

REVIEW

A reappraisal of the central effects of botulinum neurotoxin type A: by what mechanism?

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Abstract

Botulinum neurotoxin A (BoNT/A) is a metalloprotease that enters peripheral motor nerve terminals and blocks the release of acetylcholine via the specific cleavage of the synaptosomal-associated protein of 25-kDa. Localized injections of BoNT/A are widely employed in clinical neurology to treat several human diseases characterized by muscle hyperactivity. It is generally assumed that the effects of BoNT/A remain localized to the injection site. However, several neurophysiological studies have provided evidence for central

effects of BoNT/A, raising the issue of how these actions arise. Here we review these data and discuss the possibility that retrograde axonal transport of catalytically active BoNT/A may explain at least some of its effects at the level of central circuits.

Keywords: botulinum neurotoxins, movement disorders, neuromuscular junction, retrograde axonal transport, synaptic transmission, synaptosomal-associated protein of 25-kDa. *J. Neurochem.* (2009) **109**, 15–24.

Botulinum neurotoxins are produced by anaerobic bacteria of the genus *Clostridium* and are the most potent toxins known (Schiavo *et al.* 2000; Meunier *et al.* 2002; Montecucco and Molgo 2005). There are seven serotypes of BoNTs, indicated with letters from A to G. Each toxin is composed of a heavy (H, 100 kDa) and a light chain (L, 50 kDa) linked by a disulphide bond and non-covalent interactions (Turton *et al.* 2002). The carboxy-terminus of the heavy chain (H_C) binds with extraordinary specificity to nerve terminals. Following receptor-mediated endocytosis and acidification of the endosome, the amino-terminal portion of the heavy chain (H_N) translocates the L chain across the vesicular membrane into the cytosol (Koriazova and Montal 2003). The L chain acts as Zn²⁺-dependent endopeptidase to cleave essential protein components of the neurotransmitter release machinery, the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins. This results in disruption of Ca²⁺-triggered fusion of synaptic vesicles (SVs) with the plasma membrane (Schiavo *et al.* 2000).

The L chains of the seven BoNTs are remarkably specific proteases. BoNT/B, /D, /F and /G cleave vesicle-associated

membrane protein/synaptobrevin, but each at different sites. BoNT/A and /E cleave synaptosomal-associated protein of 25-kDa (SNAP-25) at two different sites, and BoNT/C attacks both syntaxin and SNAP-25. There are also remarkable differences in the duration of action of the different BoNT serotypes. For example, BoNT/A and BoNT/E cleave the same substrate SNAP-25 (removing respectively 9 and 26 aminoacids from the carboxy terminus), but they cause synaptic blockade with very different properties. Indeed, neuromuscular paralysis triggered by BoNT/E is short-lived, while the blockade induced by BoNT/A lasts for much longer (Eleopra *et al.* 1998; Keller *et al.* 1999; Foran *et al.* 2003b). Factors dictating the short duration of BoNT/E action include limited

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Abbreviations used: BoNT, botulinum neurotoxin; FGF, fibroblast growth factor; SNAP-25, synaptosomal-associated protein of 25-kDa; SV, synaptic vesicle; TeNT, tetanus neurotoxin.

stability of this protease within nerve endings and speedy replenishment of BoNT/E-cleaved SNAP-25 (Keller *et al.* 1999; Adler *et al.* 2001; Foran *et al.* 2003b). The long duration of BoNT/A is due (i) to persistent catalytic activity of the protease inside nerve terminals (Keller *et al.* 1999; Antonucci *et al.* 2008), and (ii) to the slow replacement of BoNT/A-truncated SNAP-25 (Eleopra *et al.* 1998; Foran *et al.* 2003a; Meunier *et al.* 2003), which interferes with neuroexocytosis by acting as a dominant-negative factor (Foran *et al.* 2003a; Meunier *et al.* 2003; Montecucco *et al.* 2005).

