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Botulinum toxin type A in motor nervous system: unexplained observations and new challenges

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ABSTRACT

In the motor system, botulinum toxin type A (BoNT/A) actions were classically attributed to its well-known peripheral anticholinergic actions in neuromuscular junctions. However, enzymatic activity of BoNT/A, assessed by detection of cleaved synaptosomal-associated protein 25 (SNAP-25), was recently detected in motor and sensory regions of the brainstem and spinal cord after toxin peripheral injection in rodents. In sensory regions, the function of BoNT/A activity is associated with its antinociceptive effects, while in motor regions we only know that BoNT/A activity is present. Is it possible that BoNT/A presence in central motor nuclei is without any function? In this brief review we analyze this question. Limited data available in the literature warrant further investigations of BoNT/A actions in motor nervous system.

Keywords: botulinum toxin type A; Synaptosomal-associated protein 25; motor regions; central effect

Background

Botulinum toxin serotype A (BoNT/A) is a presynaptic neurotoxin synthesized by anaerobic gram-positive rod-shaped bacterium *Clostridium botulinum*. Intoxication with neurotoxin causes botulism, the disease characterized by prevention of acetylcholine release at neuromuscular junctions resulting in flaccid paralysis of skeletal muscle, and paralysis of autonomic cholinergic synapses (Matak and Lacković, 2014). BoNT/A targets synaptosomal associated protein SNAP-25, an integral part of heterotrimeric soluble N – ethylmaleimidesensitive factor attachment protein receptor (SNARE) complex of proteins required for synaptic vesicle exocytosis (Blasi et al., 1993). A widely accepted belief is that BoNT/A actions in the motor nervous system are confined to peripheral nerve endings, which may explain its classical therapeutic and toxic paralytic effects. Symptoms of botulism are confined mostly to muscular and autonomic paralysis, and central effects similar to the ones exerted by tetanus toxin are not present. However, in the last decade, mounting experimental and clinical data suggest that BoNT/A may reach central nervous system (CNS) after toxin peripheral injection (Mazzocchio and Caleo, 2015). Up to now, the significance of BoNT/A axonal transport and enzymatic activity in central motor neurons is unknown. In present review we will discuss this question.

Evidence for axonal transport of BoNT/A

Starting point in this review are observations that cleaved SNAP-25 (cSNAP-25) product of BoNT/A enzymatic activity appears in facial nucleus in the brain stem (Antonucci et al., 2008, Restani et al., 2012b) or motor regions of the spinal cord after peripheral injections of the toxin (Matak et al., 2012; Koizumi et al., 2014). Stringent analysis would reveal that evidence in favor of central effect of BoNT/A in motor regions of central nervous system (CNS) are indirect (Mazzocchio and Caleo 2014.). Ideally, the direct evidence would be to

isolate the BoNT/A molecule from CNS motor regions after toxin peripheral injection, demonstrate that it is enzymatically active, and that it has some pharmacological effect, which has not been done yet. However there are many types of indirect but convincing evidence, which will be discussed in following sections.

Early experiments of Habermann (1974) and Wiegand et al. (1976) after toxin injection into calf muscle in rats and cats with I125-radioiodinated BoNT/A, demonstrated the gradual radioactivity ascent along the sciatic nerve, followed by appearance of radioactivity in motor ventral roots and spinal cord. Radioactivity redistribution into the axonal compartment ofmotor nerve was found after incubation of diaphragm and frog leg muscles with the toxin (Black and Dolly, 1986). The drawback of these experiments is that it is not known if radioactively labelled BoNT/A retained its enzymatic activity in the axonal compartment and central motor regions.

