The binding of botulinum neurotoxins to different peripheral neurons

THE BINDING OF BOTULINUM NEUROTOXINS TO DIFFERENT PERIPHERAL NEURONS

O. Rossetto

Dipartimento di Scienze Biomediche, Università di Padova, Via U. Bassi 58/B, 35121 Padova, Italy

Correspondence: ornella.rossetto@unipd.it

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Abstract

Botulinum neurotoxins are the most potent toxins known. The double receptor binding modality represents one of the most significant properties of botulinum neurotoxins and largely accounts for their incredible potency and lethality. Despite the high affinity and the very specific binding, botulinum neurotoxins are versatile and multi-tasking toxins. Indeed they are able to act both at the somatic and at the autonomic nervous system.

In spite of the preference for cholinergic nerve terminals botulinum neurotoxins have been shown to inhibit to some extent also the noradrenergic postganglionic sympathetic nerve terminals and the afferent nerve terminals of the sensory neurons inhibiting the release of neuropeptides and glutamate, which are responsible of nociception. Therefore, there is increasing evidence that the therapeutic effect in both motor and autonomic disorders is based on a complex mode of botulinum neurotoxin action modulating the activity of efferent as well as afferent nerve fibres.

Introduction

Botulinum Neurotoxins (BoNTs) are a large group of bacterial protein exotoxins produced by phylogenetically distinct strains of the genus *Clostridium*, and they are the causative agents of botulism characterized by a flaccid neuromuscular paralysis (Rossetto et al., 2014; Williamson et al., 2016; Pirazzini et al., 2017). A recent and ongoing major revolution in the BoNT field is the identification of dozens and dozens of novel BoNT isoforms (Peck et al., 2017; Montecucco and Rasotto, 2015). Many toxin variants named

subtypes have been identified within serotypes (distinguished using an alpha-numeric code BoNT/A1, /A2,….. BoNT/B1, /B2 etc.) and many more are expected to be reported soon (Montecucco and Rasotto 2015; Peck et al. 2017). BoNTs are the most potent toxins known and display lethal doses in the low ng/kg range (Pirazzini et al., 2017). For this reason and due to the lack of immunological protection in the population, BoNTs are included as a Category A selected agents by the U.S. Centers for Disease Control and Prevention. At the same time, thanks to scientific and clinical research BoNTs have been developed as therapeutics for the treatment of human disorders characterized by muscular hypercontraction and also of autonomic conditions of glandular hypersecretion (Pirazzini et al., 2017; Hallett et al., 2013; Naumann et al., 2013).

Despite the existence of a high number of variants, all BoNTs are structurally similar and consist of a light chain (L, 50 kDa) and a heavy chain (H, 100 kDa). The complete crystallographic structure reveals three different domains, each with different function in the intoxication process: the L chain is a zinc-metalloprotease that specifically cleaves the three SNARE proteins necessary for neurotransmitter exocytosis; the N-terminal H_N domain assists the translocation of the L chain across the membrane of intraneuronal acidic vesicles into the cytosol; the C-terminal H_C domain is responsible for presynaptic binding and endocytosis and consists of two sub-domains (H_{CN} and H_{CC}) with different folding and membrane binding properties.

The key aspect to explain the extreme toxicity of BoNTs is their unique mode of binding and the ensuing selectivity targeting peripheral nerve terminals. This will be the focus of the present article.

Binding

BoNTs bind with high affinity to the presynaptic plasma membrane of skeletal and autonomic cholinergic nerve terminals in numbers estimated to be, for BoNT/A or /B, in the order of hundreds of molecules per square micrometers at the rat neuromuscular junction (NMJ) (Dolly et al., 1984). Indeed they have evolved a unique binding mode based on the sequential interactions, with two independent receptors (Montecucco, 1986; Rummel, 2016). With no exceptions, all BoNTs initially bind to polysialogangliosides (PSGs). The glycan part of ganglioside receptors provides abundance and specificity and accumulates the toxins onto unmyelinated areas of nerve endings thus facilitating the interaction with a second receptor (Rummel, 2016; Hamark et al., 2017). The second interaction provides a high affinity binding and is thus responsible for toxin internalization and the ensuing

