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review

Entering neurons: botulinum toxins and synaptic vesicle recycling

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Botulinum toxins are metalloproteases that act inside nerve terminals and block neurotransmitter release through their cleavage of components of the exocytosis machinery. These toxins are used to treat human diseases that are characterized by hyperfunction of cholinergic terminals. Recently, evidence has accumulated that gangliosides and synaptic vesicle proteins cooperate to mediate toxin binding to the presynaptic terminal. The differential distribution of synaptic vesicle protein receptors, gangliosides and toxin substrates in distinct neuronal populations opens up the possibility of using different serotypes of botulinum toxins for the treatment of central nervous system diseases caused by altered activity of selected neuronal populations.

Keywords: botulinum toxins; gangliosides; SV2; synaptic vesicles; synaptotagmin

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Introduction

Several anaerobic bacteria of the genus *Clostridium* produce botulinum neurotoxins (BoNTs), which enter nerve terminals and cause a prolonged blockade of neuroexocytosis. This results in an impairment of muscle contraction and autonomic nerve functions; however, the effect is fully reversible provided the patient overcomes the acute phase of respiratory failure. Seven serotypes of BoNT (A–G) have been identified and are the most potent toxins known, with median lethal dose (LD50) values for mice ranging from 0.5 to 5 ng/kg depending on the serotype (Schiavo *et al*, 2000). These di-chain toxins are formed by a heavy (H) and a light (L) chain, linked by a single disulphide bond (Schiavo *et al*, 2000). BoNTs are adsorbed in the gastrointestinal tract, and, after entering the general circulation, bind to the presynaptic membrane of cholinergic nerve terminals and prevent the release of their neurotransmitter acetylcholine (Simpson, 2000). Intoxication proceeds through a four-step mechanism comprising binding, internalization, membrane translocation and proteolytic action (Schiavo *et al*, 2000). To accomplish this multistep process, BoNTs have a modular organization, with the carboxy-terminal domain of the H chain (H_C) responsible for neurospecific binding, and the amino-terminal 50 kDa domain of the H chain (H_N) involved in membrane translocation of the L chain. The L chain is a metalloprotease specific for components of the exocytosis machinery: BoNT/B, BoNT/D, BoNT/F and BoNT/G cleave synaptobrevin/vesicle-associated membrane protein (VAMP), which is an integral protein of the synaptic vesicle (SV) membrane; BoNT/C, BoNT/A and BoNT/E cleave the synaptosomal-associated protein of 25 kDa (SNAP-25), which is a protein located on the cytosolic face of the presynaptic membrane; and BoNT/C also cleaves the plasma membrane protein syntaxin (Schiavo *et al*, 2000). The combined properties of neurospecificity, potency and reversibility account for the use of BoNT/A in the treatment of various human diseases caused by the hyperfunction of cholinergic terminals, such as blepharospasm, spasticity, dystonia, hypersalivation and hyperhidrosis (Montecucco & Molgo, 2005). The number of human syndromes that are known to benefit from BoNT treatment is increasing, and a promising new area is the treatment of diseases involving the central nervous system (CNS). Indeed, when centrally administered, BoNTs can also block the release of other neurotransmitters, including glutamate, glycine, noradrenaline, dopamine, serotonin and neuropeptides (Schiavo *et al*, 2000). It is therefore important to elucidate the mechanisms that mediate their binding and internalization in neurons. Here, we review recent studies indicating that, to enter neurons, BoNTs parasitize the physiological process of SV recycling. Indeed, BoNTs bind to the inner surface of SVs during their exposure to the external medium, and are internalized by vesicle endocytosis. This mechanism of penetration probably favours internalization in hyperactive nerve terminals, which are treated with BoNTs to reduce acetylcholine release in human dystonias.

