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Matak, Ivica; Lacković, Zdravko

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Botulinum neurotoxin type A: actions beyond SNAP-25?

[1]Ivica Matak, [1]Zdravko Lacković

[1]Laboratory of Molecular Neuropharmacology, Department of Pharmacology and Croatian Brain Research Institute, University of Zagreb School of Medicine, Šalata 11, 10000 Zagreb, Croatia

Corresponding author:

Zdravko Lacković, Laboratory of Molecular Neuropharmacology, Department of Pharmacology and Croatian Brain Research Institute, University of Zagreb School of Medicine

Address: Šalata 11, 10000 Zagreb, Croatia;

tel. no. : 00385 1 4566843;

e-mails: lac@mef.hr; lackoviczdravko@gmail.com

[1] Abbreviations: BoNT/A, Botulinum neurotoxin type A; HC, heavy chain; LC, light chain; SNAP-25, Synaptosomal-Associated Protein of 25 kDa; SV2, synaptic vesicle protein 2; SNARE, Soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, Vesicle-associated membrane protein; CGRP, Calcitonin gene-related peptide; PC-12, Pheochromocytoma-12; PLA2, Phospholipase A2; AA, Arachidonic acid; LPA, lysophosphatidic acid; NMJ, neuromuscular junction

Abstract

Botulinum neurotoxin type A (BoNT/A), the most potent toxin known in nature which causes botulism, is a commonly used therapeutic protein. It prevents synaptic vesicle neuroexocytosis by proteolytic cleavage of Synaptosomal-Associated Protein of 25 kDa (SNAP-25). It is widely believed that BoNT/A therapeutic or toxic actions are exclusively mediated by SNAP-25 cleavage. On the other hand, *in vitro* and *in vivo* findings suggest that several BoNT/A actions related to neuroexocytosis, cell cycle and apoptosis, neuritogenesis and gene expression are not necessarily mediated by this widely accepted mechanism of action. In present review we summarize the literature evidence which point to the existence of unknown BoNT/A molecular target(s) and modulation of unknown signaling pathways. The effects of BoNT/A apparently independent of SNAP-25 occur at similar doses/concentrations known to induce SNAP-25 cleavage and prevention of neurotransmitter release. Accordingly, these effects might be pharmacologically significant. Potentially the most interesting are observations of antimitotic and antitumor activity of BoNT/A. However, the exact mechanisms require further studies.

Keywords: Botulinum toxin type A; Synaptosomal-associated protein of 25 kDa; arachidonic acid, neuritogenesis, apoptosis

1. Introduction

Botulinum toxin type A (BoNT/A) is a protein derived from Gram (-) anaerobic bacterium *C. botulinum*. BoNT/A and other botulinum toxin serotypes (B-G) cause a neuroparalytic disease called botulism in both animals and humans. At the same time in small doses it is widely used for treatment of pathological muscle contractions and autonomic hyperactivity. So far, BoNT/A has been registered for treatment of facial wrinkles, different movement disorders (strabism, blepharospasm, hemifacial spasm, cervical dystonia, upper limb spasticity), and autonomic disorders (primary axillar hyperhidrosis and neurogenic detrusor

overactivity). Chronic migraine is the only pain disorder with approved BoNT/A use (Chen, 2012). In addition, there many other clinical conditions with reported BoNT/A efficacy. In small doses BoNT/A is safe and usually does not induce adverse distant effects, however, it is contraindicated in disorders with pronounced muscle weakness such as myasthenia gravis, Lambert-Eaton syndrome and lower motoneuron disease (Wheeler and Smith, 2013). BoNT/A enters neurons by endocytosis into synaptic vesicles, mediated by toxin's heavy chain (HC) interaction with membrane protein acceptors (SV2 and FGF3) and gangliosides (Dong et al., 2006; Jacky et al., 2013; Rossetto et al., 2014). After the BoNT/A light chain (LC) is translocated into the synapse cytosol, it enzymatically cleaves a peptide bond on synaptosomal-associated protein of 25 kDa (SNAP-25), a synaptic protein localized on the inner side of plasma membrane (Blasi et al., 1993). SNAP-25 and two other proteins: syntaxin, and vesicle-associated membrane protein (VAMP)/Synaptobrevin forms heterotrimeric soluble N-ethylmaleimide-sensitive attachment protein receptor (SNARE) complex, which mediates the fusion of vesicular and synaptic membrane. BoNT/A -mediated SNAP-25 protein cleavage prevents the membrane fusion and neurotransmitter exocytosis (Rossetto et al., 2014). In the periphery BoNT/A paralyses the neuromuscular junctions and autonomic synapses, while in central neurons it inhibits the excitatory neurotransmission (Rosetto et al., 2014; Verderio et al., 2007; Kato et al., 2013). Apart from synaptic neurotransmitter release, BoNT/A may prevent other SNARE-dependent physiological functions in both neuronal and non-neuronal cells (reviewed by Matak and Lacković, 2014).