The natural target of BoNTs is represented by the neuromuscular junction, where BoNT poisoning results in flaccid paralysis because of blockade of acetylcholine release. Several experimental studies have shed light on the sequence of events that follow poisoning with BoNT/A (reviewed in Meunier *et al.* 2002; Foran *et al.* 2003a). Blockade of exocytosis triggers a remarkable synaptic remodelling in motoneurons, with extensive sprouting and *de novo* synaptogenesis (de Paiva *et al.* 1999; Meunier *et al.* 2002; Morbiato *et al.* 2007). This sprouting network is responsible for re-establishing functional communication between motoneurons and muscle fibers (de Paiva *et al.* 1999; Foran *et al.* 2003a). Importantly, sprouts are eliminated when the original parent terminal recovers its ability to release neurotransmission (de Paiva *et al.* 1999; Meunier *et al.* 2003).

The extended duration of BoNT/A effects at the neuromuscular junction is one of the key features that has driven the widespread use of this serotype as a therapeutic agent. Biopharmaceutical preparations of BoNT/A have been used in different neurological diseases (blepharospasm, hemifacial spasm, cervical dystonia, writer's cramp and muscle spasticity) with the aim at weakening contraction in overactive muscles (Jankovic 2004). Importantly, the toxin appears to be taken up preferentially by hyperactive nerve terminals, consistent with the finding that nerve stimulation accelerates BoNT/A poisoning (Hughes and Whaler 1962; Eleopra *et al.* 1997; Keller *et al.* 2004; Dong *et al.* 2006). The peripheral effect of BoNT/A can be monitored by using electrophysiological measures of motor function, such as the maximum direct motor response (Mmax), elicited by peripheral nerve stimulation, which reflects the activation of all motor axons. BoNT/A injections lead to a consistent reduction of Mmax size in parallel with clinical improvement of function. However, this is not always so. It has been observed that the onset and peak of clinical response can be delayed by several days to a week compared to the electrophysiological findings, and the clinical benefit by BoNT/A injection may last much longer than the duration of the neuroparalysis induced by the toxin (Priori *et al.* 1995; Ziemann *et al.* 1998; Hardie 2000). On the other hand, patients may request retreatment with BoNT/A in spite of significant residual neuromuscular blockade (Hamjian and Walker 1994). A

reduction in the severity of leg spasticity has been reported after BoNT/A injection in spite of little neuromuscular blockade (Mazzocchio *et al.* 2007). There is also evidence for an improvement of function in muscles acting as antagonists to the injected one (Gracies *et al.* 2001; Miscio *et al.* 2004). All these observations are consistent with the suggestion that BoNT/A produces central effects when injected at therapeutic doses in humans (Currà *et al.* 2004; Abbruzzese and Berardelli 2006).

Evidence for central effects of BoNT/A

This issue has been addressed by many electrophysiological studies in humans and animal models. Here we briefly review the main findings. Neurophysiological effects of BoNT/A have been found to involve (i) spinal cord circuitry, (ii) brainstem, and (iii) motor cortex.

(i) Spinal cord circuitry. A first set of studies has been conducted in patients with essential tremor or dystonia, two conditions characterized by a dysfunction in the pattern of agonist/antagonist coordinated activity (Rothwell 1995; Ziemann *et al.* 1998). These patients exhibit reduced presynaptic inhibition of Ia terminals between flexor and extensor forearm muscles (Nakashima *et al.* 1989; Priori *et al.* 1995). Presynaptic inhibition is restored to normal levels after injection of BoNT/A in the wrist flexors of these patients (Priori *et al.* 1995).

A second set of experiments suggests a possible effect of intramuscular BoNT/A on intraspinal recurrent inhibition (Renshaw inhibition). Renshaw cells are glycinergic inhibitory interneurons that receive input from motor axon collaterals and synapse in turn on the somata of motoneurons in a negative-feedback fashion (Alvarez and Fyffe 2007). Evidence for reduced motoneuron input to Renshaw cells after peripheral BoNT/A injection has been obtained from morphological and physiological studies in the rat (Sanna *et al.* 1993; Gonzalez-Forero *et al.* 2005; Clowry *et al.* 2006). However, a cat study showed no effect of intramuscular BoNT/A on the discharge pattern of individual Renshaw cells (Hagenah *et al.* 1977). Another report demonstrated a decreased recurrent inhibitory activity after BoNT/A muscle injection, resulting in an increase in motoneuron excitability (Wiegand and Wellhoner 1977). These findings were attributed to an action of BoNT/A on the inhibitory synapse between the Renshaw cells and the α -motoneurons (Wiegand and Wellhoner 1977). Studies in patients with lower limb spasticity also point to a reduction in the strength of recurrent inhibition following intramuscular BoNT/A (Mazzocchio *et al.* 2007).