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Table 1 | Receptors for BoNT/A, BoNT/B and BoNT/G

Botulinum neurotoxin serotypes	Receptor	K_d/potency	Lipid requirement	Receptor expression at the neuro-muscular junction	Receptor expression in hippocampus/cortex excitatory neurons	Receptor expression in hippocampus/cortex inhibitory neurons
BoNT/B	Syt-I (Nishiki *et al*, 1996; Dong *et al*, 2003)	2.3 nM (Nishiki *et al*, 1996)	Yes (Nishiki *et al*, 1996; Dong *et al*, 2003)	No (Li *et al*, 1994; Marqueze *et al*, 1995)	Yes (Geppert *et al*, 1991)	Yes (Geppert *et al*, 1991)
BoNT/B	Syt-II (Nishiki *et al*, 1996; Dong *et al*, 2003)	0.23 nM (Nishiki *et al*, 1996)	Yes (Nishiki *et al*, 1996; Dong *et al*, 2003)	Yes (Li *et al*, 1994, Marqueze *et al*, 1995)	Yes (subpopulation; Geppert *et al*, 1991; Marqueze *et al*, 1995)	To be defined
BoNT/G	Syt-I (Rummel *et al*, 2004a)	Syt-I peptide 66% inhibition (Rummel *et al*, 2004a)	No (Rummel *et al*, 2004a)	See above	See above	See above
BoNT/G	Syt-II (Rummel *et al*, 2004a)	Syt-II peptide 37.9% inhibition (Rummel *et al*, 2004a)	No (Rummel *et al*, 2004a)	See above	See above	See above
BoNT/A	SV2C (Dong *et al*, 2006; Mahrhold *et al*, 2006)	Binding activity SV2C>SV2A>SV2B (Dong *et al*, 2006)	Yes, all isoforms (Dong *et al*, 2006)	Yes (Dong *et al*, 2006)	No Bajjalieh *et al*, 1994; Janz & Sudhof, 1999; Dong *et al*, 2006)	Yes (subpopulation only; C.V., C.G., M.M., unpublished data)
BoNT/A	SV2A (Dong *et al*, 2006)	Binding activity SV2C>SV2A>SV2B (Dong *et al*, 2006)	No (Mahrhold *et al*, 2006)	Yes (Dong *et al*, 2006)	Yes (Bajjalieh *et al*, 1994; Janz & Sudhof, 1999)	Yes (Bajjalieh *et al*, 1994; Janz & Sudhof, 1999)
BoNT/A	SV2B (Dong *et al*, 2006)	Binding activity SV2C>SV2A>SV2B (Dong *et al*, 2006)	No (Mahrhold *et al*, 2006)	Yes (Dong *et al*, 2006)	Yes (Bajjalieh *et al*, 1994; Janz & Sudhof, 1999)	No (Bajjalieh *et al*, 1994; Janz & Sudhof, 1999; C.V., C.G., M.M., unpublished data)

BoNT, Botulinum neurotoxin; K_d, dissociation constant; SV, synaptic vesicle protein; Syt-I and -II, synaptotagmin I and II.

Presynaptic binding and entry of BoNTs

Gangliosides are sialylated glycosphingolipids that are involved in the development, function and maintenance of the nervous system. Complex gangliosides are particularly enriched at the presynaptic membrane (Hansson *et al*, 1977). Polysialogangliosides are thought to be involved in BoNT binding as a result of the following observations: BoNTs bind to polysialogangliosides, particularly to G_{D1b}, G_{T1b} and G_{Q1b} (Schiavo *et al*, 2000), and the H_C fragments of BoNT/A and B bind to G_{T1b} through the conserved peptide motif H…SXWY…G (Rummel *et al*, 2004b); pre-incubation of BoNTs with polysialogangliosides partly prevents the poisoning of the neuromuscular junction; pre-treatment of cultured cells with polysialogangliosides increases their sensitivity to BoNT/A, whereas depletion of gangliosides in neuroblastoma cells impairs BoNT/A internalization; treatment of membranes with neuraminidase, which removes sialic-acid residues, decreases BoNT binding; and knockout mice defective in the production of polysialogangliosides show reduced sensitivity to BoNT/A and BoNT/B (Bullens *et al*, 2002; Kitamura *et al*, 2005; Schiavo *et al*, 2000; Yowler *et al*, 2002). More recently, polysialogangliosides G_{D1b} and G_{T1b} and phosphatidylethanolamine were reported to be the functional receptors of BoNT/C and BoNT/D, respectively (Tsukamoto *et al*, 2005).