Up to now, SNAP-25 is the only accepted BoNT/A target molecule, and it is widely believed that the therapeutic and toxic actions of BoNT/A are exclusively mediated by prevention of synaptic neurotransmitter release induced by SNAP-25 cleavage (Wheeler and Smith, 2013; Rossetto et al., 2014). Only recently it is being investigated if some of the BoNT/A actions might be mediated by other SNARE-dependent physiological functions, such as the involvement of membrane trafficking of receptors in the antinociceptive action of BoNT/A (Shimizu et al., 2012). However, in addition to BoNT/A actions mediated by SNAP-25,

several observations suggest the actions apparently unassociated with SNAP-25 cleavage, linked to: (1) arachidonic acid pathway, (2) neuritogenesis, (3) cell cycle and apoptosis, and possibly (5) gene expression. In present review we summarize these observations and evaluate their significance.

2. BoNT/A and arachidonic acid-mediated neuroexocytosis

Arachidonic acid (AA) is formed from membrane phospholipids by different phospholipase A2 (PLA2) isoenzymes. In addition to its involvement in the synthesis of eicosanoids, AA promotes neurotransmitter release. BoNT/A effects on AA–mediated neuroexocytosis have been initially explored in a model of neuronal growth factor-differentiated pheochromocytoma-12 (PC-12) cell line (Ray et al., 1993). BoNT/A applied to the cell culture reduced the K^+-stimulated acetylcholine and arachidonic acid release. The toxin action was prevented by addition of PLA2 itself, by PLA2 activators mellitin and mastoparan (peptides from wasp venom) or by exogenous AA (Ray et al., 1993). Depletion of SNAP-25 by antisense oligonucleotides did not prevent neuroexocytosis evoked by PLA2, mastoparan and K+ (Ray et al.,1999). Accordingly, it was concluded that PLA2-medated neurotransmitter release, as well as the effect of BoNT/A, might be independent of SNAP-25 (Ray et al., 1999). Recently, intracellular delivery of PLA2 activator mastoparan-7 via heavy chain fragment of BoNT/A (mas-7-HC construct) was shown to prevent the BoNT/A-mediated inhibition of glycine release from spinal cord cells by 40%. The effect seems to be unrelated to SNAP-25, since the mas-7-HC did not alter the cleaved SNAP-25 levels (Zhang et al., 2013).

Similarly to AA, it was reported that BoNT/A prevents the neurotransmitter release evoked by lysophosphatidic acid (LPA), another phospholipid product of PLA2 (Ishida et al., 2004). The effect of LPA on neurotransmitter release involves reorganization of actin mediated by Rho cytosolic GTP-ases. It was found that BoNT/A reduced the RhoB protein expression and

prevented the LPA-evoked actin reorganization. Reduction of RhoB protein expression was prevented by proteasome inhibitors. Overexpression of RhoB protein prevented the BoNT/A-mediated inhibition of LPA-induced neurotransmitter release and actin reorganization. It was concluded that BoNT/A effect are mediated by increased RhoB proteasomal degradation, which occurs via unknown signaling pathway (Ishida et al., 2004).

3. BoNT/A and neuritogenesis

A well known property of BoNT/A action is the induction of transient sprouting at the motor nerve terminals in the proximity of neuromuscular junctions (NMJ). Due to the similar time course of sprouting and paralysis, it has been assumed that BoNT/A effects on neuritogenic outgrowth at the NMJ represent a secondary response to synaptic paralysis mediated by SNAP-25 cleavage (Morbiato et al., 2007; Harrison et al., 2011; Jiang et al., 2014)

The apparent association of sprouting activity of BoNT/A with SNARE-mediated proteolytic activity was questioned in a study of cultured embryonic motor neurons (Coffield and Yan, 2009). The authors reported the dose-dependent increase in neurite sprouting at lower toxin doses. Surprisingly, at higher BoNT/A doses, the effect on neurite sprouting was suppressed. Moreover, HC fragment of BoNT/A induced a dose-dependent biphasic neuritogenic effect similar to BoNT/A holotoxin. *Triticum vulgaris* lectin which binds to polysialogangliosides prevented the neuritogenic actions of BoNT/A and HC. Thus, binding to HC domain acceptors, which initiates yet unknown signaling pathways, may be responsible for BoNT/A neurogenic effect (Coffield and Yan, 2009). However, transfection of motor neurons with BoNT/A-resistant SNAP-25 reduced the neuritogenic action of BoNT/A *in vivo*, suggesting that the role of SNAP-25 cannot be excluded (Ragunath et al., 2008).