In vitro several authors found that fluorescently labelled BoNT/A heavy chain and holotoxin are axonally transported in compartmentalized cultured neurons, with minor differences about which cellular compartment is responsible for toxin transport (Restani et al., 2012b; Wang et al., 2015; Bomba-Warczak et al., 2016). In compartmentalized culture of motor neurons Restani et al. (2012b) showed retrograde axonal transport of full toxins (BoNT/A and BoNT/E) and their heavy chains within non-acidic axonal compartments, which also transported tetanus toxin and neurotrophins. This study suggests that axonally transported BoNT/A might employ well known routes of retrograde axonal transport similar to the ones used by other toxins and signaling molecules. A similar study with fluorescently labeled BoNT/A heavy chain (Hc) was performed by Wang et al. (2015) in compartmentalized culture of hippocampal motor neurons. By employing mCherry-labelled Hc, they demonstrated retrograde movement of the toxin within retrogradely transported organelles.

After injection into the mouse hind-limb, occurrence of fluorescently-labelled Hc was also demonstrated in soma of spinal cord motoneurons. Unlike Restani et al. (2012b), they demonstrated the presence of toxin within acidic axonal compartments corresponding to autophagosomes, and subsequent movement into lysosomal compartments of the cell soma (Wang et al., 2015). The weakness of these experiments is that it is not known whether the movement of labelled toxin represents only the movement of BoNT/A Hc alone, as the light chain might be translocated into the cytoplasm. The argument that BoNT/A is transported in a functional form in both mentioned studies is the immunodetection of cSNAP-25 in the neuronal soma compartment (Restani et al., 2012b; Wang et al.2015).

Recently, Bomba-Warczak et al (2016) provided *in vitro* evidence that retrogradely transported BoNT/A, BoNT serotype D, and tetanus toxin are secreted from the soma into the extracellular fluid, from where they can be recaptured and enter second-order neurons. The authors used microfluidic chambers with separated axons and soma of cultured neurons and employed labeled toxin molecules and cSNAP-25 detection. Toxins were applied to the axon chamber. Transsynaptic passage of BoNT/A and other toxins was inhibited by adding excess of their respective Hc or neutralizing antibodies. Thus, during the transsynaptic passage, BoNT/A is exposed to the extrasynaptic milieu. Entering the second order neurons by receptor moiety dependent process requires that the entire molecule is transported and released. This study suggests the existence of cellular mechanisms for the axonal transport of BoNT/A holotoxin and transcytosis within neurons. However, it has to be noted that for transcytosis experiments the authors employed high concentration of BoNT/A (30 nM) in the microfluidic chamber containing the axons (Bomba-Warczak et al., 2016). This concentration is higher than picomolar concentrations needed to produce synaptic blockade and SNAP-25 cleavage *in vitro* (Scherf et al., 2014; Hubbard et al., 2015).

Detection of truncated SNAP-25 fragments in CNS after peripheral toxin injection

In vivo convincing evidence of axonal transport of active BoNT/A molecules was demonstrated with detection of cSNAP-25 in central motor regions. When BoNT/A was injected into the rat whisker pad, hind paw, gastrocnemius, or sciatic nerve, cSNAP-25 was detected in the corresponding motor regions mostly surrounding the primary motor neurons (Antonucci et al., 2008; Restani et al., 2012b; Matak et al., 2012; Koizumi et al., 2014). By employing detection of cSNAP-25 fragment by Western blot and immunohistochemistry, Antonucci et al. (2008) showed that after BoNT/A injection into the rat whisker pad (135 pg of 150 kDa toxin), a strong signal of cSNAP-25 immunoreactivity was detected in rat ipsilateral facial nucleus. Importantly, after BoNT/A and tetanus toxin application into the rat whisker pad both toxins proteolytic activity appeared in the brainstem with a similar time course that paralleled the peripheral spastic paralysis for tetanus toxin, but lagged behind the onset of flaccid paralysis for BoNT/A. This demonstrates similarity of axonal transport of both toxins but difference in appearance of peripheral effect, and finally behavioral effect of BoNT/A activity in CNS reminded unknown (Restani et al., 2012b). The study of Antonucci et al. (2008) was met with skepticism because of the dose employed, the use of nontherapeutic toxin preparation, and the lack of antibody characterization for specificity to cSNAP-25 (Aoki and Francis, 2011). Subsequently it was demonstrated that the antibody employed in study of Antonucci et al. (2008) was indeed specific for cSNAP-25, since it bound to the 24 kDa Western blot band corresponding to cSNAP-25 in toxin-injected tissue (Matak et al., 2011). The appropriate position of 24 kDa band was confirmed with referent antibody which recognizes both uncleaved and cSNAP-25 (Matak et al., 2011). Moreover, in further study BoNT/A-cSNAP-25 fragments were detected in dorsal horn, and much stronger signal was detected in the ventral horn of the spinal cord after low dose toxin injections into