However, polysialogangliosides alone do not account for the neurospecificity of binding of all BoNT serotypes. Indeed, SV proteins also participate in BoNT binding (Table 1). BoNT/B and BoNT/G interact with the luminal domain of the SV proteins synaptotagmin I (Syt-I) and Syt-II. BoNT/B has a higher affinity for Syt-II (Nishiki *et al*, 1996), whereas BoNT/G interacts preferentially with Syt-I (Dong *et al*, 2003; Nishiki *et al*, 1996; Rummel *et al*, 2004a). The following experimental evidence suggests that this binding is physiologically relevant: Syt-II-transfected Chinese hamster ovary cells bind BoNT/B after treatment with polysialogangliosides (Nishiki *et al*, 1994, 1996); entry of BoNT/B (but not BoNT/A or BoNT/E) into PC12 cells is dependent on Syt-I and Syt-II (Dong *et al*, 2003); mice are partly protected from BoNT/B toxicity by fragments corresponding to the luminal domain of Syt-II (Dong *et al*, 2003); BoNT/B and BoNT/G interact with Syt-I and Syt-II in pull-down assays; and, finally, peptides derived from the Syt-I and Syt-II luminal domains are able to partly inhibit neurotoxicity at the phrenic neuromuscular junction (Rummel *et al*, 2004a).

More recently, the specific interaction of BoNT/A with the luminal domain of SV2 has been reported (Dong *et al*, 2006; Mahrhold *et al*, 2006). SV2 is highly glycosylated and has 12 putative transmembrane regions (Fig 1), similar to transporters with cytosol-exposed amino and carboxy termini (Bajjalieh *et al*, 1992). The function of SV2 is not clearly defined, although it has been shown to be involved in vesicle recruitment to the plasma membrane in endocrine cells (Iezzi *et al*, 2005). SV2 isoforms bind to Syt-I (Schivell *et al*, 2005), providing an explanation for the spurious isolation of Syt-I as a BoNT/A-binding protein (Li & Singh, 1998). Vertebrates contain three distinct *SV2* genes encoding homologous proteins called SV2A, SV2B and SV2C (Bajjalieh *et al*, 1992, 1994), which are also expressed by endocrine cells (Buckley & Kelly 1985; Iezzi *et al*, 2005; Portela-Gomes *et al*, 2000).

Consistent with the BoNT/A–SV2 interaction detected by pull-down assays, fragments of the SV2 domain that interact with BoNT/A inhibit the binding of toxin to neurons (Dong *et al*, 2006). Furthermore, in hippocampal neurons deficient in SV2A and SV2B, which are the main isoforms expressed in the hippocampus (see