(ii) Brainstem. A series of studies by the group of Delgado-Garcia (Moreno-Lopez *et al.* 1994, 1997a,b; Pastor *et al.* 1997) has demonstrated dose-dependent changes in abducens motoneurons following delivery of BoNT/A into the lateral rectus muscle in the cat. Specifically, excitatory

and inhibitory synaptic transmission to abducens motoneurons is strongly affected several days after BoNT/A injection (Pastor *et al.* 1997). Moreover, motoneurons show modifications in their firing pattern, as demonstrated by the appearance of an abnormally low discharge rate (Moreno-Lopez *et al.* 1994). These changes are accompanied by ultrastructural modifications, such as synaptic stripping at the level of motoneuron somata (Pastor *et al.* 1997). There is also a reduction in the number of clear vesicles in the terminals impinging onto motoneurons, indicative of an impairment in neuroexocytosis (Pastor *et al.* 1997). It is worth noting that these changes are dose-dependent (Moreno-Lopez *et al.* 1994, 1997b). Electrophysiological alterations are modest or absent with doses corresponding to 50–70 mouse units, and become prominent with doses of about 600 mouse units (Moreno-Lopez *et al.* 1994, 1997b). Human studies revealed no modification of the hyperexcitable brainstem pathways in patients with cranial dystonia (blepharospasm) treated with BoNT/A in the orbicularis oculi muscle (Valls-Sole *et al.* 1991; Girlanda *et al.* 1996; Grandas *et al.* 1998; but see Behari and Raju 1996). In addition, BoNT/A injections for hemifacial spasm and craniocervical dystonia failed to show any effect on brainstem auditory evoked potentials (Behari and Raju 1996; Ce 2000). However, it may well be that the pathways tested by this particular technique are unaffected by BoNT/A central effects. On the other hand, unilateral thyroarytenoid muscle injections of BoNT/A for adductor spasmodic dysphonia led to hypoactivity also in the non-injected muscle, suggesting that speech improvement may in part reflect changes at brainstem level (Bielamowicz and Ludlow 2000).

(iii) Motor cortex. Corticomotor representation of the hand and forearm muscles was shown to be altered in patients with writer's cramp, and these changes could be reversed by BoNT/A injection into the affected muscles (Byrnes *et al.* 1998). Cortical changes induced by BoNT/A were completely reversible and disappeared at the completion of BoNT/A effects (Byrnes *et al.* 1998). Contrasting findings have been obtained for the studies concerning motor cortical excitability following BoNT/A. In one study in patients with upper limb dystonia, the clinical benefit of BoNT/A correlated with a return of cortical inhibition to the levels seen in normal subjects (Gilio *et al.* 2000), but restoration of inhibition has not been confirmed by a later report (Borojerdi *et al.* 2003). Finally, abnormally high cortical potentials, evoked by peripheral nerve stimulation, suggestive of increased cortical excitability, were significantly reduced after successful treatment of cervical dystonia with intramuscular BoNT/A (Kanovsky *et al.* 1998).

Altogether, these data provide evidence that intramuscular BoNT/A produces central effects. The following section addresses the possible mechanisms by which these actions arise.

Possible mechanisms for the central action of BoNT/A

Botulinum neurotoxin A might affect central circuits by at least three mechanisms, that are schematically summarized in Fig. 1.

(1) BoNT/A-mediated blockade of gamma motor endings, with consequent reduction of spindle afferent input from the injected muscle.