the gastrocnemius muscle or sciatic nerve (5-10 mouse LD50 doses per kg (U/kg)). The immunoreactivity to cSNAP25-was situated in nerve terminals in contact with α-motoneuron

cell bodies and in more distant neuronal processes (Matak et al., 2012).The cSNAP-25 collocalised with cholinergic terminals surrounding the motoneuronal cells after BoNT/A injection into the hind paw i.e. near plantar muscles (Matak et al., 2012). Since α-motoneurons innervating plantar muscles do not have recurrent axon collaterals (Cuilheim and Kellert, 1978), we hypothesize that cholinergic terminals exhibiting cSNAP-25 did not reside in motor neurons innervating the plantar muscles. Thus, BoNT/A-cleaved SNAP-25 immunoreactivity might be situated in second order synapses within the ventral horn, indicating possible transsynaptic effect of of BoNT/A. By employing high dose of BoNT/A (10 U), Koizumi et al. (2014) similarly demonstrated the occurrence of BoNT/A-cleaved SNAP-25 in corresponding spinal cord segment. The cSNAP-25 was visible both in ipsilateral and contralateral ventral and dorsal horn. This finding implies transcytosis and neuronal spread through commissural interneurons to contralateral side; however, at high peripheral doses (10 U). In our experiments (Matak et al., 2012), occurrence of cSNAP-25 was restricted to ipsilateral segment of the spinal cord. cSNAP-25 was also observed in parts of CNS distant to the site of toxin central injection: a. after unilateral intrahippocampal toxin injection, cSNAP-25 was found in contralateral hippocampus, which was accompanied by suppression of neuronal activity on that side. (b.) detection of cSNAP-25 in retina after toxin injection into superior colliculus, accompanied by suppression of cholinergic transmission in retina (Antonucci et al., 2008; Restani et al., 2012a), and occurrence of cSNAP-25 in superior colliculus after BoNT/A injection into retina (Restani et al. 2011).

Investigating the transport of BoNT/A Wang et al. (2015) mentioned the possibility that cSNAP-25 could also be transported from periphery to soma for degradation. Thus,

occurrence of cSNAP-25 in the CNS might not necessary reflect BoNT/A activity but cSNAP25 transported from periphery. However, immunohistochemically, most of cSNAP-25 was found in nerve fibers and not in cell soma where degradation of cSNAP-25 could be

expected (Antonucci et al., 2008; Matak et al., 2012). Moreover, according to already described experiments of Caleo group (Antonucci et al. 2008; Restani et al., 2012a), it would imply that such truncated protein is transcytosed and axonally transported not only retrogradely but for example anterogradely from retina along the optic nerve, and transcytosed to second-order synapses in the superior colliculus. For now there is no experimental evidence for that. In experiments by Antonucci et al. (2008), optic nerve transection followed by application of shortly acting BoNT serotype E in the eye transiently depleted the BoNT/Acleaved
SNAP-25 epitope in the retina. Upon completion of BoNT/E effects, BoNT/Atruncated SNAP-25 re-appeared in the distant synapses, suggesting the long-term presence of axonally transported BoNT/A protease (Antonucci et al., 2008; Restani et al., 2011). Similar experiments have not yet been performed in the motor system.