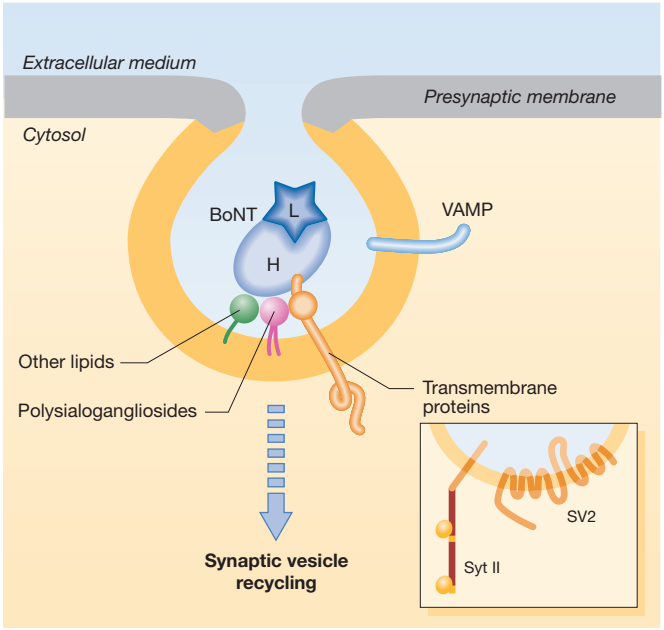

Fig 1 | Binding of botulinum neurotoxins to the luminal surface of synaptic vesicles exposed to medium on neuroexocytosis. The illustration depicts the combined role of polysialogangliosides (purple) and a synaptic vesicle (SV) protein (SV2 or synaptotagmin; orange) in mediating botulinum neurotoxin (BoNT)-neurospecific binding and entry into neurons after the retrieval of the vesicle. It suggests additional low-affinity interactions with other molecules (lipids and/or proteins) of the SV membrane (green; modified from Montecucco *et al*, 2004). Multiple interactions with molecules of the SV membrane would allow almost irreversible neuron binding, which would then become completely irreversible on vesicle fission from the presynaptic membrane. The yellow and grey areas denote the different compositions of the SV and presynaptic membranes, respectively. H, heavy chain of BoNT; L, light chain of BoNT; VAMP, vesicle-associated membrane protein.

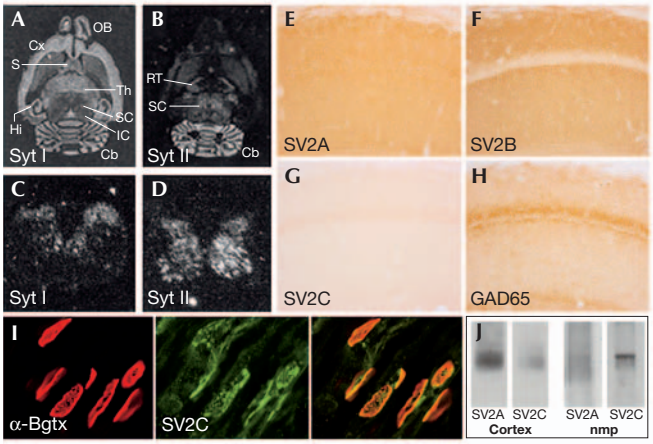

Fig 2 | Localization of Syt-I and Syt-II, and SV2A, SV2B and SV2C messenger RNA and protein. (**A**–**D**) *In situ* hybridization for synaptotagmin I (Syt-I) (**A**,**C**) and Syt-II (**B**,**D**) in rat brain (**A**,**B**) and spinal cord (**C**,**D**; modified from Berton *et al*, 1997). Note that Syt-II messenger RNA is restricted to the cerebellum (**B**) and is concentrated in the ventral horns of the spinal cord (**D**). (**E**–**H**) Immunoperoxidase staining of adult rat hippocampal sections for synaptic vesicle protein 2A (SV2A), SV2B and SV2C. SV2C immunoreactivity is largely undetectable (**G**), whereas SV2B (**F**) is excluded from inhibitory terminals, as identified by antibodies to glutamate decarboxylase 65 (GAD65; **H**). (**I**) Rat hemidiaphragms stained for α-bungarotoxin (α-Bgtx), which binds to postsynaptic nicotinic receptors, and for SV2C. Note the bright labelling for SV2C at the motor neuron terminals (see also Dong *et al*, 2006). (**J**) Western blot image of cortex and nerve muscle preparation (nmp) homogenates for SV2A and SV2C. Note the higher expression of SV2A compared with SV2C in rat cortex. The opposite situation is detectable in nerve muscle preparations.