One of the most accredited hypothesis to explain the central physiological effects observed after intramuscular injection of BoNT/A is a concurrent indirect effect on spinal and cortical circuitry, through the action of BoNT/A on the intrafusal, beside the extrafusal, neuromuscular junction (see Currà *et al.* 2004 for references). Acetylcholine is also the neurotransmitter of γ -motoneuronal endings in intrafusal muscle fibers, and BoNT/A blockade of intrafusal fibers has been documented in animal studies (Filippi *et al.* 1993; Rosales *et al.* 1996). Specifically, at spinal level, lesser muscle afferent input from the wrist flexor (the injected muscle) would decrease the presynaptic gating of the extensor Ia terminals, resulting in a larger presynaptic inhibition of the Ia afferents from the wrist flexors (Priori *et al.* 1995). At cortical level, reduced muscle afferent input from the injected muscle would lead to a temporary reorganization of the altered sensorimotor interaction (Byrnes *et al.* 1998), which is a distinctive feature of writer's cramp (Marsden and Sheehy 1990).

(2) Plastic changes following BoNT/A-mediated blockade of neuromuscular transmission.

Theoretically, two sites of plasticity can be envisaged following blockade of neuromuscular transmission: one regarding the motoneuron itself, and the other concerning the target muscle. Indeed, there is evidence for rearrangements at both sites.

The functional and structural properties of motoneurons are known to vary following blockade of neuromuscular transmission with BoNT/A. At the functional level, changes in rheobase current, input resistance, time constant, and duration of the after-hyperpolarization are observed in spinal motoneurons of cats treated with BoNT/A into the medial gastrocnemius muscle (Pinter *et al.* 1991). Metabolic changes have been observed in rat hypoglossal motoneurons after intramuscular BoNT/A (Watson 1969). Blockade of synaptic transmission triggers changes in the expression of numerous genes in spinal motoneurons (Jung *et al.* 1997). In addition, significant reductions in the density of gephyrin, a glycine receptor clustering protein, on the membrane of cat abducens motoneurons have been reported after BoNT/A injection in their target muscle (Moreno-Lopez *et al.* 1998). This confirms alterations in synaptic input to motoneurons poisoned with BoNT/A.

A second site of significant plasticity is represented by the injected muscle. To our knowledge, not much emphasis has

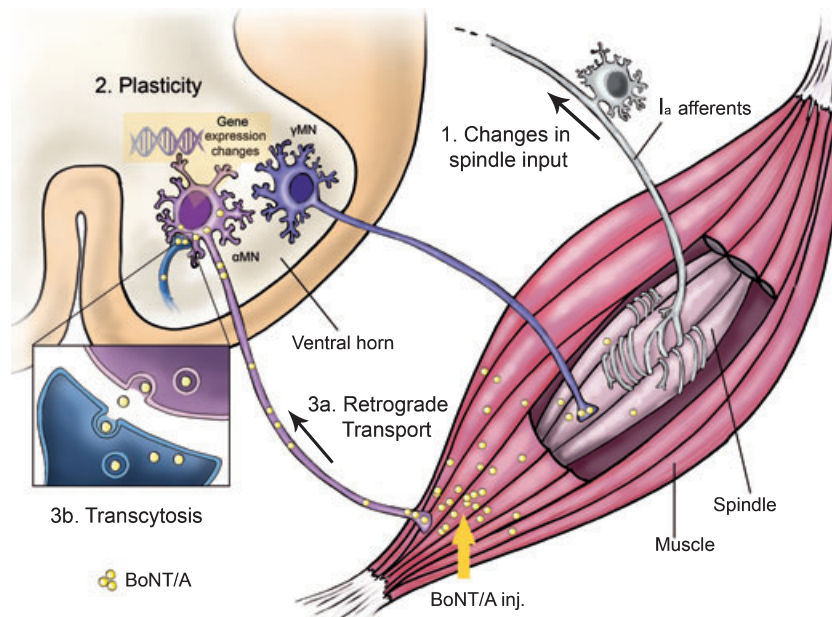


Fig. 1 Sketch of the extra- and intra-fusal muscle fibers with their afferent and efferent innervation and possible mechanisms for the central action of BoNT/A. (1) Presynaptic blockade of the neuromuscular connection between γ -motoneuronal endings and intrafusal muscle fibers with consequent reduction of input from Ia afferents. This may cause changes in the excitability of spinal pathways mediating presynaptic inhibition of Ia afferents to antagonist muscles, as well as transitory changes in motor maps at cortical level. (2) Pre-

synaptic blockade of the neuromuscular connection between α -motoneuronal endings and extrafusal muscle fibers may cause plastic changes in the motoneuron (e.g. changes in gene expression). (3) Retrograde transport along the motor axon to the motoneuron (3a) and transcytosis to afferent synapses (3b). Such possibility allows for direct central effects of BoNT/A. α MN, α -motoneuron; γ MN, γ -motoneuron.