4. Effects on cell cycle and apoptosis

Karsenty et al. (2009) studied the effect of commercially available BoNT/A in human prostate cancer cell lines and prostate cancer xenografts in mice. BoNT/A dose-dependently reduced the mitotic index and increased the apopototic index in LNCaP cell lines at low BoNT/A concentrations (0.25-1 U/ml). In xenografts, BoNT/A reduced the tumor size and serum PSA levels (Karsenty et al., 2009). Proietti et al. (2012) performed similar experiments in LNCaP and PC-3 prostatic cancer cell lines, and at somewhat higher BoNT/A concentrations (1 U-5 U/ml) observed a similar reduction of mitotic index in LNCaP and PC-3 line. In addition, BoNT/A increased the expression of phosphorylated PLA2 (phospho-c-PLA2). It was suggested that BoNT/A inhibits the expression of activated PLA2, which may reduce the synthesis of arachidonic acid and eicosanoids (Proietti et al., 2012). PC-3 cell line expresses neither SNAP-25 transcript nor protein (http://www.proteinatlas.org/ENSG00000132639-SNAP25/cell/HPA001830, assessed June 2015).

In epithelial breast cancer cells (T-47 D) BoNT/A has antiproliferative and cytotoxic effects associated with increased caspase 3/7 activity (Bandala et al. (2013). Interestingly, in non-cancerogenous breast epithelial cell line (MCF-10 A) BoNT/A did not induce any proapoptotic effects, suggesting that the BoNT/A proapoptotic activity may be selective for cancer cells.

In contrast to BoNT/A proapoptotic activity in non-neuronal cancer cells, it was observed that BoNT/A complex alone and one of its auxiliary complex proteins (hemaglutinin-33) may reduce the apoptosis in SH-SY5Y human neuroblastoma cells (Kumar et al., 2012).

Another toxin action possibly independent of SNAP-25 may be linked to the observed BoNT/A clinical benefit in reducing keloids and hypertrophic scars (Shaarawy et al., 2015). In one study BoNT/A had no effect on keloid fibroblast proliferation and cytokine expression (Haubner et al., 2014). However, other authors observed that BoNT/A alters the cell cycle and reduces proliferation of cultured fibroblasts isolated from hypertrophic scars (Zhibo and Miaobo, 2008). In addition, BoNT/A reduces the expression of cytokines and growth factors known to participate in scar forming (Xiao et al., 2010; Xiao et al., 2011; Xiaoxue et al.,

2013). In cultured human fibroblasts, BoNT/A upregulated the collagen type I forming and decreased the expression of matrix metalloproteinases (Oh et al., 2012). Since fibroblasts do not express SNAP-25, observed BoNT/A effects might be mediated by other target molecules. The authors hypothesized that BoNT/A may produce its beneficial effects either by reducing the tensile force exerted on the keloids by surrounding muscle, or by altering cellular proliferation and dynamics of extracellular matrix (Roh et al., 2013).

5 *In vitro* and *in vivo* effects on gene expression

In line with possibility that the BoNT/A molecule may induce a more complex host response within the interacting cells, two studies employed a microarray analysis of gene expression in toxin-exposed cell cultures (Thirunavukkarasusx et al., 2011; Scherf et al., 2014). To mimic the food-borne toxin exposure, Thirunavukkarasusx et al. (2011) employed human intestine epithelial cell line (HT-29) (lacking SNAP 25) and human neuroblastoma cell line (SH-SY5Y). In HT-29 cells, 167 genes were significantly upregulated, and 60 genes were down-regulated (min. 2-fold change) after 6 h exposure to BoNT/A complex. Altered genes were related to focal adhesion/cell adhesion molecules, ubiquitin-proteasome pathway, p450-medated xenobiotic metabolism etc. In SH-SY5Y cells (72 h toxin exposure) 223 genes were up-regulated, while 18 genes were down-regulated. Altered genes are involved in inflammatory pathways, phosphatidyl inositol-related signaling, proteasomal degradation, Huntington's disease, calcium signaling pathway etc. The authors suggested a link between altered gene expression and proteins involved in synaptic remodeling and actin reorganization.