below and Fig 2), BoNT/A binding was abolished but was restored by the expression of *SV2A*, *SV2B* or *SV2C*. Finally, mice that lack SV2B have a reduced sensitivity to BoNT/A (Dong *et al*, 2006). Interestingly, BoNT/A has a much stronger interaction with the luminal L4 loop of SV2C than with the corresponding loops of SV2A and SV2B (Dong *et al*, 2006), although the latter proteins contribute to BoNT/A binding to hippocampal neurons in culture and to the neuromuscular junction (Dong *et al*, 2006). Possibly owing to the reduced binding of BoNT/A to SV2A and SV2B compared with SV2C, Mahrhold and colleagues found a specific interaction of BoNT/A with SV2C, but not with SV2A and SV2B (Mahrhold *et al*, 2006). A peptide comprising the intravesicular domain between transmembrane domains 7 and 8 specifically reduced the neurotoxicity of BoNT/A to phrenic nerve preparations, supporting the physiological relevance of this interaction (Mahrhold *et al*, 2006). Although it has been reported that either lipids or proteins alone are sufficient to account for BoNT-neurospecific binding (for a discussion, see Montecucco, 1986), these findings can be rationalized in terms of a double-receptor model in which BoNTs interact with both polysialogangliosides and SV proteins (Fig 1, Table 1; Montecucco, 1986). Indeed, the existence of protein–lipid microdomains has been shown in neurons (Tsui-Pierchala *et al*, 2002).

BoNT activity in the central nervous system

Given that BoNTs do not cross the blood–brain barrier, their action is normally limited to peripheral nerve terminals. However, when injected directly into the brain, BoNTs can intoxicate neurons of the central nervous tissue. Indeed, SV proteins, ganglioside receptors and BoNT substrates are expressed in the CNS, although they have specific patterns of distribution (see above). The finding that BoNTs bind to protein receptor isoforms with different affinities opens up the possibility that the sensitivity of distinct terminals to BoNTs might depend on the isoforms expressed, and possibly on their glycosylation pattern. Motor neuron terminals express the protein receptor isoforms that bind more strongly to BoNTs, such as Syt-II (Juzans *et al*, 1996; Li *et al*, 1994; Marqueze *et al*, 1995) and SV2C (Fig 2; Janz & Sudhof, 1999). Conversely, high-affinity isoforms are poorly expressed in CNS neurons and terminals, in which Syt-I, SV2A and SV2B are the prevalent isoforms (Fig 2; Geppert *et al*, 1991; Marqueze *et al*, 1995). SV2C has been reported in only a small subset of neurons in the basal forebrain and brainstem, and was undetectable in the hippocampus (Janz & Sudhof, 1999) except for a subpopulation of inhibitory neurons (C.V., C.G. & M.M., unpublished data). The preferential expression of the high-affinity receptors at the neuromuscular junction might underlie the high efficiency of BoNT/A and BoNT/B intoxication at motor neuron terminals. Interestingly, BoNT/G, which binds preferentially to Syt-I, was shown to be far less efficient than BoNT/B

Table 2 | The distribution of synaptosomal-associated protein isoforms at the neuromuscular junction and in different neuronal populations in the central nervous system

SNAP isoform expression at the neuromuscular junction	SNAP isoform expression in the hippocampus/cortex excitatory neurons	SNAP isoform expression in the hippocampus/cortex inhibitory neurons
SNAP-25 (Washbourne *et al*, 2002)	SNAP-25 (mainly Vglut-1; F Conti, L Bragina, G Fattorini, S Giovedi, F Benfenati, C Candiracci, unpublished data)	SNAP-25 (largely undetectable; Verderio *et al*, 2004; Frassoni *et al*, 2005; F Conti, L Bragina, G Fattorini, S Giovedi, F Benfenati, C Candiracci, unpublished data, but see Tafoya *et al*, 2006)
SNAP-23 (to be defined)	SNAP-23 (mainly Vglut-2; F Conti, L Bragina, G Fattorini, S Giovedi, F Benfenati, C Candiracci, unpublished data)	SNAP-23 (subpopulation only; F Conti, L Bragina, G Fattorini, S Giovedi, F Benfenati, C Candiracci, unpublished data)
SNAP-47 (to be defined)	SNAP-47 (Holt *et al*, 2006)	SNAP-47 (to be defined)
SNAP-29 (to be defined)	SNAP-29 (Pan *et al*, 2005)	SNAP-29 (to be defined)