been given to the role of muscle fiber type in the long term changes following BoNT/A injection. Muscles contain a characteristic mixture of slow fibres, innervated by tonically firing motoneurons and generating relatively little force but easily recruited and resistant to fatigue, and fast fibres, innervated by phasically active motoneurons which generate larger amounts of force (Henneman and Mendell 1991). Each fibre type expresses characteristic slow or fast myosin heavy chain with differing metabolic properties appropriate to the functional class of the muscle fibre (Pette and Staron 2000). Motoneurons innervating slow muscle fibres fire many more action potentials on a daily basis than do motoneurons that innervate fast muscle fibres (Hennig and Lomo 1985). Thus, nerve paralysis will eliminate proportionately more evoked acetylcholine release among slow than fast motoneurons. This may explain the differential effects of BoNT/A on motoneuron electrical properties according to fibre type (Pinter *et al.* 1991) and, perhaps, why the terminals of slow motoneurons sprout more vigorously than those of fast motoneurons after BoNT/A (Duchen 1970; Tonge 1974; Brown 1984; Frey *et al.* 2000). Muscle fibre phenotype is largely governed by motor axon activity patterns (Navarrete and Vrbova 1993), and so slow motor axons could take over fast fibres by collateral sprouting following blockade of

neuromuscular transmission by BoNT/A. Indeed, there is evidence for a change in muscle fibres' phenotype, as estimated by an increased expression of slow myosin heavy chain, in BoNT/A-treated muscles (Inagi *et al.* 1999; Frey *et al.* 2000; Dodd *et al.* 2005; Clowry *et al.* 2006). These findings may explain the clinical observation that BoNT/A injections occasionally leave the injected muscle disproportionately weak: if slow motor axons take over fast fibres, the result will be less tension and a weaker muscle. This notion may have important functional consequences for the treatment of the spastic muscle, which shows differential and time-dependent changes in its contractile properties (Hufschmidt and Mauritz 1985; Gracies 2005).

(3) Retrograde transport and transcytosis of BoNT/A following uptake at the neuromuscular junction.

Botulinum neurotoxin A applied in the periphery could directly affect central circuits via retrograde transport and transcytosis (Antonucci *et al.* 2008). The notion that BoNT/A could reach the central nervous system by retrograde axonal transport was initially supported by experiments with radiolabelled BoNT/A. It was found that the toxin is transferred to the ventral roots and adjacent spinal cord segments upon intramuscular injection in the cat (Habermann 1974; Wiegand *et al.* 1976). Black and Dolly (1986)

observed radiolabelled BoNT/A within the axoplasm of myelinated axons after peripheral injection of the toxin in mice, suggesting a retrograde intra-axonal transfer of the toxin. However, it has been argued that the retrograde axonal transport was so slow that the applied BoNT/A was likely to be inactivated before it reached the cell soma (Black and Dolly 1986; Dressler and Adib Saberi 2005). Contrary to these arguments, it has been recently shown that BoNT/A is also capable of long-distance effects after application at the neuromuscular junction. 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.* 2008).

An important issue is whether the central effects of BoNT/A depend on dosage. First, it is important to point out that several neurophysiological studies in patients indicate central actions of BoNT/A when injected intramuscularly at therapeutic doses (see above). Second, evidence from animal experiments has provided proof-of-principle for a direct central effect of BoNT/A via retrograde transport, especially at high doses (Wiegand *et al.* 1976; Moreno-Lopez *et al.* 1994; Pastor *et al.* 1997; Antonucci *et al.* 2008). For example, Antonucci *et al.* (2008) reported retrograde transport after injecting about 5–6 BoNT/A units into the whisker pad of adult rats (i.e., 15 units/kg body weight, taking into consideration that an adult rat weighs 350–400 g). Total dosage of up to 800 units can be employed for the treatment of dystonia and spasticity in patients (e.g. Garner *et al.* 1993; Wohlfarth *et al.* 2001; Jankovic 2004). Considering an average body weight of 70 kg, this leads to a maximum dose of about 11.5 units/kg in humans, which is slightly lower than the amount used in Antonucci *et al.* (2008). However, a direct comparison of animal and human data should be regarded with caution, as retrograde transport of BoNT/A is likely to depend not only on the total amount of injected toxin, but also on type and size of the muscle, density of innervation, and levels of expression of toxin receptors in the specific pathways under consideration.