Evidence for central effect of BoNT/A after its peripheral application: central antinociceptive activity of BoNT/A

For now, physiological or behavioral effect of BoNT/A in central motor system after toxin peripheral injection is not known. So far the most convincing evidence for functional consequences of axonally transported of BoNT/A arose from behavioral studies or combination of behavioral and immunohistochemical experiments in sensory system. In number of behavioral experiments we (Bach-Rojecky et al., 2005, 2008, 2009, 2010a, 2010b, Bach Rojecky and Lacković 2009; Matak and Lacković 2014; Drinovac et al., 2016) and the

group of Pavone and Luvisetto (Luvisetto et al, 2006, 2007, 2015; Marinelli et al., 2012; Pavone and Luvisetto 2010; Mika et al., 2011) demonstrated that antinociceptive effect of BoNT/a cannot be explained solely by its peripheral action. For example, nerve application of axonal transport blocker colchicine prevented antinociceptive effect of BoNT/a in different models of peripheral pain or intrathecal application of BoNT/a is more effective than peripheral one. Futhermore, bilateral pain associated with experimental diabetes (Bach-Rojecky et al., 2010) or cytostatic induced neuropathy (Favre-Guilmard et al., 2009), acidic saline (Bach-Rojecky and Lacković, 2009) or carrageenan-induced mirror pain (Drinovac et al., 2016) can be bilaterally reduced by unilateral injection of BoNT/A in rats. Most convincing are the experiments demonstrating that behavioral influence of BoNT/A is closely associated with appearance of cSNAP-25 in the CNS (Matak et al., 2011; Filipović et al., 2012; Wu et al., 2016). In trigeminal regions we found that extracranially injected BoNT/A prevents neurogenic inflammation in the cranial dura, which is associated with appearance of cSNAP-25 collocalised with calcitonin gene-related peptide (CGRP) in nerves innervating the dura mater.

Physiological effects of BoNT/A in motor nervous system possibly related to its direct central effects

Experimental studies in cats suggested BoNT/A distant effects in abducens motoneuron nucleus upon toxin injection into the lateral rectus muscle (Moreno-Lopez et al., 1997; Pastor et al., 1997). BoNT/A dose of 3 ng inhibited the discharge of motoneurons and induced synaptic alterations such as build-up of presynaptic vesicles in synapses connected to motoneuron cell bodies. Interestingly, 0.3 ng BoNT/A dose, which paralyzed the muscle, exerted only subtle changes in the motoneuron discharge pattern (Moreno-Lopez et al., 1997; Pastor et al., 1997). The authors hypothesized that BoNT/A underwent axonal transport in

abducens motor neurons upon high intramuscular doses, and then enters synapses connected to motor neuronal cell bodies.

Surprisingly, suggestions of possible BoNT/A central effects in motor regions came from studies in patients (Mazzocchio and Caleo 2015). Some subtle toxin effects on H reflex, suggesting action on the CNS, have been observed in individuals suffering from botulism (Tyler et al., 1963).

Wohlfarth et al., (2001) reported reductions in F-wave measured in distant, non-injected muscles in patients treated for cervical dystonia, indicating distant motoneuronal changes in excitability. The authors hypothesized that mentioned results might result from BoNT/A effect upon muscle spindles, however, it was unlikely that BoNT/A was reaching the remotes spindles in sufficient amounts to reduce their activation (Wohlfart et al., 2001). As another option, the authors hypothesized a central effect of BoNT/A upon motoneuronal cell bodies, which reduces their excitability (Wohlfarth et al., 2001).

More recently, Marchand-Pauvert et al. (2013) measured recurrent inhibition in distant muscles of patients treated for lower limb spasticity. In their setup, recurrent inhibition was evoked by stimulation of toxin-uninjected muscles. They discovered that BoNT/A injected into soleus muscle reduces the recurrent inhibition in biceps femoris muscle. They argued that peripheral effects on muscle spindles in injected muscle would have minimal effect on recurrent inhibition in distant muscle. A more proximal, central site of action was suggested since recurrent collaterals of BoNT/A injected soleus α-motoneurons innervate the Renshaw cells which control the biceps femoris motoneurons. Thus, it is suggested that BoNT/A, following its axonal transport, blocked the cholinergic synapse between recurrent collaterals of soleus α- motoneurons and Renshaw cells innervating the biceps femoris (Marchand-Pauvert et al., 2013; Mazzocchio and Caleo 2015). Similar findings were obtained in quadriceps muscle of stroke-related spastic patients after BoNT/A injection into soleus muscle

(Aymard et al., 2013).