trafficking. In most cases it is the luminal domain of a synaptic vesicle (SV) protein, which is serotype-specific. The SV calcium sensors Synaptotagmin I/II (Syt-I and Syt-II) were identified as specific receptors for BoNT/B (Nishiki et a., 1994; Dong et al., 2003), /G (Rummel et al., 2004; Dong et al., 2007) and the mosaic serotype /DC (Peng et al., 2012). This protein binding site is located adjacent but non-overlapping to the ganglioside binding pocket at the C-terminal tip of the H_C-subunit. Binding is mediated by hydrophobic interactions but also by one major salt bridge. *In vitro* binding experiments have shown that the affinity of BoNT/B and BoNT/G to the luminal domain of Syt-I and Syt-II decreases in the order B-Syt-II>>G-Syt-I>G-Syt-II>>B-Syt-I (Rummel et al., 2007). Remarkably, it has been recently shown that human Syt-II is not a high affinity receptor for BoNT/B and G due to a phenylalanine to leucine mutation in its luminal domain, which eliminates one of the three major interactions between Syt-II and BoNT/B (Peng et al., 2012; Strotmeier et al., 2012). This mutation is present only in humans and chimpanzees and might explain the observed disparity of BoNT/B potency in human and mice (Tao et al., 2017). Noteworthy Syt-II is present in every endplate in diaphragm muscle whereas only a subpopulation of NMJs additionally expresses Syt-I (Pang et al. 2006). The F54L mutation in human Syt-II, together with the low expression at the NMJ of Syt-I, explains why high doses of BoNT/B are required to achieve therapeutic effects in neuromuscular disorders. In contrast, the predominant presence of Syt-I in autonomic and sensory neurons (Li et al. 1994) might explain the observed autonomic effects of BoNT/B and the lower BoNT/A:BoNT/B ratio for autonomic indications (Kranz et al. 2011).

In addition to species-specificity differences in receptor binding, also subtype binding dissimilarities have been reported. Kozaki and co-workers identified a type B BoNT (now named BoNT/B2) produced by the strain 111 isolated from a case of infant botulism in Japan (Kozaki et al., 1998). This neurotoxin has a significant amino acid difference in the receptor binding domain with respect to BoNT/B1 (Ihara et al. 2003). Indeed, the latter binds both Syt-I and II, while BoNT/B2 binds only Syt-II. Therefore toxin subtypes may have different receptor recognition sites targeting different nerve terminals (e.g. autonomic versus motor neurons) depending on the receptor isoform expressed.

Other BoNTs use synaptic vesicle proteins different from synaptotagmins. BoNT/A and /E interact with a luminal loop of the glycoprotein SV2, a multi-spanning integral protein of synaptic vesicles of unknown function (Dong et al., 2006; Mahrhold et al., 2006; Binz and Rummel, 2009; Benoit et al., 2014, Yao et al., 2016). This loop is transiently exposed on the surface of nerve terminals upon SV exocytosis and becomes available for toxin binding.

Three isoforms of SV2 (A, B, C) are expressed at motor nerve terminals, but SV2C appears to be the one binding BoNT/A (Mahrhold et al., 2006; Benoit et al., 2014; Mahrhold et al., 2016) whilst BoNT/E binds isoforms A and B, but not C (Dong et al., 2008). In addition to the protein-protein H_C/A-SV2C contacts, which involve mostly the backbones of the two proteins (Benoit et al., 2014), protein-glycan interaction is required for high affinity binding of BoNT/A1 to SV2C and the glycan linked to asparagine N559 is specifically involved (Mahrhold et al., 2016; Yao et al., 2016).

Glutamine 1292 is a key residue in the SV2 glycan-binding site of the toxin and mutation G1292R severely decreases the toxicity in BoNT/A1 (Weisemann et al. 2014; Yao et al., 2016). This residue is conserved in seven of the eight BoNT/A subtypes and, notably, BoNT/A4, which is the only one with an arginine residue (R1292) has ~1,000-fold reduced biological activity with respect to BoNT/A1 (Whitemarsh et al., 2013; Montecucco and Zanotti, 2016).