Evidence for retrograde transport and transcytosis of catalytically active BoNT/A

Experiments in the visual system have provided conclusive evidence that at least a fraction of the injected BoNT/A undergoes retrograde axonal transport in neurons and is then transcytosed in a catalytically active form to afferent synapses (Antonucci *et al.* 2008). These studies consisted in injections of BoNT/A into the rat superior colliculus, a midbrain area that receives a massive, unidirectional projection from ganglion cells in the retina (see Fig. 2a). Three days (but not one day) after injection, BoNT/A-truncated SNAP-25 could be detected in retinal synapses impinging

onto ganglion cells (Antonucci *et al.* 2008; Fig. 2a). Importantly, cleavage of SNAP-25 was only apparent in neuronal populations directly connected to the superior colliculus, and appearance of truncated SNAP-25 in the retina was blocked by the axonal transport blocker colchicine, thus ruling out a systemic spread (Antonucci *et al.* 2008). Appearance of BoNT/A-altered SNAP-25 in retinal synapses suggested retrograde axonal transport and transcytosis, but it was at least theoretically possible that the cleaved SNAP-25 circulated, rather than the intact toxin. To prove retrograde transfer and transcytosis of catalytically active BoNT/A, retinal ganglion cell axons were cut (to prevent further transport from the colliculus) and BoNT/E was injected intraocularly (Fig. 2b). It is well known that BoNT/A removes the last nine residues from the C-terminus of SNAP-25, while BoNT/E cleaves a larger 26 residue fragment from the same region of SNAP-25; thus, the intraocular injection of BoNT/E removed BoNT/A-truncated SNAP-25 from retinal synapses (Fig. 2b; Lawrence *et al.* 1997; Keller *et al.* 1999; Adler *et al.* 2001). This loss of BoNT/A-truncated SNAP-25 should be permanent if the cleaved substrate is transported, as there is no way of generating new BoNT/A-altered SNAP-25 in the retina. Conversely, if active BoNT/A is transported from the colliculus, its action would reappear when BoNT/E effects are extinguished (Keller *et al.* 1999; Adler *et al.* 2001). Indeed, BoNT/A-mediated cleavage reappeared at the completion of BoNT/E effects (Fig. 2b). The return of BoNT/A-truncated SNAP-25 demonstrates that BoNT/A is retrogradely transported from the optic tectum and persists in a catalytically active form in retinal terminals.

BoNT/A retrograde transport: possible mechanisms and implications

To reach distant synapses, BoNT/A has to be loaded onto retrogradely moving organelles, escape degradation in the cell soma, be released at postsynaptic sites, undergo a second cycle of uptake, and finally be released into the cytosol to exert its proteolytic activity. This pathway is typical of tetanus neurotoxin (TeNT), another member of the clostridial family of toxins (Lalli *et al.* 2003; Rind *et al.* 2005). TeNT is internalized in neurons via a process of endocytosis that requires the activity of dynamin and other classical clathrin endocytic adaptors, and is then sorted towards the retrograde transport route (Deinhardt *et al.* 2006a,b). It will be important in future studies to determine if there is some overlap in the intracellular trafficking events that involve TeNT and BoNT/A. Another issue is whether there are differences in trafficking between BoNT/A and other BoNT serotypes. Ultrastructural analysis has revealed radiolabelled BoNT/B within myelinated axons after peripheral injection of the toxin, indicative of retrograde axonal transport (Black and Dolly 1986). Other experiments have failed to detect

