family of clostridial neurotoxins comprises tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNTs, from A to G). They are the causative agents of tetanus and botulism, respectively. Tetanus is characterized by spastic paralysis due to a TeNT-mediated blockade of inhibitory circuits in the spinal cord. In contrast, intoxication by BoNT results in flaccid paralysis due to a blockade of acetylcholine (ACh) release at the neuromuscular junction (NMJ). Due to their extreme potency and specificity, TeNT and BoNTs have also been exploited for scientific and therapeutic applications. In experimental settings, they represent valuable tools for the study of cellular physiology. In addition, localized minute injections of BoNTs are increasingly being used in the clinic for the treatment of several human diseases characterized by hyperexcitability of peripheral nerve terminals (Davletov et al., 2005; Montecucco and Molgo, 2005; Naumann et al., 2008; Simpson et al., 2008a,b).

Structurally, TeNT and BoNTs share a common organization, with a heavy (H, 100 kDa) and a light chain (L, 50 kDa) linked by a disulphide bond and non-covalent interactions. The carboxy-terminus of the heavy chain (H_C) binds with extraordinary affinity and specificity to nerve terminals. Following internalization, the amino-terminal portion of the heavy chain (H_N) inserts into the membrane of the endosome at acidic pH and assists the translocation of the L chain into the cytosol. Finally, the L chain is endowed with a zinc-endopeptidase activity specific for SNARE proteins. SNARE proteins are involved in the fusion of synaptic vesicles with the plasma membrane and therefore the catalytic activity of the light chain is to prevent exocytosis and neurotransmission (Jahn and Sudhof, 1999).

Poisoning by TeNT and BoNTs occurs via a sequential mechanism comprising cell binding, internalization, trafficking, translocation into the neuronal cytosol and catalytic cleavage of protein substrates (Meunier et al., 2002; Turton et al., 2002). The neuronal binding domain resides in the C-terminal portion of the heavy chain (H_C). Specifically, there is evidence that the H_C of clostridial neurotoxins binds to both polysialogangliosides and membrane proteins, thus providing support for the "double-receptor model" (Montecucco, 1986). In particular, it has been proposed that TeNT and BoNTs are first captured by an antenna consisting of the oligosaccharide portion of

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polysialogangliosides. This step yields a membrane concentration that facilitates the subsequent interaction with protein receptor molecules on the plasma membrane (Montecucco et al., 2004). Indeed, it appears that both polysialogangliosides and protein receptors should be present for efficient and productive binding of clostridial toxins to membranes (Dong et al., 2007; Rummel et al., 2007). Interaction with polygangliosides is mediated by an oligosaccharide-binding pocket in the H_C portion of all clostridial toxins (known as the lactose site). TeNT possesses an additional carbohydrate-binding site which has been shown to dock sialic acid (Rummel et al., 2003). Protein receptor molecules have been so far identified only for a subset of clostridial toxins (Dong et al., 2003; Rummel et al., 2004; Dong et al., 2006; Mahrhold et al., 2006). BoNT/B and BoNT/G have been found to bind to synaptotagmins (Syts) I and II, two proteins integral to the synaptic vesicle membrane (Dong et al., 2007; Rummel et al., 2007). The Syt domain recognized by BoNT/B is inside the synaptic vesicle lumen and it is therefore exposed at the synaptic terminal during exocytosis (Schiavo, 2006). This accounts for the well known activity-dependent uptake of BoNTs by synaptic terminals. Another synaptic vesicle protein, SV2, acts as the protein receptor for BoNT/A (Dong et al., 2006; Mahrhold et al., 2006). Again, binding of BoNT/A occurs within a lumenal loop of SV2, accounting for accelerated uptake of the toxin following nerve stimulation (Hughes and Whaler, 1962; Keller et al., 2004; Dong et al., 2006). In spite of these recent discoveries, many questions about the membrane binding of BoNTs remain unanswered. In addition to the lack of functional information on the receptors of BoNT/E and F, protein receptors do not seem to play a major role in the binding of BoNT/C and D (Tsukamoto et al., 2005). Furthermore, recent data indicate that BoNTs may interact with more than one single protein ligand. Indeed, BoNT/A and BoNT/B appear to associate with synaptic vesicle protein complexes comprising SV2, synaptotagmin, synaptophysin, VAMP-2, and several subunits of the vesicular ATPase (Baldwin and Barbieri, 2007). There is also evidence that a growth factor receptor, the fibroblast growth factor (FGF) receptor 3 can bind BoNT/A (Fernandez-Salas et al., 2008). The situation is even less clear regarding the neuronal receptors of TeNT. Indeed, TeNT binds the glycoprotein Thy-1 but this interaction is unlikely to be crucial for the biological activity of TeNT as Thy-1 knockout mice retain sensitivity to this toxin (Herreros et al., 2001). Furthermore, a recent report suggested the possibility that both the oligosaccharide and sialic binding sites of TeNT recognize different ganglioside species (Chen et al., 2008).