Scherf et al. (2014) conducted a microarray analysis of BoNT/A effect on neuronal transcriptome. BoNT/A and BoNT/A atoxic derivative (BoNT/A ad) were employed to address the contribution of cellular mechanisms unrelated to SNAP-25 cleavage. Atoxic BoNT/A, which is unable to cleave SNAP-25 due to point mutation in LC active site, enters neurons

similarly to BoNT/A molecule (Pellet et al., 2011). Human neuronal culture consisting of glutamatergic and GABA-ergic neurons derived from differentiated human pluripotent stem cells was employed as a model system. The cells were exposed for 48 h to BoNT/A, its atoxic derivative, and the culture medium as a control. The cells exposed to toxin were washed and then kept for another 2 or 14 days in culture medium. At 2 days post BoNT/A exposure there was little difference in gene expression of all three treatments. However, at 14 days post exposure several hundred of genes were differentially expressed in BoNT/A and BoNT/A ad compared to control cells. Interestingly, there was a very similar change in gene expression upon cell exposure to either BoNT/A holotoxin or BoNTA ad. Several most highly upregulated genes are involved in neurite outgrowth and Ca2+ channel sensitization. The authors interpreted the BoNT/A actions as consequences of additional delayed responses within the host cells, induced by long term intracellular presence of toxin molecule. Apparently, only the experiment on human intestine epithelial cell line (HT-29) lacking SNAP-25, indicate BoNT/A action independent of SNAP- 25. In neuronal cell cultures the role of SNAP-25 cannot be completely ruled out.

BoNT/A effects on gene expression have also been reported *in vivo*. There are several reports of altered expression of different genes in sensory ganglia, parasympathetic ganglia and spinal cord after BoNT/A injection in the periphery (Zhang et al., 1993; Humm et al., 2000; Jung et al., 1997, Gomez-Pinilla et al., 2004, Bossowska and Majewski, 2012; Lepiarczyk et al., 2015). These distant effects remained largely unexplained, and, at the time, have been interpreted as indirect consequences of toxin peripheral neuroparalytic action. The site and mechanism of BoNT/A interaction with gene expression *in vivo* remain unknown.

6. Relevance of BoNT/A interaction with targets beyond SNAP25

Molecular interactions leading to SNAP-25-unrelated events may be related to either known of unknown BoNT/A binding abilities (Fig. 1). BoNT/A actions on neuritogenesis are most likely mediated via BoNT/A HC-binding molecules (SV2, FGF3, and polysialoglangliosides) which mediate its entrance into neurons (Coffield and Yan, 2009). Hypothetically, BoNT/A endopeptidase might cleave additional SNARE or non-SNARE proteins: BoNT/A at high concentration may cleave a non-neuronal SNAP-25 protein isoform SNAP-23 in rat cell cultures (Banerjee et al., 2001). BoNT/A LC interaction with membrane cytoskeletal proteins septins mediates the LC ability to evade cytosolic proteasomal degradation and induces septin assembly (Vagin et al., 2014).This interaction is dependent on dileucine motif situated outside of endopeptidase active site, suggesting the existence of additional binding sites at the LC (Vagin et al., 2014). Lastly, there might also be additional, yet unindentified active binding sites and target molecules.

Figure 1

When assessing potential pharmacological relevance of non SNAP-25-mediated effects of BoNT/A, it is important to compare the employed doses with the doses that produce effects mediated by SNAP-25 cleavage. In table 1 we summarized the doses and concentrations producing *in vitro* and *in vivo* effects that are difficult to explain by cleavage of SNAP-25.

In vivo *studies*. Doses producing *in vivo* effect possibly not associated with SNAP-25 are comparable with doses required to produce SNAP-25 related effects. For example, the dose (100 U) which induced alterations in neuropeptide expression after injection into pig bladder (Bossowska and Majewski, 2012; Lepiarczyk et al., 2015) is equal to the dose used for treatment of neurogenic detrusor overactivity (100 U) (Kuo et al., 2015). The 5 U BoNT/A dose (cca 15 U/kg) which induces CGRP and enkephalin expression in the rat spinal cord (Humm et al., 1997; Jung et al., 2000) is higher than the minimal doses required to produce local muscular (0.4 U/kg) or antinociceptive effects (3.5 U/kg) (Akaike et al., 2013; Bach-

Rojecky et al., 2005). Importantly, this dose is below systemic toxic doses which induce generalized muscle weakness (30 U/kg) in rats; Cui et al., 2004).