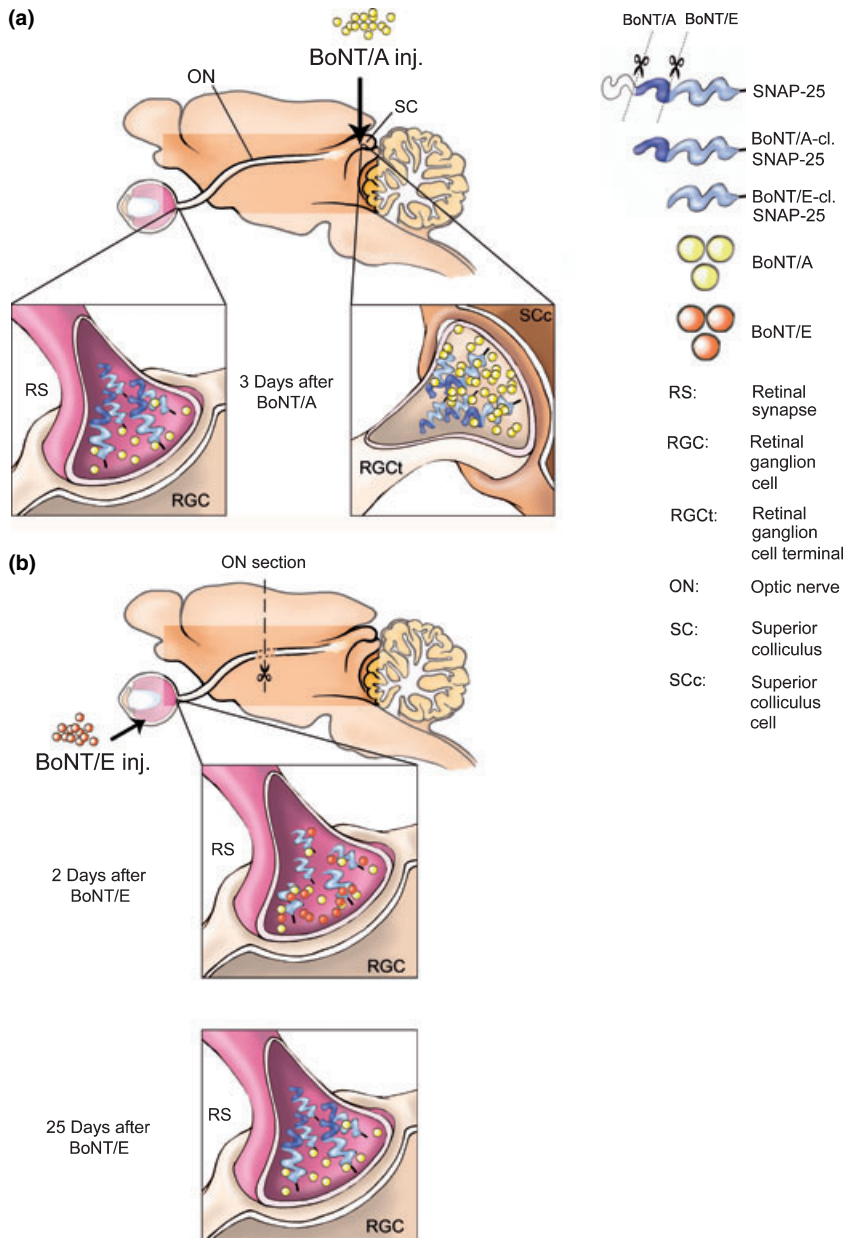


Fig. 2 Experimental design for the demonstration of retrograde transport of active BoNT/A in the visual system. (a) BoNT/A is injected into the superior colliculus (SC) of rats, where it exerts most of its proteolytic activity. Three days after toxin delivery, significant amounts of BoNT/A-truncated SNAP-25 are also detectable in retinal terminals impinging onto retinal ganglion cells. (b) To demonstrate retrograde transfer and transcytosis of active BoNT/A, retinal ganglion cell axons are cut (to prevent additional transport from the colliculus) and BoNT/E is injected intraocularly (top panel). BoNT/E removes BoNT/A-truncated SNAP-25 from retinal synapses and generates BoNT/E-altered SNAP-25 (middle panel). However, BoNT/A-mediated cleavage reappears at the completion of BoNT/E effects (25 days post-BoNT/E, lower panel), demonstrating persistent catalytic activity of BoNT/A within the retina.

retrograde transport and transcytosis of BoNT/E, at least in hippocampal neurons (Antonucci *et al.* 2008).