Following systemic intoxication, the initial target of TeNT and BoNTs is the NMJ. TeNT is internalized via a clathrin-dependent mechanism (Deinhardt et al., 2006a) and transported back to the motor neuron (MN) soma via retrograde axonal transport. It is then further transcytosed to inhibitory interneurons making synaptic contact with MNs, where it exerts its toxic effects (see below). In contrast, BoNTs mainly remain at the NMJ. They are internalized in synaptic vesicles and after acidification of the vesicle lumen are translocated into the neuronal cytosol, where they cleave their target SNAREs. However, several central nervous system (CNS) effects have been reported following peripheral administration of BoNT/A, raising the issue of how these central actions might arise. The next two paragraphs examine in detail the fate of TeNT and BoNTs upon application at the NMJ and their remote effects on brain circuits.

1. Retrograde transport of TeNT in motor neurons

Following peripheral administration, TeNT has been reported to enter the CNS very efficiently. The development of fluorescently-tagged versions of the TeNT H_C binding fragment has provided one important tool for characterizing the mechanisms of the retrograde transport of TeNT in MNs (Lalli et al., 2003a). Indeed, TeNT and TeNT H_C are internalized and transported within MNs in morphologically identical organelles with overlapping speed distributions (Lalli et al., 2003b).

TeNT enters synaptic terminals of MNs via clathrin-coated pits and axolemmal infoldings associated with lipid microdomains (Roux et al., 2005). Specifically, TeNT H_C binds to a lipid-protein receptor complex containing the ganglioside GD1b. TeNT is then laterally sorted to clathrin-coated pits and, during this sorting event, GD1b is excluded from the toxin receptor complex (Deinhardt et al., 2006a). TeNT endocytosis requires the activity of dynamin and a subset of classical clathrin endocytic adaptors, including AP-2, and AP180, but it is independent of epsin-1 (Deinhardt et al., 2006a). After clathrin-mediated endocytosis, TeNT is sorted towards the retrograde transport route. Recent data indicate that two small GTPases, Rab5 and Rab7, are required in a sequential manner for the sorting steps preceding retrograde translocation of TeNT-positive cargoes (Deinhardt et al., 2006b). TeNT appears to first transit through a Rab5-positive, stationary compartment, and then progresses to a Rab7-positive moving compartment. Functional knockdown of either Rab5 or Rab7 completely abolishes TeNT retrograde transport in MNs (Deinhardt et al., 2006b).

The progression of TeNT-positive organelles along axons requires the concerted activity of both cytoplasmic dynein (a microtubule-based motor) and myosin Va (an F-actin based motor) (Lalli et al., 2003b). Interestingly, the transport pathway of TeNT is shared by the neurotrophin receptors $p75^{NTR}$ and TrkB, as well as their ligands nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Deinhardt et al., 2006b). TeNT-positive axonal carriers also contain phosphorylated Erk1/2, indicating that they might have signalling capability (Deinhardt et al., 2006b; G.S., unpublished results). This is consistent with the observation that TeNT and TeNT H_C mediate activation of intracellular signalling pathways involving neurotrophin receptors (Gil et al., 2003), although the details of this transactivation mechanism remain unclear.

It is interesting to note that retrogradely moving TeNT-positive carriers exhibit a neutral pH, which is kept constant during transport (Bohnert and Schiavo, 2005). This is in contrast to the situation found in the classical endosomal pathway, which undergoes rapid acidification upon internalization. This specialized pH regulation of TeNT carriers is likely to be due to the exclusion of the

vacuolar H+ ATPase (vATPase) from TeNT-positive moving organelles (Bohnert and Schiavo, 2005). The entry of TeNT into a neutral endocytic compartment is functionally important, as acidic pH triggers a conformational change of TeNT (and BoNTs) that causes their membrane insertion and the translocation of the L chain into the cytosol (Koriazova and Montal, 2003). Thus, the neutral pH of the retrogradely moving TeNT organelles allows entrapment of TeNT in the lumen of the carrier during axonal transfer to MN somas.