SNAP, synaptosomal-associated protein.

in phrenic nerve intoxication, although high concentrations of recombinant BoNT/G were used in the study (Rummel *et al*, 2004a). Varying sensitivity to BoNTs might also result from specific patterns of polysialogangliosides and other lipids, expressed by selected neurons. It is therefore possible that high-affinity receptors for BoNTs might be localized specifically to those neurons that represent the *in vivo* cellular target of each BoNT serotype. The predominant expression of the low-affinity protein receptors (Syt-I, SV2A and SV2B) in the brain might explain the findings that BoNTs are less potent in hippocampal neurons than the CNS-acting tetanus neurotoxin, which penetrates through SV recycling (Matteoli *et al*, 1996; Schiavo *et al*, 2000; Verderio *et al*, 1999; C.V., C.G. & M.M., unpublished data).

The mechanisms of toxin penetration in brain neurons deserve thorough investigation, owing to the promising beneficial effect of BoNTs in CNS pathologies and in the treatment of pain (Montecucco & Molgo, 2005). Direct microinjection of BoNTs into the rat CNS can be used to inhibit neurotransmission reversibly and to control inflammatory pain centrally (Luvisetto *et al*, 2003, 2006). Another promising application of BoNTs is in the treatment of drug-resistant epilepsies. Indeed, the delivery of BoNT/E to the rat hippocampus markedly reduces both focal and generalized kainic-acid-induced seizures, supporting its possible applications as an anti-ictal and anti-epileptogenic agent (Costantin *et al*, 2005). Although a synaptic protein receptor for BoNT/E has not yet been identified, the possibility of an activity-dependent internalization in the CNS could provide the basis for a preferential penetration of the toxin in the hyperactive neurons of the epileptic foci. These neurons would indeed expose more receptors because of their high rate of SV exo/endocytosis.

Further aspects to be considered in relation to possible differential sensitivity of distinct nerve terminals to BoNTs are the expression and accessibility of toxin substrates (Table 2). For example, it is known that γ-aminobutyric acid (GABA)ergic terminals are less sensitive to BoNT/A and BoNT/E than glutamatergic terminals (Ashton & Dolly, 1988; Bigalke *et al*, 1981; Verderio *et al*, 2004). The molecular basis for this differential sensitivity is still undefined. However, the finding that mature hippocampal inhibitory terminals express undetectable levels of SNAP-25 (Verderio *et al*, 2004), together with the observation that inhibitory synapses express BoNT/A receptors that allow toxin penetration (C.V., C.G. & M.M., unpublished data) suggest that the relative resistance of GABAergic terminals to BoNT/A and BoNT/E could result from one of two scenarios: a BoNT/A-resistant SNAP, such as SNAP-23, SNAP-47 or SNAP-29 (Galli *et al*, 1998; Holt *et al*, 2006), might be involved in GABA exocytosis; or low amounts of SNAP-25 at inhibitory synapses might be segregated in such a way as to be inaccessible to the BoNT L chain. Irrespective of the molecular mechanism involved, the preferential action of BoNT/A and BoNT/E at excitatory terminals could be the basis for the use of these serotypes to treat pathologies that result from imbalances between excitatory and inhibitory terminals (Costantin *et al*, 2005). A similar concept could be extended to other BoNT substrates. For example, synaptobrevin/VAMP1, which, at least in the rat, is resistant to BoNT/B, has a selective distribution in the brain and is restricted to nuclei involved in modulating somatomotor functions (Raptis *et al*, 2005; Trimble *et al*, 1990). The distribution of different syntaxin isoforms in the brain still needs to be thoroughly investigated, particularly in relation to their sensitivity to BoNT/C. Once a more detailed picture of the distribution of toxin receptors and substrates is achieved, it will be possible to use different BoNTs for the therapy of neurological disorders caused by the unbalanced activity of selected neuronal populations.

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