This novel host recognition strategy, which in addition to protein–protein interactions uses the simultaneous recognition of an N–glycan leads to several implications:

- N-glycans vary from individual to individual and could contribute to explain the different clinical response, in term of onset and duration of neuroparalysis, among different human patients treated with the same dose of injected BoNT/A1.
- as invertebrate and vertebrate N-glycans are different (Moremen et al., 2012), this may contribute, together with the absence of gangliosides, to the lack of sensitivity of invertebrates to BoNTs.
- different laboratories use various cell models, which may have SV2 with a different glycosylation pattern and this may have an impact on the biological activity of the toxin tested in a toxicity assay.

The double receptor binding accounts for the extreme potency of BoNTs but does not explain their apparent selectivity for cholinergic nerve terminals, which may be provided by additional receptor(s) still to be identified (Montecucco et al., 2004).

BoNTs affect different peripheral neurons

The natural target of BoNTs is represented by the neuromuscular junction, where BoNT poisoning results in flaccid paralysis due to the blockade of acetylcholine release. When injected into skeletal muscles, BoNT/A acts at the extrafusal as well as intrafusal neuromuscular junctions (Filippi et al., 1993; Rosales et al., 1996; Currà et al., 2004). Actually, it blocks the acetylcholine release of both the α-motorneuronal endings (extrafusal)

and the γ-motorneuronal endings (intrafusal), i.e. the efferent nerve fibres of the somatic nervous system supplying skeletal muscles. Moreover, BoNT/A has been shown to block cholinergic pre and post-ganglionic nerve terminals of the parasympathetic and sympathetic autonomic nervous system supplying smooth muscle or secretory glands. The action of BoNT at both the somatic and the autonomic nervous system explains its efficacy in the treatment of movement disorders as well as autonomic disorders as demonstrated in several randomized clinical trials (Naumann et al., 2015).

Although the preference for cholinergic nerve terminals have been shown, BoNTs bind also to the afferent nerve terminals of the sensory neurons inhibiting the release of neuropeptides such as calcitonin gene-related peptide (CGRP) (Durham and Cady, 2004; Meng et al., 2007; Dolly and O'Connell 2012), substance P (Welch et al., 2000) and glutamate (Cui et al., 2004) and thus causing the analgesic effect reported in animal models of inflammatory and neuropathic pain (Marinelli et al., 2010; Aoki and Francis, 2011).

The ability of BoNT/A to affect efferent as well as afferent nerves is exemplified by its beneficial effect in different bladder control disorders (Chancellor et al., 2008).

Versatility of BoNT/A: The Bladder Paradigm

The bladder is innervated by parasympathetic (cholinergic) and sympathetic (adrenergic) efferent nerve terminals and by sensory (peptidergic) afferent nerve terminals. Bladder storage and voidance involve a complex interplay of efferent and afferent signals in a way that parasympathetic, sympathetic, somatic, and sensory nerves can work synergistically (Ikeda et al., 2012). Functional impairment at various levels may result in bladder control disorders, which can be roughly classified as disturbances of storage and disturbances of emptying. Different human bladder disorders, such as the Overactive Bladder (OAB) or the Neurogenic Detrusor Overactivity (NDO) characterized by urinary incontinence greatly benefit of the treatment with BoNT/A.

The inhibitory effect of BoNT/A on different bladder nerve terminals derives from the presence, at different extent, of BoNT/A high affinity binding sites. Indeed SV2 is expressed in all type of terminals with the highest expression in the parasympathetic nerve terminals followed by the sympathetic and sensory ones (Coelho et al., 2010). Accordingly, expressed SNAP25 is cleaved in almost all cholinergic parasympathetic neurons and in half of adrenergic sympathetic and sensory neurons after intramural BoNT/A injection throughout the whole bladder (Coelho et al., 2012a and 2012b).

In the treatment of OAB or NDO, the effect of BoNT/A on efferent signalling (mainly via acetylcholine and ATP) is thought to dampen detrusor smooth muscle contractions, thereby limiting detrusor overactivity (if present) and reducing the sensation of urgency (Ikeda et al., 2012). Moreover, increasing evidence shows that BoNT/A inhibits also afferent sensory signalling in the bladder (Kanai et al., 2011; Ikeda et al., 2012). In fact it can reduce afferent sensitization by inhibiting CGRP and SP release from peripheral afferent nerve endings projecting to this organ (Rapp et al., 2006; Coelho et al., 2014).