Once it has reached the cell body, TeNT must escape degradation in lysosomes and be sorted to synaptic sites for further trafficking. Indeed, TeNT is released from the MN to afferent inhibitory terminals via a process of synaptic transcytosis. The mechanisms and compartments involved in this process are still poorly defined (von Bartheld, 2004). Work with neurotrophic factors has shown that some of the retrogradely transported factors (i.e. NGF) are degraded in lysosomes, while others (i.e. BDNF) are localized to multivesicular bodies within the proximal dendrites of MNs and then released at synaptic sites (Rind et al., 2005). TeNT is specifically transcytosed to inhibitory terminals via a process likely to involve synaptic vesicle uptake (Matteoli et al., 1996). TeNT is finally translocated into the cytosol of inhibitory terminals where it cleaves its specific substrate VAMP/synaptobrevin. The details on how this remarkably specific transcytosis is achieved are presently unknown. Obviously, further elucidation of the membrane binding partners of TeNT would help in clarifying this issue.

The ability of TeNT to undergo retrograde transport and transcytosis has been exploited for tracing neural circuits and drug delivery in experimental settings. For tracing purposes, the non-toxic TeNT H_C has been chemically conjugated or genetically fused with horseradish peroxidase, beta-galactosidase, or green-fluorescent protein (von Bartheld, 2004; Bilsland and Schiavo, 2008). Such hybrid neuronal tracers maintain the ability to be transferred trans-synaptically (Miana-Mena et al., 2003; Perreault et al., 2006) and have been used successfully in several experimental systems. TeNT H_C has also been used as a delivery vehicle for drugs and ligands to brain neurons (Bizzini et al., 1980; Francis et al., 1995). Although this approach is elegant, its translation to clinical practice is likely to be impractical using wild type TeNT H_C due to the presence of antibodies directed against TeNT in most individuals. Protein re-engineering using TeNT H_C mutants lacking immunodominant epitopes might be of use for the future exploitation of this delivery strategy.

2. Central effects of BoNTs following peripheral administration

In the previous section, we have described the mechanisms underlying the long-distance journey of TeNT from the NMJ to the CNS. In contrast to TeNT, it is generally assumed that the effects of BoNTs remain restricted to the peripheral nervous system. However, there is substantial evidence that BoNTs, especially at high doses, can affect higher structures in the brain. One of the first demonstrations of a central effect of BoNTs was provided by Tyler (1963) that reported alterations of the H reflex in a man with botulism, indicative of alterations at the spinal level. Polley et al. (1965) showed changes in cortical activity in BoNT-poisoned monkeys, and pyramidal signs have been described in a case of human botulism (Santini et al., 1999). Recent neurophysiological studies also indicate a central effect of BoNT/A following intramuscular administration (reviewed by Curra et al., 2004; Gracies, 2004; Abbruzzese and Berardelli, 2006). For example, studies on the reciprocal inhibition between forearm muscles reveal that intramuscular BoNT/A changes the excitability of spinal cord circuitry (Priori et al., 1995). Interestingly, many distant effects of BoNT/A have been reported (Garner et al., 1993; Wohlfarth et al., 2001). In particular, there is a significant prolongation of the latency and a reduction in the persistence of F-waves in segments remote from the injection site (Wohlfarth et al., 2001), again pointing to a modification of MN excitability. In addition, two studies have demonstrated alterations at the level of motor cortex in subjects treated with BoNT/A. Specifically, corticomotor representation was shown to be altered in patients with dystonia, and BoNT/A treatment in the dystonic limb re-established normal cortical maps (Byrnes et al., 1998). Intracortical inhibition was also found to be defective in subjects with upper limb dystonia, and the clinical benefit of BoNT/A correlated with a return of cortical inhibition to the levels seen in normal subjects (Gilio et al., 2000). It is worth noting that these cortical changes were completely reversible and disappeared at the completion of BoNT/A effects. Thus, BoNT/A can transiently affect the excitability of cortical areas.

Studies in cats by the group of Delgado-Garcia indicate an effect of BoNT/A also on brainstem circuitry. These authors analyzed the spiking activity and ultrastructure of abducens MNs following delivery of BoNT/A to the lateral rectus muscle. There were clear alterations in the discharge pattern of these MNs (Moreno-Lopez et al., 1997a). Interestingly, these changes were dose-dependent and only minor modifications of MN firing were observed with a low amount of BoNT/A (Moreno-Lopez et al., 1994, 1997b). At the anatomical level, high doses of BoNT/A affected the synaptic inputs to MNs, with signs of synaptic stripping and a reduction in the number of clear vesicles near the active zone, indicative of an impairment in neuroexocytosis (Pastor et al., 1997). Altogether, these data provide compelling evidence that intramuscular BoNT/A produces central effects in a dose-dependent manner (Moreno-Lopez et al., 1994).