In vitro *studies***.** BoNT/A concentrations employed in *in vitro* studies (Table 1) are in the range of IC50 values of BoNT/A activity on SNAP-25 cleavage and prevention of neurotransmitter release. In human PC-12 cells, 0.2-2 nM doses employed by Ray et al. (1993) and Ishida et al., (2004) are similar to the reported BoNT/A IC50 for evoked neurotransmitter release (2 nM) (McInnes and Dolly, 1990). In SH-SY5Y human neuroblastoma cells, 6 nM concentration which induces alterations of gene expression (Thirunavukkarasux et al., 2011) is similar to the reported BoNT/A IC50 (5.56 nM) for prevention of neurotransmitter release (Keller et al., 2004). Keller and Neale (2001) reported IC50 value for SNAP-25 cleavage of 0.5 nM in neuronal cell cultures, which is higher than the concentration which induces alterations of gene expression (10 pM) (Scherf et al., 2014). Primary spinal cord cultures seem to be more sensitive to BoNT/A: the IC50 of BoNT/A for 50% SNAP-25 cleavage is around 3 pM, while the IC50 for 3H-glycine spinal cord release is 0.1-0.3 pM. BoNT/A 1 pM concentration, whose effect on 3H-glycine spinal cord release was reduced by PLA2 activation (Zhang et al. (2013), is in the range of mentioned IC50 values.

7. Conclusion

The effects of BoNT/A described in the present review point to the existence of additional toxin actions unrelated to SNAP-25, occurring at pharmacologically relevant BoNT/A doses/concentrations. Proapoptotic and antitumor activity might be the most important novel BoNT/A action. Therefore, identification of additional mechanisms and target molecules might elucidate other pharmacological or toxic effects of BoNT/A.

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FIGURE

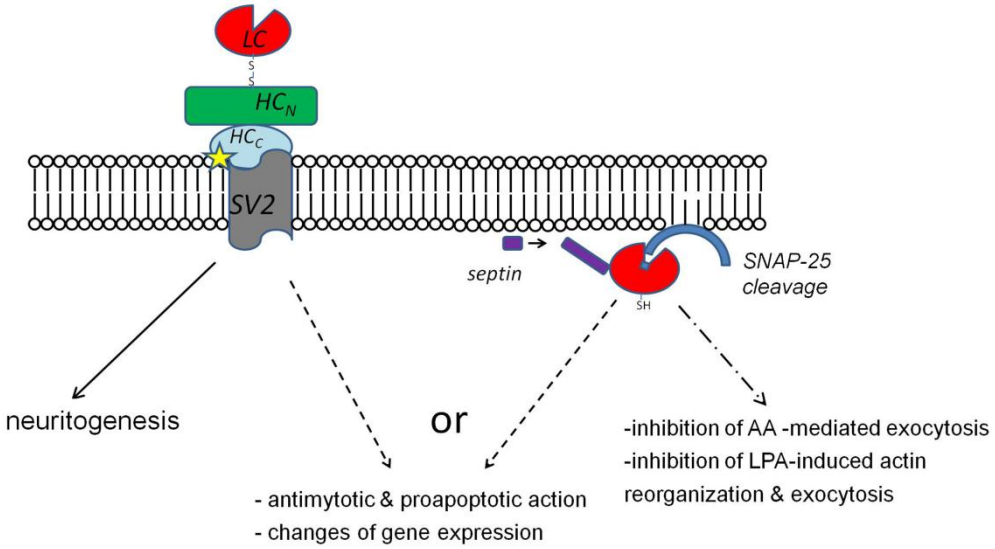

Figure 1 Possible BoNT/A cellular actions unrelated to SNAP-25. Left side: BoNT/A heavy chain c terminal domain (HC_C) interaction with membrane acceptors SV2 and polysialoganglioside (yellow star) leads to neuritogenesis. Middle: either LC action or HC interaction with membrane acceptors mediate the antimytotic and proapoptotic actions of BoNT/A, and changes of gene expression.

Right side: on the inner side of the membrane, light chain (LC, red) binds septins and induces their polymerization. LC probably mediates the inhibition of arachidonic acid (AA) and lysophosphatidic acid-mediated neurotransmitter release.