In addition, BoNT/A injection for overactive bladder treatment is also associated with a significant decrease of purinergic receptors P_2X_3 and capsaicin receptors TRPV1, most probably by reducing the SNARE dependent receptor trafficking (Apostolidis et al., 2005; Dolly and Lawrence, 2014; Liu et al., 2014). These two receptors are involved in nociception in the suburothelial sensory nerve fibres and these inhibitory mechanisms mediated by BoNT/A are expected to impair nociceptive input from urinary bladder.

The proof of concept that BoNT/A has a strong activity on sensory terminals is that intrathecal administration of toxin has a predominant effect on sensory fibres and is enough to control neurogenic detrusor overactivity in chronic spinal cord injured rats (Coelho et al., 2016).

Duration of action

It is well established that the clinical effect of BoNT is markedly longer in autonomic disorders than in motor indications. The effect of BoNT/A injected in skeletal muscle lasts 3 to 4 months whereas the effect of the neurotoxin injected in the bladder lasts 6 to 9 months. Even longer is the duration of action of BoNT/A in the treatment of hyperhidrosis (up to 30 months) (Naumann and Lowe, 2001; Heckmann, 2001). The reason why autonomic disorders have prolonged clinical benefit compared with movement disorders is unclear but different hypothesis can be made:

1) the longer duration of action in the autonomic disorders could result from the impairment of both pre- and postganglionic neurons and the different time of recovery of the two neurons which increases the probability of maintaining inhibition of parasympathetic or sympathetic function. Comparative studies on the persistence of cleaved SNAP-25 in preganglionic and postganglionic fibers are necessary to test this hypothesis. (Coelho et al., 2012a).

2) the mechanisms of the recovery of motor and of the autonomic nerve terminals could be different and could depend on different mediators. It is long known that the BoNT poisoned NMJ undergoes a profound remodelling with novel nerve terminals, which sprout from their

unmyelinated motor axon terminal and, to a lesser extent, from the first node of Ranvier (Duchen, 1971; Juzans et al., 1996; Meunier et al., 2002). The sprouts are guided by proliferating perisynaptic Schwann cells and eventually reach novel muscle fibres to form new nerve-muscle contacts even though are poorly efficient in ACh release (Rogozhin et al., 2008), providing a limited contribution to the recovery of the neurotransmission from nerve to the muscle fibre. With time sprouts degenerate and the original NMJ reacquires full function. In contrast, no information is available on the events that take place after the blockade of an autonomic nerve terminal.

3) the toxin life-time could be different in motor and autonomic nerve terminals. The long lifetime of the LC protease of BoNT/A within the nerve cytosol is one of the major determinants of its duration of action. It has been recently shown that BoNT/A L chain is extraordinarily stable because it escapes the action of ubiquitin ligases, by recruiting de-ubiquitinases, i.e. specialized enzymes that remove polyubiquitin chains (Shoemaker and Oyler, 2013; Tsai et al., 2017). No comparative studies of the toxin lifetime in the motor and in the autonomic nerve terminal are available.

Concluding remarks

It has been known for a long time that botulinum neurotoxins block acetylcholine release from nerve terminals, and therefore leads to cessation of somatic motor and/or parasympathetic transmission. Recently, it has been found that BoNTs also interferes with sensory transmission and this has opened new avenues in their clinical application for different pain conditions. A number of studies have examined the potential of targeting BoNT/A to afferent nerves through modification of its protein structure (Duggan et al., 2002; Meng et al., 2009), which would offer increased efficacy and a reduction in adverse side effects (e.g., urinary retention, antibody formation). Indeed, there is a growing area of research that aims at changing binding specificity, affinity, and duration of BoNT action to obtain tailor-made therapeutic agents. In addition, the identification of many different BoNT variants with different binding properties and nerve terminal specificity could potentially represent a natural goldmine for novel therapeutic applications.

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