The mechanisms involved in retrograde transport and transcytosis of BoNT/A remain to be defined. Only some hypotheses can be drawn at this stage. Entry of BoNT/A into neurons is known to be mediated via SV recycling (Verderio *et al.* 2006). Specifically, BoNT/A interacts with the luminal domain of SV2 (Dong *et al.* 2006; Mahrhold *et al.* 2006). 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 SVs shuttle between synaptic terminals and display considerable motility in axons (Darcy *et al.* 2006).

Botulinum neurotoxin A has also been reported to bind the fibroblast growth factor (FGF) receptor 3 (Fernandez-Salas *et al.* 2008). Thus, another possibility is that BoNT/A is trafficked retrogradely following interaction with the FGF receptor 3, as FGF is well known to undergo axonal transport in neurons (Mufson *et al.* 1999).

One crucial determinant of the long-distance effects of BoNT/A is the type(s) of synapses that are affected in remote

areas following retrograde transport of the toxin. For example, tetanus toxin is retrogradely transported to spinal cord motoneurons and selectively transcytosed into inhibitory cells, thus resulting in spastic paralysis. Some specificity in transcytosis appear to exist also for BoNT/A. Indeed, in the study of BoNT/A trafficking cited above (Antonucci *et al.* 2008), BoNT/A effects were mainly observed in cholinergic synapses of the retina following injection of the toxin into the superior colliculus. Interestingly, retinal cholinergic terminals express high levels of SV2C (Wang *et al.* 2003; Antonucci *et al.* 2008), the SV2 isoform that exhibits the most robust BoNT/A binding activity (Dong *et al.* 2006; Mahrhold *et al.* 2006). Thus, retinal cholinergic cells might take up BoNT/A more efficiently because of SV2C receptor expression.

The central synapses that are targeted by BoNT/A after retrograde transport in motoneurons remain to be determined. It is conceivable that such direct central actions may act synergistically with the peripheral blockade (for example in the case of the silencing of an excitatory input to the motoneuron, that would reinforce the peripheral effect). In other cases, additional circuits may be affected. Indeed, *in vitro* and *in vivo* studies demonstrate that BoNT/A has the potential to interfere with release of several neurotransmitters from central neurons (Ashton and Dolly 1988; Luvisetto *et al.* 2003, 2004; Bozzi *et al.* 2006; Verderio *et al.* 2007). Wiegand and Wellhoner (1977) postulated a direct action of intramuscular BoNT/A on inhibitory connections between Renshaw cells and alpha-motoneurons. Cholinergic inputs from motor axon collaterals to Renshaw cells might be affected as well (Sanna *et al.* 1993; Gonzalez-Forero *et al.* 2005; Clowry *et al.* 2006; but see Hagenah *et al.* 1977). The physiological data in cats injected with BoNT/A into the lateral rectus muscle are consistent with a specific blocking effect of the toxin on vestibular and reticular afferents onto abducens motoneurons (Moreno-Lopez *et al.* 1994, 1997b).

Concluding remarks

From a clinical point of view, BoNT/A is an excellent drug for the treatment of neuromuscular pathologies, such as dystonia and spasticity. In both pathologies, treatment with BoNT/A has advantageously replaced surgical procedures. The range of clinical applications of BoNT/A is continuously increasing to include treatment of a variety of ophthalmological, gastrointestinal, urological, orthopaedic, dermatological, secretory, painful, and cosmetic disorders. Despite this widespread use, relatively little is known on BoNT/A intracellular trafficking and potential central effects. It is therefore important to fully characterize the spectrum of actions of this neurotoxin. There is substantial evidence that intramuscular injection of BoNT/A results in central nervous system effects. These findings have been usually ascribed to plastic rearrangements subsequent to the peripheral blockade. The finding of a

retrograde transport of catalytically active BoNT/A suggests that BoNT/A may also have direct central effects, especially at high doses. It is not yet clear whether these central effects actually contribute to the therapeutic efficacy of BoNT/A. A more detailed understanding of the central actions of BoNT/A will provide valuable information for present and future uses of this neurotoxin in clinical practice.

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