BoNT/A effects on gene expression in motor regions

In motor regions, BoNT/A might induce additional effects at pharmacologically relevant doses, not necessarily connected only to prevention of neurotransmitter release (Matak and Lacković, 2015). There is a possibility that long-term presence of BoNT/A molecule in neurons might enable an indirect or direct BoNT/A interaction with gene expression and other cellular mechanisms (Scherf et al., 2014; Mazzocchio and Caleo, 2015). Bilateral upregulation of CGRP and enkephalin m-RNA expression in corresponding spinal cord segments and more distant spinal cord regions has been observed after local intramuscular BoNT/A injection of sub-systemic doses (Humm et al., 2000; Jung et al., 1997; Zhang et al.,1993). Increased nitric oxide synthase activity was observed in facial motoneurons after toxin whisker pad injection (Mariotti and Bentivoglio, 1996). After BoNT/A injection into the soleus, a widespread reduction of brain-derived neurotrophic factor (BDNF) and increase of neurotrophin-3 (NT3) mRNA and protein was observed in the ipsilateral spinal cord (Gomez-Pinilla et al., 2004). BoNT/A induced a reduced expression of synapsin I mRNA in the ipsilateral spinal cord (Gomez-Pinilla et al., 2004). Up to now, it remains completely unknown how does peripheral BoNT/A injection induce such widespread changes on gene expression, and whether any of these effects contribute to toxin's activity in the motor nervous system. However, critical comment to possible effect of BoNT/A on gene expressions is that it is widely known that every denervation induces plastic changes in the central nervous system. Thus, specificity of mentioned effects requires further investigations.

Possible effects of BoNT/A on peripheral γ-motor nerve endings and muscle spindle afferents mediating indirect central actions

BoNT/A used for treatment of focal muscle hyperactivity movement disorders induces muscle relaxation and relief of spastic or involuntary contractions. General opinion is that BoNT/A induced local effects fully account for its beneficial activity in these disorders. However, the beneficial duration of action of BoNT/A on spastic or involuntary contractions does not necessarily concur or follow the muscle-relaxing effects, suggesting a more complex mechanism of action (Rosales and Dressler, 2010; Kaňovský and Rosales, 2011; Mazzocchio and Caleo 2015). One of the suggested mechanisms of action is modification of stretch reflex by toxin's indirect action on firing of muscle spindle afferents which provide proprioceptive control for the muscle length and tension (Rosales and Dressler, 2010;Kaňovský and Rosales, 2011). This is based on only few experimental studies assessing the BoNT/A effects on muscle spindles. Filippi et al. (1993) examined spindle afferent discharge in trigeminal mesencephalic nucleus shortly after injecting the toxin into the rat massetermuscle area densely populated with muscle spindles. They observed a fast onset reduction of afferent discharge, but jaw muscle tension was not affected during the measurement (up to 20 min after the BoNT/A injection). As explanation of these observations, it was proposed that BoNT/A blocked the intrafusal fibers, but not the extrafusal fibers mediating the muscle tension. However, the results of this study have to be considered with caution, since authors used high single dose of BoNT/A (140 mouse lethal LD50 dose units (U)).