The mechanism by which these modifications arise is not completely understood. These central effects may result from an alteration in sensory inputs, specifically in the spindle afferent inflow directed to spinal MNs (Curra et al., 2004; Abbruzzese and Berardelli, 2006). In support of this idea, there is evidence that BoNT/A injected in the muscle acts onto intrafusal junctions, blocking γ-motor endings and thereby reducing the spindle afferent discharge (Filippi et al., 1993). A second possibility envisages central plastic rearrangements subsequent to deafferentation. Indeed, substantial reorganization of the motor system is observed following denervation (Ziemann et al., 1998). Although these two possibilities have been previously favoured to others, such as the direct crossing of the

intact blood–brain barrier at specific sites (Boroff and Chen, 1975), a third alternative has emerged. Recently, data supporting a direct central action of BoNT/A via retrograde axonal transport in MNs have been provided. Experiments with radiolabelled BoNT/A have shown that the toxin is transferred to the ventral roots and adjacent spinal cord segments upon intramuscular injection (Habermann, 1974; Wiegand et al., 1976). It was unclear, however, whether the toxin was transported in a catalytically active form. Recently, Antonucci et al. (2008b) conclusively demonstrated that catalytically active BoNT/A (but not BoNT/E) undergoes retrograde axonal transport and transcytosis in different neurons, albeit at comparatively higher doses than those currently used in human therapy. Whilst most of the BoNT/A effects remained restricted to the injection site, there were signs of toxin activity also in distant synapses. Specifically, cleavage of the BoNT/A substrate SNAP-25 was detected in the rat facial nucleus following delivery of BoNT/A to the whisker pad (Antonucci et al., 2008b). Thus, retrograde axonal transport of active BoNT/A might account for some of the distant effects observed with this serotype.

The mechanisms and cellular compartments involved in retrograde transport of BoNT/A remain to be defined. Only a few points are worth mentioning at this stage. As we described above, entry of BoNT/A into neurons is mediated via synaptic vesicle recycling, specifically through the interaction with the luminal domain of SV2. Acidification of the recycled vesicle should then lead to release of the L chain of BoNT/A, thus preventing retrograde axonal transport. Interestingly, however, it is known that BoNT/A-containing vesicles acidify quite slowly as compared to BoNT/E-containing endosomes (Keller et al., 2004; Wang et al., 2008). This delayed translocation of BoNT/A might allow the BoNT/A cargoes to be loaded onto the axonal transport machinery. Indeed, there is evidence that synaptic vesicles shuttle between synaptic terminals and display considerable motility in axons (Darcy et al., 2006).

3. Direct central delivery of TeNT and BoNTs

As we have discussed above, TeNT and BoNTs do not normally cross an intact blood brain barrier. However, several investigators have deliberately introduced these toxins into the brains of experimental animals to interfere with central neurotransmission.

It has long been known that injection of TeNT into the brain produces epileptic foci (reviewed in Jefferys and Walker, 2006). Indeed, delivery of TeNT results in spontaneous seizures that may recur for the rest of the life of the animal. Most of the literature has examined the consequences of injecting TeNT either into the hippocampus or cortex of the rat (Brener et al., 1991; Jiang et al., 1998; Nilsen et al., 2005). In both cases, spontaneous seizures appear a few days after delivery of the toxin. In particular, administration of TeNT into the adult rat motor cortex results in focal seizures with features typical of those observed in clinical cases of epilepsia partialis continua (Nilsen et al., 2005). The epileptic syndrome produced by TeNT likely results from the suppression of inhibitory neurotransmission. Indeed, there is evidence for a loss of inhibition in both hippocampal and neocortical models of TeNT-induced epilepsy (reviewed by Jefferys and Walker, 2006).