The effect of BoNT/A on both extrafusal and intrafusal fibers was studied histologically by Rosales et al. (1996). Type I slow and type II fast extrafusal muscle fibers, as well as Bag1 and Bag2 intrafusal muscle fibers (weakly and strongly stained for acidic and alkali ATP-ase) exhibited a reduction in fiber diameters after 14 days but not on day 4 post toxin injection

compared to control. Both extrafusal and intrafusal muscles had increased end-plate length 14 days post BoNT/A. The authors concluded that the changes in extrafusal and intrafusal muscles are similar, and occur at the same time. However, Gracies (2004) pointed out that muscle spindles are encapsulated and it is not known whether large BoNT/A molecule (900 kDa complex) can penetrate it. Overall, based on available data it was questioned whether BoNT/A actually affects the γ-motor neurons, or the described results are an indirect consequence of blockade of α-motor neuron activity (Gracies, 2004).

It is also not known whether BoNT/A might affect Ia and II type afferents directly, rather than indirectly through its effect on intrafusal fibers from γ-motoneurons. A recent study by Caron et al. (2015) suggests that the mechanosensitive function of type Ia and II primary afferents recovers in parallel with the regain of muscular function, while the impaired function of metabosensitive sensory afferents (e.g. such as the ones detecting lowered pH upon fatigue) persists longer than the muscular recovery period. Thus, for now there is no definite evidence of BoNT/A direct action upon Ia and II proprioceptive afferents.

In addition, it was suggested that BoNT/A might have indirect effects on cortical excitability via muscle spindle fibers (Kaňovský and Rosales, 2011). BoNT/A normalizes the impaired cortical and subcortical representations of the treated muscles in dystonic patients, and also in other CNS regions, such as supplementary motor areas and dorsal premotor cortex (Opavský et al., 2011). However, similar cortical changes are not visible in first time-treated dystonic patients, even if the first BoNT/A application is beneficial (Nevrly et al., 2014). This suggests that robust cortical changes develop with delay after long-term regular BoNT/A treatment (Nevrly et al., 2014).

To explain observations about BoNT/A action on central motor system some authors assumed that BoNT/A affects the functional organization of the motor (Curra et al., 2004) and sensory system (Aoki and Francis, 2011) in the CNS indirectly through peripheral mechanisms. Caleo

et al. (2009) and Mazzocchio and Caleo (2014) hypothesize that BoNT/A action in the CNS could contribute to those observations. Bearing in mind the brain ability for plastic changes this remains a possibility. On the other hand, discoveries *in vitro* that neurons possess mechanisms for axonal transport of BoNT/A holotoxin and transcytosis (Bomba-Warczak et al., 2016) and discoveries *in vivo* that after peripheral application BoNT/A appears in motor as well as sensory regions of the CNS (Antonucci et al., 2008; Matak et al., 2012) cannot rule out direct action of peripherally applied BoNT/A on CNS. However, for motor system evidence for physiological effect of BoNT/A is indirect and thus, it remains unknown whether cortical changes play a causative role in muscle-relaxing actions of BoNT/A, or what might be the long term effects of gene expression in motor regions.

Conclusion

Already a large number of behavioral and immunohistochemical experiments or combination of the two demonstrate axonal transport of BoNT/A from periphery to central sensory system and its association with antinociceptive effect of BoNT/A. Concerning motoric system strongest evidence suggesting central effect of BoNT/A after peripheral application is immunohistochemical demonstration of its enzymatic activity in motoric regions. Presence of cSNAP-25 primarily in nerve fibers and not in cell bodies, evidence *in vitro* and *in vivo* for axonal transport and transcytosis suggest that SNAP-25 is indeed cleaved in the CNS and not transported by autophagosome or other mechanism from peripheral nerve endings to be destroyed in cell soma. Here we reviewed some indirect evidence for such functions. Application of BoNT/A to experimental animals and to millions of people suggest that BoNT/A effect in the CNS does not interfere with its basic effect on muscular paralysis. Most probably central BoNT/A participate in some long term effect or in fine tuning of motor activity and we hypothesize that it might be specifically important in pharmacotherapy of

different movement disorders. Finally, to put it most simple; it seems difficult to imagine that abundant presence of botulinum toxin enzymatic activity in motor regions of the CNS is without a function.

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