Several studies *in vitro* have shown that BoNTs block exocytosis at central synapses with potencies and durations matching those observed for peripheral nerve terminals (Williamson et al., 1996; Foran et al., 2003). Entry of BoNTs into central neurons depends on synaptic vesicle recycling (Verderio et al., 2006). At the functional level, BoNTs have been found to inhibit the release of several neurotransmitters. For example, BoNT/A suppresses the release of ACh, glutamate, noradrenaline, glycine, serotonin and dopamine from synaptosomes (reviewed in Bozzi et al., 2006). GABA secretion appears to be more resistant to the action of BoNT/A and BoNT/E, i.e. high toxin concentrations are required to achieve inhibition (Ashton and Dolly, 1988; Verderio et al., 2007). This relative resistance seems to be due to the almost complete absence of SNAP-25, the molecular target of BoNT/A and BoNT/E, from inhibitory terminals (Verderio et al., 2004, 2007; Garbelli et al., 2008; but see Tafoya et al., 2006). At the electrophysiological level, BoNT/A has been shown to prevent the occurrence of both spontaneous and evoked excitatory postsynaptic potentials in hippocampal neurons (Capogna et al., 1997; Sutton et al., 2004). Consistent with these data, administration of BoNT/A or BoNT/E to the rodent hippocampus *in vivo* blocks glutamate release and spontaneous spiking activity of CA1 pyramidal neurons (Costantin et al., 2005; Antonucci et al., 2008a).

There is a growing interest in the use of BoNTs as an experimental tool for the study of brain function and plasticity. For example, Sutton et al. (2004) have exploited BoNT/A to investigate a role for spontaneous synaptic activity in dendritic protein synthesis. *In vivo*, different BoNTs represent ideal tools to block neuronal communication for varying durations (Costantin et al., 2005; Caleo et al., 2007; Antonucci et al., 2008b). In our view, this property holds tremendous promise for experimental studies in the neurosciences. One important application is the dissection of the areas involved in different types of behaviour. Delivery of BoNT/B to the entorhinal cortex of the adult rat has been reported to cause substantial impairments in several memory tasks (Ando et al., 2002). Injection of BoNT/E into the hippocampus leads to a marked deficit in spatial learning, which is fully reversible (Costantin et al., 2005).

Recently, injections of BoNT/E were exploited to investigate the role of synaptic activity in the development of the visual cortex during the so-called "critical period" (Caleo et al., 2007). It is well known that the visual cortex in mammals is immature at birth and gradually develops its functional properties during an early postnatal period. The experiment consisted of a transient, BoNT/E-mediated unilateral blockade of synaptic activity in rat pups during the critical period. The data demonstrate that visual function is permanently impaired in the blocked hemisphere. Interestingly, this effect extends equally to the contralateral, uninjected side, demonstrating a fundamental role for interhemispheric connections in cortical maturation (Caleo et al., 2007).

Another very interesting option is the use of engineered toxins that allow delivery of the catalytic light chain into selected neuronal populations. For example, conjugation of lectins to BoNT/A lacking its native receptor-binding domain enables the selective blockading of neurotransmitter release in nociceptive neurons (Duggan et al., 2002; Chaddock et al., 2004). Extension of this approach will allow neurophysiologists to investigate circuit function and behaviour under selective silencing of specific subsets of neurons.

BoNTs have also been proposed for the treatment of CNS pathologies. Luvisetto et al. (2006) have demonstrated antinociceptive effects of centrally administered BoNT/A in a mouse model of inflammatory pain. The blockade of synaptic activity by BoNTs could also be exploited in the treatment of focal onset, pharmacologically-resistant epilepsies such as mesial temporal lobe epilepsy (Pitkanen and Sutula, 2002). In these epileptic syndromes, the only cure is surgical removal of the seizure focus (Spencer, 2002). Therefore, there is a renewed interest in novel antiepileptic drugs with long-lasting action that could be delivered directly into the epileptic focus. In this context, intrahippocampal infusion of BoNT/E proved very effective in the control of acute limbic seizures (Costantin et al., 2005). BoNT/E also displays an anticonvulsant action on chronic seizures in a mouse model of mesial temporal lobe epilepsy (Antonucci et al., 2009). Furthermore, administration of BoNT/E protects from seizure-induced neuronal loss (Costantin et al., 2005; Antonucci et al., 2008a). This neuroprotective action likely depends on the inhibition of the release of glutamate and other excitotoxic neurotransmitters, and occurs via downregulation of proapoptotic proteins (Manno et al., 2007).

4. Concluding remarks

TeNT and BoNTs represent extremely specific and valuable tools for both basic and clinical applications. From the basic point of view, the efficient retrograde transport of TeNT allows us to gain insights into the mechanisms controlling intracellular and trans-synaptic trafficking in neurons, and BoNTs are now used for studying brain function and plasticity. From a clinical point of view, BoNTs are excellent drugs for the treatment of movement disorders, autonomic disorders, and some forms of pain. There is now evidence that intramuscular injection of BoNT/A, especially at high doses, might result in CNS effects. A more detailed understanding of these central actions' effects of BoNTs will provide valuable information for present and future uses of these neurotoxins in clinical practice.

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Conflict of Interest

None declared.

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